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Morphological and trophic specialization in a subterranean amphipod assemblage

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SUMMARY

1. Sympatric species are expected to exhibit specialisation that reduces interspecific competition in environments with food resources that exhibit little spatial or temporal variation in availability, while sympatric species in more unpredictable environments should exhibit generalised feeding strategies to exploit a wide range of variable resources.

2. We combined stable isotopes and quantitative mouthpart morphometric data to investigate trophic structure among sympatric species in a diverse subterranean amphipod assemblage in the Edwards Aquifer, Texas, U.S.A.

3. Seven amphipod species occupied different regions of isotopic (δ^{13}C and δ^{15}N) space, suggesting the use of different food resources. Trophic position, measured as δ^{15}N, was negatively correlated with planar area of the mandible and number of molar ridges, while it was positively correlated with incisor width. Reduced molar size and robust incisors are generally associated with predatory feeding strategies in non-subterranean amphipods. δ^{13}C exhibited non-significant relationships with mouthpart morphology although one species, *Texiweckeliopsis insolita*, had significantly different mouthpart and δ^{13}C values. Another species, *Stygobromus russelli*, had more generalised mouthparts, but isotope values indicate that it had a unique and unidentified feeding method and food source, illustrating the obfuscating effect of phylogeny on form–function relationships.

4. Intraspecific relationships between body size and isotope values indicate that amphipod species showed little to moderate ontogenetic shifts in trophic position. Furthermore, body size did not predict trophic position when data were combined across species, suggesting that larger amphipods do not necessarily feed at higher trophic levels in this community.

5. Our results indicate that sympatric subterranean amphipod species can exhibit specialised feeding strategies, suggesting that competition among species is driving niche partitioning. These results contradict the assumption that variable resource availability in groundwater habitats selects for trophic generalists among sympatric species.

Keywords: Edwards Aquifer, generalist and specialist, niche partitioning, stable isotope, stygobiont

Introduction

Understanding the mechanisms that promote coexistence of potential competitors in natural communities has been a long-standing goal of ecological research (MacArthur & MacArthur, 1961; Schoener, 1974; Tilman, 1982; Abrams, 1995). A central and ongoing component of this research has focused on how multiple, potentially competing species use resources (e.g. food, nutrients), via the evolution of foraging strategies and niche partitioning (Sims *et al*., 2008; Svanbäck & Schluter, 2012; Correa & Winemiller, 2014). Optimal foraging theory, competition theory and empirical evidence suggest that in ecosystems with spatially and temporally dependable food resources, species exhibit adaptations that enhance foraging efficiency and preference for a single resource or subset of available resources (resource specialisation) (Levinton, 1972; Stephens & Krebs, 1986; Correa &

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Winemiller, 2014). Furthermore, species may forage on non-overlapping resources (i.e. niche partitioning) as a mechanism to reduce interspecific competition (MacArthur & Pianka, 1966; Wilson, 2010).

In extreme and oligotrophic habitats, the importance of interspecific competition has been suggested to be a driver of specialisation (Fišer, Blejec & Trontelj, 2012), in accordance with classical models of competition theory (Pianka, 1974). However, resources in these habitats may be patchy in time and space (Gibert & Deharveng, 2002), and the ability of a species to acquire resources may be more severely limited by some aspect of the environment (environmental heterogeneity or intraspecific competition) rather than interspecific competition (Levinton, 1972; Chesson, 2000). Under these conditions, trophic generalism is expected as an adaption to maximise acquisition of varied and unpredictable resources (Levinton, 1972).

Subterranean habitats have the potential to serve as unique systems for understanding the evolution of trophic generalism and trophic specialisation. In general, subterranean habitats exhibit relatively stable environmental conditions (Tobin, Hutchins & Schwartz, 2013) and a simplified trophic base devoid of *in situ* photosynthesisers. Relative to surface habitats, subterranean communities are also species poor (Gibert & Deharveng, 2002), making analysis of a larger proportion of the community feasible.

Subterranean communities are generally assumed to be characterised by relatively short food webs comprised of generalist consumers (Gibert & Deharveng, 2002). However, most studies of subterranean ecosystems have occurred in caves accessible to humans, in which cave stream communities are dependent on surface-derived particulate organic matter (Simon, Benfield & Macko, 2003) and dissolved organic matter (DOM) (Simon, Pipan & Culver, 2007) that are both temporally and spatially patchy (Gibert & Deharveng, 2002; Poulson, 2012).

In contrast to these relatively open cave stream systems, many phreatic karst aquifers occur at substantial depths (hundreds of metres) and can be confined below non-porous rock layers that buffer them from environmental variability and disturbance. Importantly, in some phreatic karst aquifers, chemolithoautotrophic organic matter can be an important, if not dominant, food resource (Sarbu, Kane & Kinkle, 1996; Pohlman, Iliffe & Cifuentes, 1997; Humphreys, 1999; Opsahl & Chanton, 2006). This resource is potentially spatially and temporally predictable, but because of their relative inaccessibility, ecosystem studies in deep phreatic systems have been less frequent. In groundwater habitats with lower environmental variability and a more spatiotemporally stable food resource, competition and optimal foraging theories predict the development of complex food webs (Post, 2002) and specialised feeding modes that reduce interspecific competition through niche partitioning (Levinton, 1972; Pianka, 1974; Correa & Winemiller, 2014).

The Edwards Aquifer of Central Texas, U.S.A., provides a unique opportunity to investigate trophic complexity and niche partitioning among stygobionts (obligate subterranean aquatic species). Of particular interest is the aquifer's amphipod fauna, which is comprised of more than 19 species in five families (Holsinger, 1967; Holsinger & Longley, 1980; Gibson, Harden & Fries, 2008).

In the Edwards Aquifer, chemolithoautotrophic microbial communities (Engel & Randall, 2011; Gray & Engel, 2013) and organic matter with carbon isotope ratios outside the range reported for local photosynthetic organic matter (Hutchins, Schwartz & Engel, 2013) are present along a steep redox gradient between oxygenated, low-total-dissolved-solids (TDS) waters and dysoxic to anoxic, high-TDS, high hydrogen sulphide-bearing waters, referred to here as the freshwater–saline water interface. This suggests that *in situ* chemolithoautotrophic primary production, in addition to photosynthetically derived organic matter, exists as a potential source of organic matter in the groundwater food web.

For groundwater species, inaccessibility and difficulty reproducing subterranean conditions in the laboratory (i.e. pressurised and confined conditions, presence of active chemolithoautotrophic production) make direct observation of feeding behaviours difficult. Additionally, gut content analysis has limited potential for distinguishing between different feeding modes if food items are similar in appearance, and only provides a 'snapshot' that may not represent a consumer's range of potential food items (Araújo *et al.*, 2007). Stable isotope analysis, however, can provide indirect evidence of a species' trophic niche, niche overlap and (at least qualitatively) specialisation versus generalised feeding (Layman *et al.*, 2007), as well as more specific information about trophic ecology (i.e. trophic level estimates, or identification of percentage contributions of potential food items) (Layman *et al.*, 2012).

When combined with morphological data, stable isotope analysis can be used to test hypothesised form and function relationships. Based on field and laboratory observations (Saint-Marie, 1984; Coleman, 1990; Mayer *et al.*, 2009; Mekhanikova, 2010), robust relationships between mouthpart morphology and specific feeding modes have been identified for epigean freshwater and marine amphipods. Specifically, filter feeders consistently exhibit increased mouthpart setation (Cole & Watkins, 1977; Mayer *et al*., 2009), whereas increased molar grinding surfaces and dentate and comb-like spines and setae are consistently observed in species that primarily feed by scraping biofilms (Arndt, Berge & Brandt, 2005; Mayer *et al*., 2009). Predacious species consistently exhibit reduced mouthpart setation, smaller molar grinding surfaces (Haro-Garay, 2003; Arndt *et al*., 2005; Guerra-García & Tierno de Figueroa, 2009) and large, tearing or cutting incisors (Saint-Marie, 1984; Coleman, 1990; Haro-Garay, 2003; Guerra-García & Tierno de Figueroa, 2009).

In this study, we assess relationships between feeding modes, inferred through mouthpart morphology, and diet, inferred through stable isotope composition, for seven sympatric amphipod species collected from a single site in the Edwards Aquifer. Hypotheses about general feeding strategies, based on the specific form–function relationships listed above, were evaluated based on trends in δ^{13}C and δ^{15}N values among species. We predicted that adaptations to filter feeding and scraping (e.g. increased setation and dentition of spines and setae, respectively) would be associated with differences in δ^{13}C values. Furthermore, we predicted that adaptations to predation (e.g. reduced molars, reduced setation and increased incisors) would be associated with enriched δ^{15}N values. Morphometric and stable isotope data have been combined to provide insights into the trophic ecology and diet specialisation of several species of fishes (Matthews *et al*., 2010; Lujan, German & Winemiller, 2011; Svanbäck & Schluter, 2012), but to our knowledge, this integrative approach has not been applied to invertebrates. More generally, it serves as a powerful method for exploring whether the potential functional role of specific morphologies is realised as trophic niche partitioning among species inhabiting a habitat that is otherwise difficult to observe.

Methods

Organic matter collection

As part of a previous study to characterise the isotopic composition of organic matter in the Edwards Aquifer (Hutchins *et al*., 2013), coarse particulate organic matter, fine particulate organic matter (FPOM), DOM and periphyton were extensively sampled from surface streams recharging the aquifer. Additionally, wells along the freshwater–saline water interface were sampled for FPOM (including microbial mats) and DOM. Collectively, these two groups of sites represent probably photosynthetic and chemolithoautotrophic organic matter endmembers (Hutchins *et al*., 2013). FPOM and DOM were also sampled at the site from which amphipods were collected for this study.

Amphipod collection

Seventy-five individual amphipods belonging to seven species were collected for isotopic analysis, and a subset of 30 of these individuals was used for morphometric analysis. Collections were made at an artesian well on the campus of Texas State University, San Marcos Texas, U.S.A. The well is completed in the confined portion of the San Antonio pool of the Edwards Aquifer and intersects a karst conduit at 59.5 m below ground (Holsinger & Longley, 1980). Between May 2010 and July 2013, the water outflow of the well was periodically sampled using either a 100- or 250-μm mesh net. The net was checked every 24 h, and only living animals were used for analyses. Live amphipods were identified to species under a Nikon SMZ1500 dissecting microscope (Nikon Instruments Inc., Melville, NY, U.S.A.) using Holsinger & Longley (1980) and Holsinger (1967) for the genus *Stygobromus*. Body length of all sampled individuals was measured using a Digital Sight DS-5M-L1 digital microscope camera system (Nikon Instruments Inc.).

Species belonged to the families Crangonyctidae (*Stygobromus flagellatus* and *Stygobromus russelli*), Bogidiellidae (*Artesia subterranea*) and Hadziidae (*Allotexiweckelia hirsuta*, *Texiweckeliopsis insolita*, *Texiweckelia texensis* and *Holsingerius samacos*) within the suborder Gammaridea. Hadziids and bogidiellids exhibit a Tethyan distribution, which includes extant marine and brackish water species, providing strong evidence for a marine origin of the hadziid and bogidiellid species in the aquifer (Holsinger & Longley, 1980; Lowry & Fenwick, 1983). Subterranean colonisation by amphipods may have occurred via stranding following regression of marine embayments of the study area in the late Cretaceous (100.5–66.0 myBP) although a later colonisation via active dispersal through hyporheic sediments is also possible (Holsinger & Longley, 1980). Speciation after colonisation of the subterranean habitat is possible, but undetermined (Holsinger & Longley, 1980). Continental distributions in fresh waters of North America and Asia (Holsinger, 1987) suggest that the Crangonyctidae are of Laurasian freshwater origin (Holsinger & Longley, 1980). Hypotheses about the timing of colonisation of subterranean habitats by *Stygobromus* species range from Eocene to Pliocene (56.0–2.6 myBP; Holsinger, 1966; Barr

& Holsinger, 1985), and speciation via vicariant events is thought to have followed colonisation (Barr & Holsinger, 1985).

The two *Stygobromus* species are the largest bodied and most robust species at the site, followed by *A. hirsuta* and *A. subterranea.* The remaining three hadziid amphipods are medium-sized or small-bodied (*T. insolita*), fragile species with elongated appendages. Holsinger & Longley (1980) speculated that the two *Stygobromus* species are detritivores, *T. insolita* and *H. samacos* are filter feeders, and *A. subterranea* feeds on 'soft, pulpy substances'. Two additional bogidiellids, a potential third species of Stygobromus (J. R. Holsinger, unpubl. data) and a species belonging to a fourth family (Sebidae) also occur at the well, but were excluded from analysis because no individuals were collected or small body size prevented isotopic analysis of individuals.

Stable isotope data

For detailed methods of stable isotope analysis of potential food resources, including CPOM, FPOM, DOM and periphyton, see Hutchins *et al.* (2013). For stable isotope analysis of amphipods, between 4 and 29 individuals per species were analysed for stable carbon (δ^{13}C) and nitrogen (δ^{15}N) isotope composition. C and N are extensively used as complimentary elements in isotope analyses of food webs. Trophic fractionation of C is small, allowing the contribution of food sources with distinct carbon isotope compositions (C3 versus C4 plants, for example) to be traced through food webs (Peterson & Fry, 1987). N exhibits predictable trophic fractionation, although the magnitude of fractionation depends on food sources and the physiology of consumers (McCutchan *et al.*, 2003; Vanderklift & Ponsard, 2003). Comparison of C and N isotope values allows quantification of the relative contributions of potential food sources and the relative trophic position of individuals within a food web (Peterson & Fry, 1987). Although δ^{15}N values quantify the relative trophic position of the amphipods studied, they do not necessarily imply that the investigated amphipods represent predator–prey systems. At least 20 other stygobiont species occur at the study site, all of which could be potential prey items through direct predation (e.g. small species such as copepods, ostracods and bathynellids) or necrophagy (e.g. large species, such as salamanders, shrimps and isopods).

The number of individuals analysed depended on rarity and adequate body mass. Larger individuals were preferentially analysed to ensure adequate mass and correct identification. Replicate samples were included for *c.* 10% of individuals. Animals were kept alive in filtered spring water for *c.* 3 h before drying at 50 °C for 48 h. Between 0.4 and 1.2 mg were analysed for δ^{13}C and δ^{15}N at the University of California Davis Stable Isotope Facility using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, U.K.). Standard deviations for internal laboratory standards are reported at 0.2 and 0.3‰ for C and N, respectively.

Mouthpart morphometry

Between two and five individuals per each of seven species were analysed for mouthpart morphometry (see Appendix S1 in Supporting Information). As with stable isotope analysis, the number of individuals analysed depended on rarity and size. Maxillipeds, 1st and 2nd maxilla (maxilla and maxillulae), and the left mandible were dissected from the animal under a Nikon SMZ1500 dissecting scope. Amphipod mandibles are asymmetric, and the left mandible was chosen for analysis. The paragnaths and labrum were not analysed because few form–function relationships for these mouthparts exist in the literature. Although the gnathopods serve an important function in feeding (Arndt *et al.*, 2005), these were not analysed because we felt that they provided little additional information. For an overview of amphipod mouthpart morphology and the position of mouthparts in relation to one another, see Mayer *et al.* (2009). Mouthparts were dehydrated using an alcohol dehydration series, critical point dried using CO_2 and sputter coated using a gold-palladium mixture for 2 min at 20 mAmps. Mouthparts were then mounted and imaged using a Helios NanoLab 400-FEI scanning electron microscope (Nanolab Technologies, Milpitas, CA, U.S.A.). Images were analysed using IMAGEJ software (Schneider, Rasband & Eliceiri, 2012). Twenty-four morphological variables were measured, including untransformed count variables (e.g. number of denticles on setae on the distal margin of the outer plate of the 1st maxilla, hereafter denticle number) and continuous variables (e.g. planar area of molar surface) standardised by body length.

Statistical analysis

To assess potential ontogenetic shifts in trophic position within amphipod species, species-specific δ^{15}N–body length and δ^{13}C–body length relationships were assessed using simple linear regression. A global regression of δ^{13}C and δ^{15}N as functions of body length was also performed, combining all individuals from all species. For regressions, alpha was set to minimise both Type I and Type II errors at an *a priori*-defined significant effect of $R^2 = 0.3$, using the method of Mudge *et al.* (2012). A mixed-effect model, grouping by species, was not performed because of (i) variable sample sizes among species, (ii) analytical problems relating to over fitting of linear mixed-effect models with species-specific slopes and intercepts and (iii) large individuals were preferentially chosen for isotope analysis to ensure adequate body mass.

To assess potential temporal changes in resource utilisation, $\delta^{13}C$ values were visually assessed for temporal patterns. However, high-frequency, regular-interval sampling was not attempted, and an in-depth assessment of temporal variability in isotopic values is, consequently, beyond the scope of this paper.

To assess whether amphipod species occupy the same position in trophic space (as defined by the combination of $\delta^{13}C$ and $\delta^{15}N$ values for each species), multivariate analysis of variance (MANOVA) was performed to assess global differences in $\delta^{13}C$ and $\delta^{15}N$ values among species. Afterwards, *post hoc* tests (Fisher's LSD) of two separate analyses of variance (ANOVAs) on $\delta^{13}C$ and $\delta^{15}N$ values were used to define putative trophic groups (i.e. groups of species with $\delta^{13}C$ and $\delta^{15}N$ values suggesting utilisation of at least partially non-overlapping food sources). Species were assumed to represent different trophic groups if they had significantly different mean $\delta^{13}C$ and/or mean $\delta^{15}N$ values (Table 1).

To assess potential relationships between mouthpart morphology (and inferred feeding modes) and isotopic composition, literature was used to select a suite of five morphological variables used as predictors of isotope values in linear regressions. These included planar area of the mandible, number of molar ridges, incisor width, denticle number and the length of the outer plate of the 2nd maxilla (maxilla length). Planar area of the mandible and number of molar ridges are both measures of morphological adaptations to mastication, which are expected to be reduced in higher-trophic-level species (Saint-Marie, 1984; Coleman, 1990; Haro-Garay, 2003; Guerra-García & Tierno de Figueroa, 2009). Incisor width is a measure of the size of the incisor, which is expected to be larger in higher-trophic-level species (Saint-Marie, 1984; Coleman, 1990; Haro-Garay, 2003; Guerra-García & Tierno de Figueroa, 2009). Increased denticle number is a potential adaptation to scraping (Saint-Marie, 1984; Coleman, 1990; Haro-Garay, 2003; Mayer *et al.*, 2009; Guerra-García & Tierno de Figueroa, 2009). Maxilla length strongly covaries with the number of medial and distal setae on the inner and outer plates of the 2nd maxilla, respectively, which are presumed to be greater in filter-feeding species (Cole & Watkins, 1977; Mayer *et al.*, 2009). Linear regressions of the five variables against either $\delta^{13}C$ or $\delta^{15}N$ isotope values were used to assess feeding mode. Mean species values were used for both predictor (morphological) and response (isotopic) variables. The isotope used as a response variable depended on the hypothesised functional significance (Table 2), with $\delta^{13}C$ values regressed against denticle number and maxilla length and $\delta^{15}N$ values regressed against planar area of the mandible, number of molar ridges and incisor width. For each of the five comparisons, alpha was set to minimise both Type I and Type II errors at an *a priori*-defined significant effect of $R^2 = 0.3$, using the method of Mudge *et al.* (2012). Analyses with $\delta^{15}N$ as the response isotope were performed with and without *S. russelli*, which exhibited a statistically outlying $\delta^{15}N$ value in those regressions.

Redundancy analysis (RDA) was performed to assess linear combinations of the 24 measured morphological

Table 1 Mean $\delta^{13}C$ and $\delta^{15}N$ values for amphipod species and groupings based on Fishers LSD *post hoc* test of ANOVA results

Species	$\delta^{15}N$ group	$\delta^{13}C$ group	Mean $\delta^{15}N$ (‰)	Mean $\delta^{13}C$ (‰)
Artesia subterranea	a	c	14.07	−37.13
Stygobromus flagellatus	b	b,c	11.65	−35.82
Allotexiweckelia hirsuta	b,c	c	10.42	−37.96
Texiweckeliopsis insolita	c	d	9.64	−42.03
Holsingerius samacos	d	a,b	7.87	−33.47
Texiweckelia texensis	e	a,b	5.70	−31.77
Stygobromus russelli	f	a,b	1.61	−31.34

Letters represent unique groups. Species with more than one letter belong to more than one group.

Table 2 Morphometric variables chosen as predictor variables for statistical analysis and hypothesised feeding mode interpretations

Morphometric	Hypothesised interpretation
mx1sdentnum	Variable among primary consumers: low in filters and high in scrapers
mx2op	Variable among primary consumers: larger in filter feeders (associated with number of medial setae)
mdbridges	Variable among trophic levels: lower in predators
mdbarea	Variable among trophic levels: lower in predators
mdbincw	Variable among trophic levels: higher in predators/necrophages

mx1sdentnum, average number of denticles on setae of distal medial of outer plate of 1st maxilla; mdbridges, number of molar ridges on left mandible; mx2op, length of outer plate of 2nd maxilla; mdbarea, planar area of molar of left mandible; mdbincw, width of incisor of left mandible.

variables that explained linear combinations in δ^{13}C and δ^{15}N values for individuals. Morphological variables were reduced to principal components derived from a principal components analysis (PCA) that creates eigenvectors explaining variation in combinations of morphological variables. Three principal components that, when combined, explained more than 85% of inertia (i.e. variation) in morphological characters were selected as predictor variables in RDA. Missing morphological data (i.e. broken mouthparts) were estimated using body size relationships (if apparent) or species-specific averages. No individuals and no variables had >10% missing values. After testing for global significance of the RDA, forward selection was used to identify and remove non-significant variables using the criterion of Blanchet, Legendre & Borcard (2008). Significance of remaining variables and the significance of RDA axes were quantified using permutation tests ($n = 9000$) (Borcard, Gillet & Legendre, 2011).

All statistical analyses were performed in R v3.0.1 (R Core Team, 2013) using the packages AGRICOLAE (de Mendiburu, 2013) (Fishers LSD), VEGAN (Oksanen *et al.*, 2013) and PACKFOR (Dray *et al.*, 2013) (PCA, RDA and forward selection).

Results

Carbon isotopes of organic matter were analysed in detail in Hutchins *et al.* (2013) and are only briefly summarised here. In surface streams, organic matter δ^{13}C values fell within ranges typical for mixed C3 and C4 communities: $\delta^{13}C_{CPOM} = -28.20$‰ (range = −22.99 to −35.04‰), $\delta^{13}C_{FPOM} = -24.24$‰ (range = −11.47 to −33.34‰), $\delta^{13}C_{DOM} = -27.70$‰ (range = −24.80 to −32.00‰) and $\delta^{13}C_{periphyton} = -25.51$‰ (range = −9.86 to −34.43‰). δ^{13}C values of FPOM from freshwater–saline water interface wells (near the likely source of chemolithoautotrophy) were significantly more negative than surface FPOM by an average of 8.76‰ and exhibited a wide range: $\delta^{13}C_{groundwater\ FPOM} = -32.93$‰ (range = −19.91 to −58.18‰). δ^{13}C values of DOC were not significantly different between surface and groundwater sites although carbon concentrations in ground water were usually below the minimum concentrations of the analytical facility's calibration standards. At the artesian well where amphipods were collected, δ^{13}C values for organic matter overlapped with surface stream organic matter ($\delta^{13}C_{FPOM} = -28.55$‰, range = −27.39 to −29.36‰, $\delta^{13}C_{DOC} = -24.28$‰, range = −19.80 to −27.20‰).

Body length, combined across all species, was not a significant predictor of δ^{13}C values (adjusted $R2 = -001$; $P = 0.8788$, $F = 0.02_{1,72}$; optimal $\alpha = 0.02$). Furthermore, R^2 for the relationship between body length and δ^{15}N values combined across all species was below the *a priori*-defined significant effect of $R^2 = 0.3$ ($R^2 = 0.154$; $P = 0.001$ $F = 12.83_{1,64}$; optimal $\alpha = 0.02$). *A. subterranea* exhibited a modest decrease in δ^{13}C values with increasing body length ($F = 6.49_{1,6}$, $P = 0.044$, $R^2 = 0.440$, optimal $\alpha = 0.27$) (Fig. 1). *A. hirsuta* ($F = 2.941_{1,2}$ $P = 0.229$, $R^2 = 0.393$, optimal $\alpha = 0.39$) and *T. insolita* ($F = 7.313_{1,6}$, $P = 0.035$, $R^2 = 0.474$, optimal $\alpha = 0.27$) exhibited a positive relationship between δ^{15}N values and body length (Fig. 1).

The seven amphipod species displayed disparate δ^{13}C and δ^{15}N values, suggesting (i) multiple potential food sources, including autochthonous organic matter produced via chemolithoautotrophy and allochthonous organic matter produced on the surface via photosynthesis, and (ii) the presence of multiple trophic levels, including primary and secondary predators (Fig. 2). MANOVA indicated that the amphipod species occupy significantly different positions in isotope bi-plot space (Pillai's trace = 1.215, $F_{6,\ 58} = 14.964$, $P < 0.001$). *Post hoc* comparisons revealed that, with the exception of *S. flagellatus* and *A. hirsuta*, all species were significantly different from one another for at least one isotope ($P < 0.05$) (Fig. 2, Table 1). On average, *A. subterranea*, *S. flagellatus* and *A. hirsuta* had higher δ^{15}N values than other amphipods, and one species, *T. insolita*, had more negative δ^{13}C values relative to other amphipods (although some individuals from other species had equally negative δ^{13}C values). One species, *S. russelli*, had low δ^{15}N values relative to other species. Relative to suspended FPOM from the sampling site ($\delta^{13}C = -28.55 \pm 1.03$‰, $\delta^{15}N = 1.47$‰), most individuals had similar or more negative δ^{13}C values and higher δ^{15}N values.

Principal components analysis revealed strong separation of species in morphological space, with the exception of the two closely related species *S. flagellatus* and *S. russelli* (Fig. 3). The first three principal components explained 86% of variation in morphology and described a gradient between species with longer, more setose maxilla to species with reduced setae and a more robust incisor and lacinae mobilis (PC 1, inertia explained = 54%), a gradient between species with elongated, dentate lacinae mobilis and numerous, dentate distal setae (PC 2, inertia explained = 21%) and a gradient in molar development (PC 3, inertia explained = 11%). Using forward selection, principal components 2 and 3 were identified as significant predictors of δ^{13}C and δ^{15}N values (PC 2: $F = 3.35_{1,2.69}$, $P = 0.047$; PC 2: $F = 7.79_{1,6.24}$, $P = 0.005$)

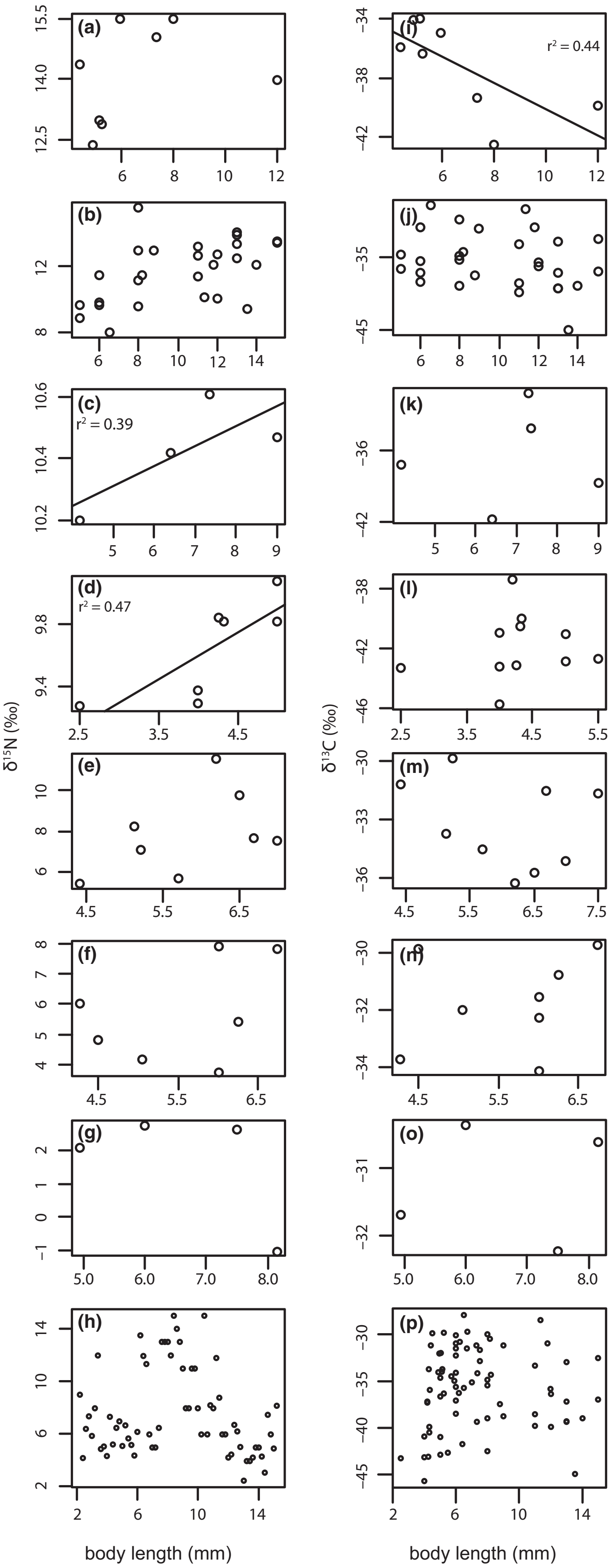

Fig. 1 Species-specific δ^{15}N–body length and δ^{13}C–body length relationships. (a, h) *Artesia subterranea*; (b, i) *Stygobromus flagellatus*; (c, j) *Allotexiweckelia hirsuta*; (d, k) *Texiweckeliopsis insolita*; (e, l) *Holsingerius samacos*; (f, m) *Texiweckelia texensis*; (g, n) *Stygobromus russelli*. Trendlines and R^2 values are shown for significant relationships.

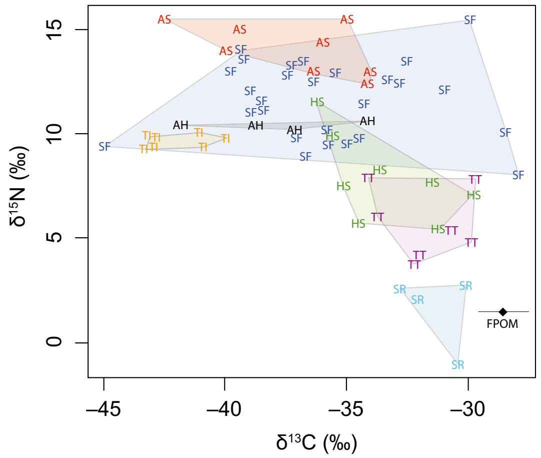

Fig. 2 Isotope biplot of individual amphipods and fine particulate organic matter (FPOM) from the sampling site. Bar shows FPOM standard deviation (none for δ^{15}N because $N = 1$). AS, *Artesia subterranea*; SF, *Stygobromus flagellatus*; AH, *Allotexiweckelia hirsuta*; TI, *Texiweckeliopsis insolita*; HS, *Holsingerius samacos*; TT, *Texiweckelia texenis*; SR, *Stygobromus russelli.*

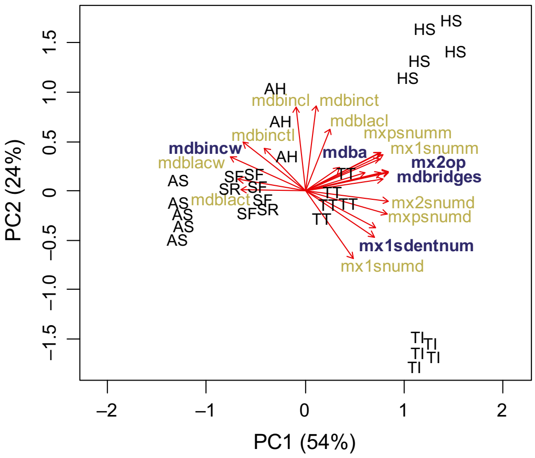

Fig. 3 Principal components analysis (PCA) biplot of amphipod positions in morphometric space. Proportion of variance explained = 54% and 21% for PC 1 and PC 2, respectively. AS, *Artesia subterranea*; SF, *Stygobromus flagellatus*; AH, *Allotexiweckelia hirsuta*; TI, *Texiweckeliopsis insolita*; HS, *Holsingerius samacos*; TT, *Texiweckelia texensis*; SR, *Stygobromus russelli.* mxpsnumd, number of setae on distal margin of inner plate of maxilliped; mxpsnumm, number of setae on medial margin of inner plate of maxilliped; mx1snumm, number of setae on medial margin of inner plate of 1st maxilla; mx1snumd, number of setae on distal margin of 1st maxilla; mx1sdentnum, average number of denticles on setae on distal margin of outer plate of 1st maxilla; mx2snumm, number of setae on medial margin of inner plate of 2nd maxilla; m2snumd, number of setae on distal margin of outer plate of 2nd maxilla; mdbridges, number of molar ridges on left mandible; mdbinct, number of incisor teeth on left mandible; mdblact, number of lacinae mobilis teeth on left mandible; mxppalp, length of four distal segments of maxilliped palp (includes tooth); mxpendite, length of inner plate of maxilliped; mx1ip, length of inner plate of 1st maxilla; mx1op, length of outer plate of 1st maxilla; mx2ip, length of inner plate of 2nd maxilla; mx2op, length of outer plate of 2nd maxilla; mx2sl, maximum length of distal setae on outer plate of 2nd maxilla; mdbarea, planar area of molar of left mandible; mdbincl, length of incisor of left mandible; mdbincw, width of incisor of left mandible; mdbinctl, maximum tooth length of incisor of left mandible; mdblacl, length of lacinae mobilis of left mandible; mdblacw, width of lacinae mobilis of left mandible; mdblactl, maximum length of lacinae mobilis tooth of left mandible. Morphometrics used in linear regressions are dark and bold.

(Fig. 4), although together they explained a small proportion of variance in isotope values (29%). Only the first RDA axis was significant (RDA 1: $F = 10.95_{1,8.77}$, $P = 0.0005$; RDA 2: $F = 0.19_{1,0.15}$, $P = 0.83$) and explained 29% of variance in isotope values. Both principal components and isotope vectors were orthogonal to one another in RDA, with PC 2 associated with δ^{13}C and PC 3 associated with δ^{15}N values.

Average denticle number (Fig. 5), hypothesised to be an indicator of scraping, was not a significant predictor of δ^{13}C values in linear regression. Although P for the regression was below optimal $\alpha = 0.29$, the strength of the relationship was below the *a priori* biologically meaningful $R^2 = 0.30$ ($F = 2.23_{1,5}$, $P = 0.20$, $R^2 = 0.17$). Despite the lack of a significant relationship, one species, *T. insolita*, was a statistical outlier, having nearly three times the denticle number as any other species. *T. insolita* was also assigned to its own carbon isotope group using Fisher LSD (Table 1). Maxilla length (Fig. 6), hypothesised to be an indicator of filter feeding, was also not a significant predictor of δ^{13}C values ($F = 0.12_{1,\ 5}$, $R^2 = -0.17$, $P = 0.73$). One species, *H. samacos*, was a statistical outlier for the number of medial setae on the outer plate of the 2nd maxilla (data not shown), but was not an outlier for maxilla length.

None of the three morphological variables that were predicted to be correlated with δ^{15}N (number of molar ridges on the left mandible, planar molar area and width of the incisor, Figs 7 & 8) were significant predictors of δ^{15}N values when *S. russelli* was included (number of molar ridges: $F = 1.16_{1,\ 5}$, $R^2 = 0.03$, $P = 0.33$; planar molar area: $F = 0.53_{1,5}$, $R^2 = -0.09$, $P = 0.50$; width of the incisor: $F = 0.85_{1,5}$, $R^2 = -0.03$, $P = 0.40$). However, all three regressions were significant when *S. russelli* was excluded (Fig. 9), with negative relationships between the two molar variables and δ^{15}N values and a positive relationship between width of the incisor and δ^{15}N value (number of molar ridges: $F = 14.28_{1,\ 4}$, $R^2 = 0.73$, $P = 0.02$; planar molar area: $F = 15.31_{1,4}$, $R^2 = 0.74$, $P = 0.02$; width of the incisor $F = 4.63_{1,4}$, $R^2 = 0.42$, $P = 0.10$).

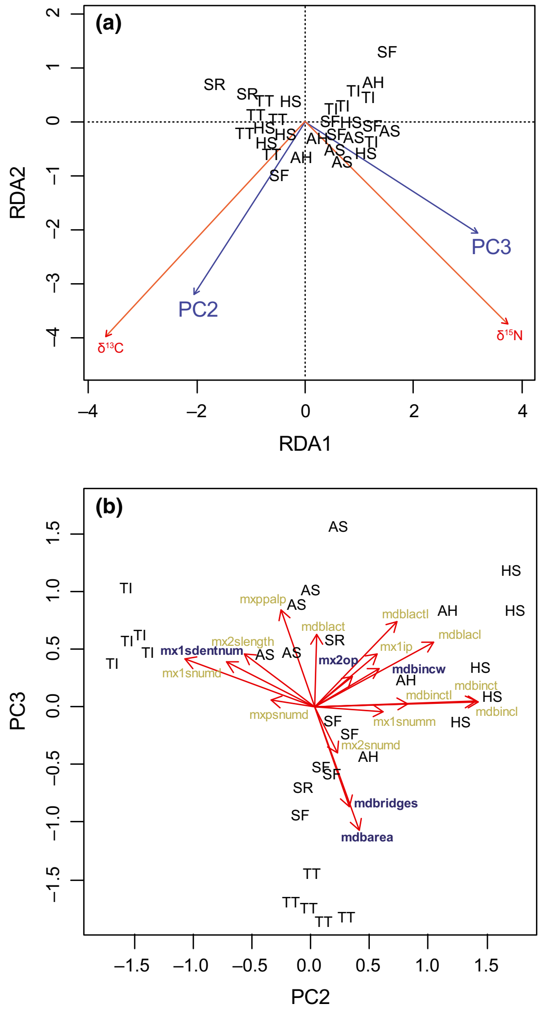

Fig. 4 Principal components analysis (PCA) and redundancy analysis (RDA) results. (a) Biplot of RDA results showing relation between significant principal components (shown in b) and stable isotope values for individuals. Only RDA axis 1 is significant, explaining 29% of variance in isotope values. RDA2 explains <1% of variance. (b) PCA biplot of morphometric data on PC 2 and PC 3. Text is as in Fig. 3.

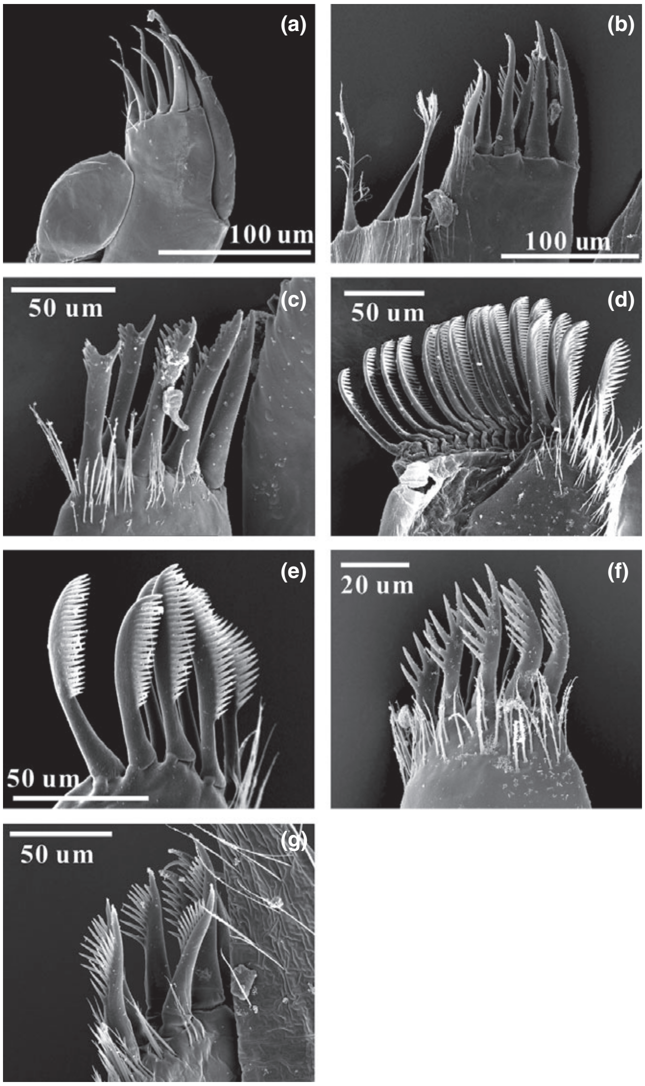

Fig. 5 SEM images of setae on the distal margin of outer plate of 1st maxilla. (a) *Artesia subterranea*; (b) *Stygobromus flagellatus*; (c) *Allotexiweckelia hirsuta*; (d) *Texiweckeliopsis insolita*; (e) *Holsingerius samacos*; (f) *Texiweckelia texensis*; (g) *Stygobromus russelli*.

Discussion

Ecological mechanisms that allow for the coexistence of species have received considerable study (Hutchinson, 1961; Schoener, 1974; Tilman, 1982; Abrams, 1995). Recent advances in our understanding of the role of biodiversity in ecosystem function and ecosystem services (Cardinale, 2011; Carroll, Cardinale & Nisbet, 2011) make such studies increasingly important. In the Edwards Aquifer, the availability of multiple sources of organic matter, including potentially temporally and spatially predictable organic matter produced *in situ*, sets the stage for resource partitioning among a diverse and ancient subterranean amphipod assemblage.

Relative to potential food sources, amphipods displayed a wide range in both δ^{13}C and δ^{15}N values. δ^{13}C values for amphipods only partially overlapped with δ^{13}C values for suspended FPOM from the sampling site, suggesting that suspended FPOM from the site contributed to the food web but was not the sole source of consumed organic matter. Importantly, isotopic data also indicate that amphipod species utilise partially non-overlapping food resources, which suggests resource partitioning among sympatric species.

Although isotopic and morphological data are consistent with resource partitioning among species, we found limited evidence of ontogenetic shifts in diet. Significant body size–δ^{15}N relationships have been observed in

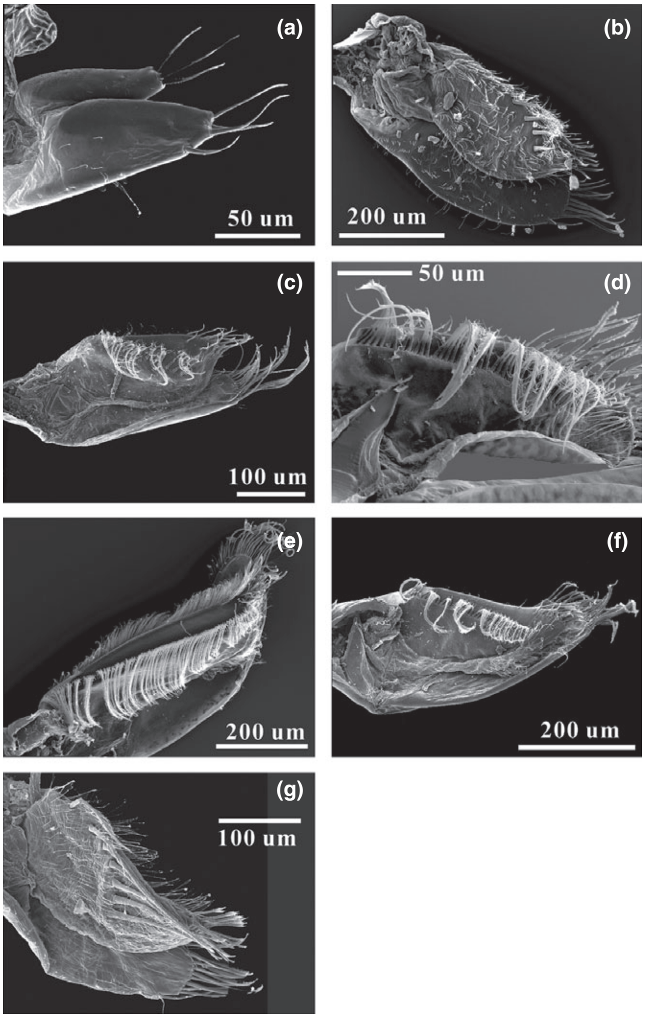

Fig. 6 SEM images of 2nd maxilla. Only inner plate shown for *Texiweckeliopsis insolita.* (a) *Artesia subterranea*; (b) *Stygobromus flagellatus*; (c) *Allotexiweckelia hirsute*; (d) *T. insolita*; (e) *Holsingerius samacos*; (f) *Texiweckelia texensis*; (g) *Stygobromus russelli.*

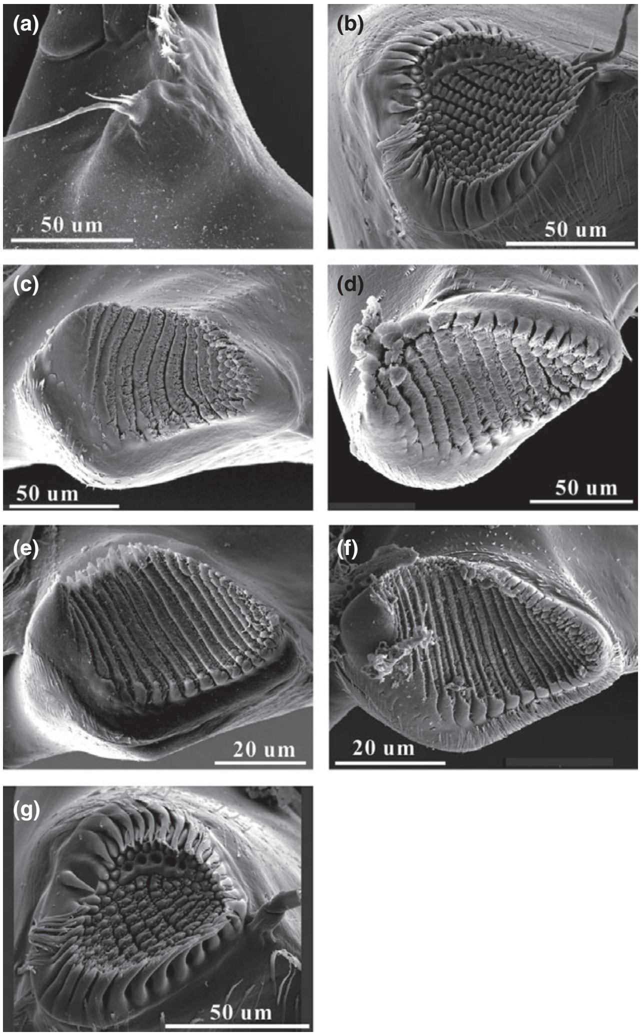

Fig. 7 SEM images of the left molar. (a) *Artesia subterranea*; (b) *Stygobromus flagellatus*; (c) *Allotexiweckelia hirsute*; (d) *Texiweckeliopsis insolita*; (e), *Holsingerius samacos*; (f) *Texiweckelia texensis*; (g) *Stygobromus russelli.*

epigean ecosystems (Jennings *et al.*, 2001), but the relationship observed in Edwards Aquifer amphipods was weak and significant for only two species. The lack of a strong body size–trophic level relationship may be due to a lack of correlation between prey body size and trophic position (Layman *et al.*, 2005), but size relationships should be interpreted with caution because samples were biased towards the largest individuals collected (see Methods).

Interspecific differences in amphipod δ^{13}C and δ^{15}N values were largely associated with independent morphological variables in RDA, suggesting that the separate components of trophic position described by δ^{15}N and δ^{13}C values (i.e. trophic position and basal food source, respectively) are influenced by largely separate mouthpart morphologies. As has been observed in marine species (Saint-Marie, 1984; Coleman, 1990; Haro-Garay, 2003; Guerra-García & Tierno de Figueroa, 2009), large, strong incisors and reduction in molar area are associated with an increasingly predatory (or necrophagous) feeding strategy (as indicated by higher δ^{15}N values) in some Edwards Aquifer amphipods, including *S. flagellatus*, *A. subterranea* and *A. hirsuta*. Lower trophic level species, such as *T. texensis*, exhibited proportionally larger and more strongly dentate mandibles, suggesting an adaptation to mastication. This has been observed in other basal consumer amphipods (Mayer *et al.*, 2009) and could be adaptive in the Edwards Aquifer for processing benthic sediments or biofilms containing carbonate mineral particles (Roberts *et al.*, 2004).

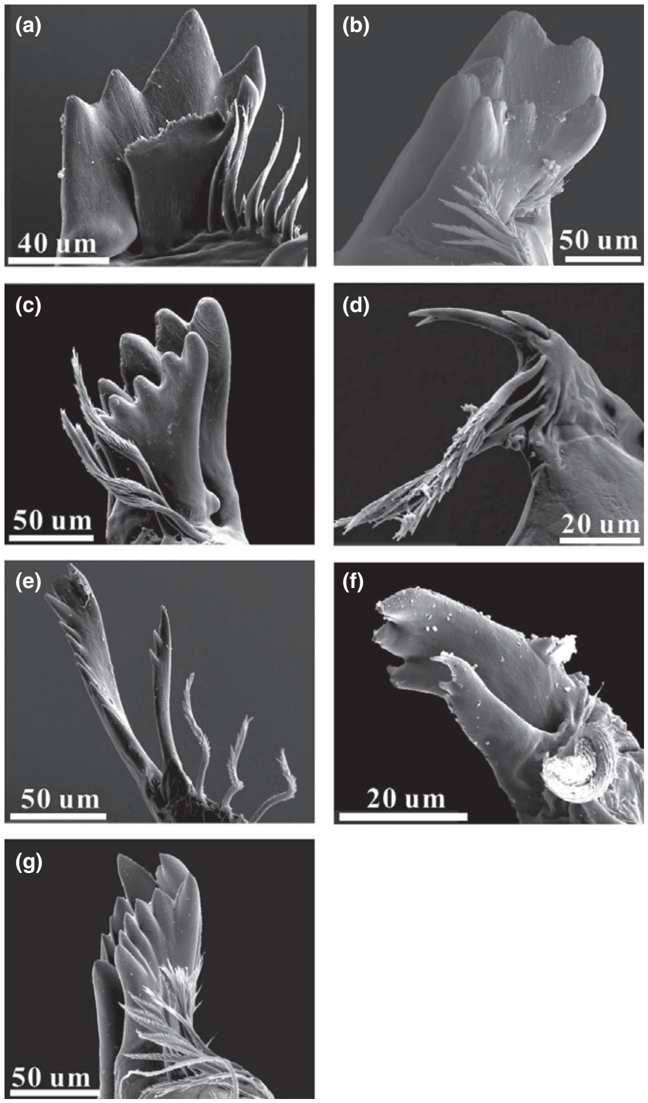

Fig. 8 SEM images of the left incisor and lacinae mobilis. (a) *Artesia subterranea*; (b) *Stygobromus flagellatus*; (c) *Allotexiweckelia hirsute*; (d) *Texiweckeliopsis insolita*; (e) *Holsingerius samacos*; (f) *Texiweckelia texensis*; (g) *Stygobromus russelli*.

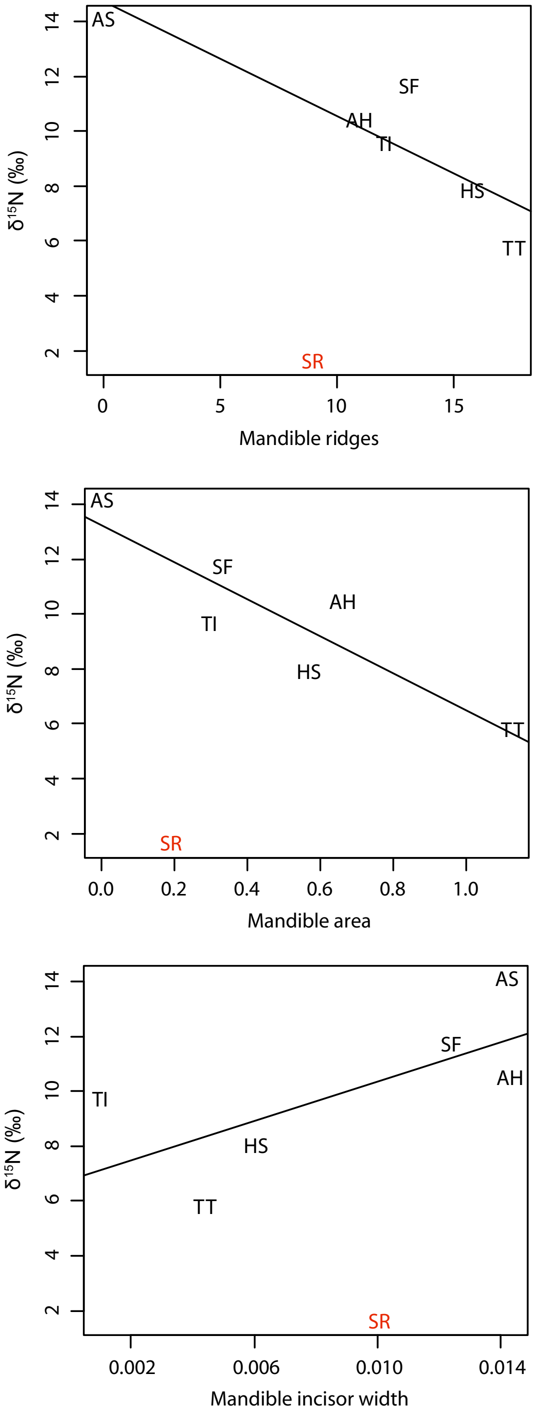

Fig. 9 Significant linear regressions of species average δ^{15}N values versus morphometric variables. *Stygobromus russelli* position is shown, but *S. russelli* was excluded from regression analysis. AS, *Artesia subterranea*; SF, *Stygobromus flagellatus*; AH, *Allotexiweckelia hirsuta*; TI, *Texiweckeliopsis insolita*; HS, *Holsingerius samacos*; TT, *Texiweckelia texensis*; SR, *Stygobromus russelli*.

Contrary to our predictions, significant relationships between δ^{13}C values and mouthpart morphometrics were not observed. Limited apparent relationships between morphology and isotope data could result from several confounding factors including phylogenetic history (Perry & Pianka, 1997), highly specialised feeding modes or flexibility in feeding behaviours. Amphipods employ diverse, highly specialised feeding behaviours, including exoparasitism (Schell, Rowntree & Pfeiffer, 2000; Mekhanikova, 2010), endoparasitism (Laval, 1978; Mekhanikova, 2010), predation (Coleman, 1990), egg predation (Mekhanikova, 2010), scraping ice algae (Arndt *et al.*, 2005) and necrophagy (Saint-Marie, 1984). The presence of species-specific morphologies associated with highly specialised feeding behaviours might not be apparent as general morphological gradients across multiple species. Furthermore, species may preferentially behave as trophic generalists, but exhibit morphological specialisations for feeding on non-preferred or suboptimal food resources to avoid interspecific competition when preferred resources are limiting (Robinson & Wilson, 1998). In this circumstance, feeding modes inferred from morphological characteristics may not be reflected in average resource use, as quantified with isotope data.

Two species had unusual combinations of isotope values and mouthpart morphology. Relative to other sampled species, *T. insolita* had more negative $\delta^{13}C$ values and a higher denticle number; a morphology that is associated with scraping in some marine and freshwater amphipods (Arndt *et al.*, 2005; Mayer *et al.*, 2009). This combination of distinct $\delta^{13}C$ values and morphology suggests that *T. insolita* may be scraping biofilms not utilised by other species and which is isotopically distinct from entrained OM sampled at the study site. Organic matter (including biofilms) present in other parts of the aquifer had more negative $\delta^{13}C$ values than both organic matter sampled in surface streams and entrained OM from the study site. Hutchins *et al.* (2013) cite three lines of evidence (organic matter isotope composition, geochemistry and microbial community composition) for chemolithoautotrophy, which may explain *T. insolita's* negative $\delta^{13}C$ values, but more research is necessary to quantify the potential role of chemolithoautotrophy in the Edwards Aquifer food web. Despite having 'generalised crangonyctid' (Holsinger & Longley, 1980) mouthparts, similar to those of *S. flagellatus*, *S. russelli* had unusually low $\delta^{15}N$ values. Because of its large size relative to other amphipods, it is unlikely that *S. russelli* is a food source for these species, which exhibit higher $\delta^{15}N$ values. Isotopic data suggest that *S. russelli* may feed on an organic matter source not utilised by other species, although the identity of the food source and how *S. russelli* feeds is unclear.

Although niche partitioning as a mechanism to reduce intraspecific competition was not formally tested in this study, we believe that it is the most likely explanation for the observed data. It is unlikely that significant interspecific differences in isotopic values result from differences in trophic fractionation among species, all of which are ammonotelic, freshwater gammaroidean amphipods (McCutchan *et al.*, 2003). Rather, differences probably result from utilisation of different food resources. There is no evidence suggesting that the investigated species occur in different habitats, which would preclude the opportunity for competition. Indeed, four of the seven species have been recorded together in a lake in a nearby cave (B. Hutchins, unpubl. data), and the majority of water discharging from the sampled well issues from a single conduit, the presumed habitat for the investigated species. Given that the species probably occur in the same habitat, feed on disparate food resources and have distinct mouthpart morphologies, we feel that intraspecific competition is consistent with the observed data and is more likely than alternative explanations, such as chance colonisation by ancestral species with disparate feeding modes. This study does not meet the six conditions proposed by Schluter (2000) to test for character displacement, and species sorting could be an alternative mechanism by which the observed isotope–morphological relationships could have evolved. However, whether these relationships have arisen through character displacement or species sorting, both are examples of interspecific competition leading to trophic specialisation in an environment generally considered to select for trophic generalists.

An integrated approach incorporating both morphological and isotopic data allowed us to test predictions about trophic generalism and specialisation in a community that would otherwise be difficult to observe. The occurrence of niche partitioning suggests that species are not employing generalist feeding strategies to cope with unpredictable food resources in this system and food resources are probably not as patchy as is often assumed for subterranean habitats. In the light of these observations, a reassessment of the assumption that subterranean systems typically possess limited trophic diversity is warranted. The observed partitioning of food resources by consumers is probably an important factor in the maintenance of high biological diversity and long food chains in the Edwards Aquifer, because it promotes coexistence of potential competitors and increasing resource exploitation (Duffy *et al.*, 2007; Finke & Snyder, 2008), which in turn increases resources available for the observed higher-trophic-level species.

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Supporting Information

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

Appendix S1. Amphipod isotopic and morphometric data for individuals measured for morphometry.

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