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Identification of trophic niches of subterranean diving beetles in a calcrete aquifer by DNA and stable isotope analyses

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Abstract. The Yilgarn calcrete aquifers in Western Australia are an interesting system for investigating the process of speciation within subterranean habitats, because of the limited opportunities for dispersal between isolated calcretes. The presence of different-sized diving beetles (Dytiscidae) in separate calcretes, including sympatric sister-species pairs, suggests that species may have evolved within calcretes by an adaptive shift as a result of ecological-niche differentiation. We have studied the potential for trophic niche partitioning in a sister triplet of diving beetles, of distinctly different sizes, from a single aquifer. Fragments of the mitochondrial COI gene, specific to known species of amphipods and copepods, were polymerase chain reaction-amplified from each of the three beetle species, indicating that there is an overlap in their prey items. Significant differences were found in the detected diets of the three species, and results showed a propensity for prey preferences of amphipods by the large beetles and one species of copepod for the small beetles. A terrestrial source of carbon to the calcrete was suggested by stable isotope analyses. The combined approach of molecular, stable isotope and behavioural studies have provided insight into the trophic ecology of this difficult-to-access environment, providing a framework for more fine-scale analyses of the diet of different-sized species to examine speciation underground.

Additional keywords: amphipods, Chiltoniidae, copepods, dietary analysis, Harpacticoida, stable isotopes, stygofauna, Yilgarn calcrete aquifers.

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Introduction

The Yilgarn calcrete aquifers (~10 m thick, porous carbonate deposits containing groundwater) in central Western Australia are a biodiversity hotspot for subterranean diving beetles (Dytiscidae; Balke *et al.* 2004; Watts and Humphreys 2004, 2009), and provide a fascinating system for studying speciation and adaptive evolution within discrete 'island' subterranean environments where opportunities for dispersal are very limited. Although the evolution of subterranean fauna is largely thought to have been the result of colonisation from surface species, there is evidence that speciation can occur underground in subterranean taxa (Juan *et al.* 2010). Of particular interest in the Yilgarn diving beetles is the presence of a repeated pattern of occurrence of sympatric species, in distinct and non-overlapping size classes, consistently co-occurring in separate calcretes (Humphreys *et al.* 2009). Phylogenetic studies of the diving beetles from the Yilgarn calcretes imply a scenario of invasion of the subterranean environment by several surface-dwelling ancestors during a period of aridification from 10 to 5 million years ago (Cooper *et al.* 2002; Leys *et al.* 2003; Guzik *et al.* 2009). On the basis of mitochondrial DNA analyses of beetles found within 13 separate calcretes, a pattern has been observed of different-sized sympatric species of diving beetles that are each other's nearest relative (henceforth, referred to as 'sister species'; Cooper *et al.* 2002; Leys *et al.* 2003; Leys and Watts 2008). Plausible hypotheses for how speciation has occurred include repeated colonisation from the same ancestral species with divergence in allopatry, intra-calcrete speciation in sympatry as a result of ecological-niche partitioning or intra-calcrete speciation through micro-allopatric processes (Cooper *et al.* 2002; Leys *et al.* 2003; Leijs *et al.* 2012). A comparison of observed and expected frequencies of sister species in the

calcretes under a model of repeated colonisation found that within-aquifer speciation best explained the pattern of sympatric sister species (Leijs *et al.* 2012). Micro-allopatric processes of evolution have been detected in several diving beetle populations (Guzik *et al.* 2009, 2011; Bradford *et al.* 2013) in individual calcretes, including the one studied here. However, there are no empirical studies on ecological differentiation within the Yilgarn calcretes, and evidence that different-sized beetle species occupy separate ecological niches is required to provide support for the alternative hypothesis of intra-calcrete speciation in sympatry. Trophic differences are a possible driver of diversification into different body sizes (Schoener 1974) and the aim of the present study was to investigate the hypothesis that different-sized beetles, and/or their larvae, occupy separate trophic niches within the calcrete.

A single calcrete at Sturt Meadows in the Yilgarn region of Western Australia (Fig. 1) was chosen to investigate trophic partitioning in diving beetles, because it contains three sympatric sister species of diving beetles that do not overlap in size, namely *Paroster macrosturtensis* (large) Watts & Humphreys 2006, *P. mesosturtensis* (medium) Watts & Humphreys 2006 and *P. microsturtensis* (small) Watts & Humphreys 2006, with total body lengths of 3.6–4.1 mm, 1.9–2.3 mm, and 1.7–1.8 mm, respectively (Leys and Watts 2008). The calcrete at Sturt Meadows is relatively simple in terms of macro-invertebrate species diversity. A previous study using DNA barcoding of a fragment of the mitochondrial Cytochrome *c* oxidase 1 gene (COI) of macro-invertebrates (>0.5-mm body length) identified that, in addition to the diving beetles, three species of chiltoniid amphipod (*Yilgarniella sturtensis*, *Scutachiltonia axfordi*, *Stygochiltonia bradfordae*; King *et al.* 2012), four divergent lineages of oligochaete worms and two species of copepod (Harpacticoidea; T. Karanovic and S. J. B. Cooper, unpubl. data) were present in the groundwater (Bradford *et al.* 2010). In addition, terrestrial troglobionts, including a single species of mite (Acari), a centipede (Chilopoda) and Collembola were identified (Bradford *et al.* 2010). Predaceous diving beetles and their larvae are likely to be the top predators in calcrete aquifer ecosystems. To determine trophic differences for each beetle species in this subterranean environment, we used a combination of molecular genetic and stable isotope analyses, and behavioural observations. Our molecular approach was to identify the source of degraded DNA that is the product of beetle digestion, from common potential prey items. Prey-specific primers were developed to be used in feeding trials and in a natural-predation study of beetles collected from the calcrete. This approach enables specific dietary items of the diving beetles to be determined, because the beetles are small and difficult to observe feeding in their natural environment and their larvae are liquid feeders. Polymerase chain reaction (PCR) amplification of food items present in the gut of whole beetle extracts using group-specific primers (gut-content assays) enables very small fragments of prey DNA to be detected, and detection times can be tested in feeding trials (King *et al.* 2008).

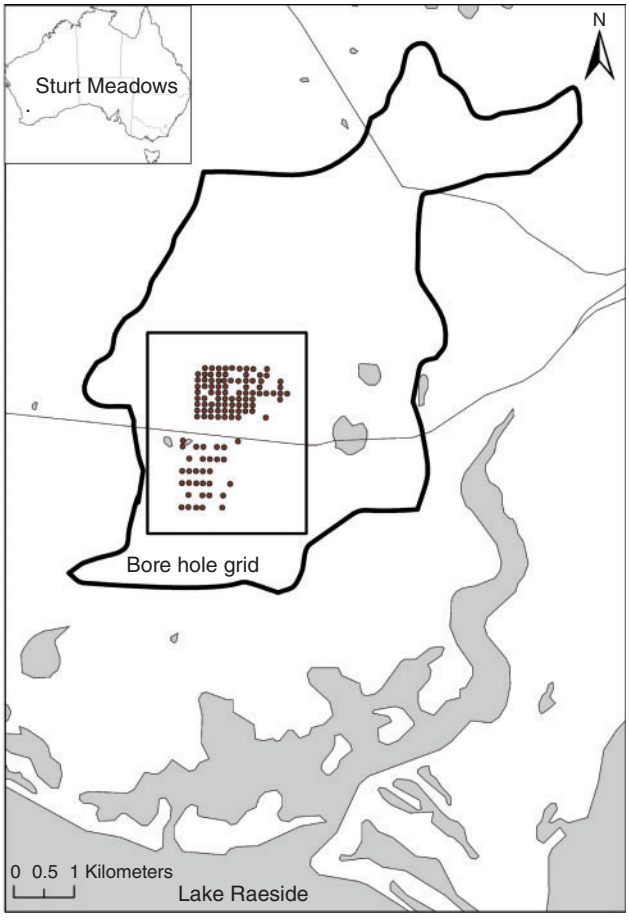

Fig. 1. Map of Australia, with the Sturt Meadows pastoral property in Western Australia indicated. The expanded diagram shows the sample site at Sturt Meadows, with the bore-hole grid north and south of the road (grey line) outlined in a black square. The shape of the calcrete is illustrated in black, and the salt lake and low-lying areas that may contain water are shown in grey.

Further information on the trophic ecology in the calcrete was sought by examining the stable isotope ratios of nitrogen and carbon of the diving beetle species, amphipods and terrestrial plants at Sturt Meadows, with trophic fractionation down the food chain considered to be 2–4‰ for nitrogen and 1–2‰ for carbon (Ehleringer *et al.* 1986; West *et al.* 2006). By investigating the trophic niche occupied by the different-sized predaceous beetles, the aim was to gain an insight into the food web of this unusual system, where direct observation is difficult, and to test the hypothesis that trophic partitioning could have been a driving force in diving beetle speciation underground. Behavioural observations of the subterranean diving beetles in captivity were carried out as part of the present study to further our understanding of the changes in behaviour that may have accompanied their adaptation to the groundwater calcrete environment.

Materials and methods

Collection of samples and DNA extraction

Adult and larval diving beetles in the Sturt Meadows calcrete aquifer were sampled by haul netting from 41 abandoned mineral exploration bore holes, from over 100 bores that are in a 3.5-km^2 grid (Fig. 1), with 10 hauls of a weighted plankton net (mesh 250 μm) through the water column in each bore found to

be the most efficient collecting method (Allford *et al.* 2008). Adults and larval beetles were sorted live, within several hours of collection, from other stygofauna present in the samples under a stereo-microscope, and were identified to species level (Watts and Humphreys 2009). Individual diving beetle adults and larvae were rinsed with rain water to prevent contamination of gut-content assays by calcrete water, which may contain decaying invertebrates. Diving beetles to be used in feeding trials were transported to the laboratory in rainwater. Specimens for the natural-predation study were blotted dry and killed by snap freezing in liquid nitrogen. Whole-body DNA extracts were prepared using the DNeasy Blood and Tissue Kit (Qiagen, Chatsworth, CA, USA), according to the manufacturer's instructions. Dytiscid larvae were sequenced for COI and typed to species level, based on clustering in phylogenetic-distance analyses (see below).

Amplification, sequencing and analysis of the mitochondrial COI gene

For the verification of larval diving beetles species, a 623-bp fragment of the mitochondrial COI gene was PCR amplified with universal primers LCO1490 (GGTCAACAAATCA TAAAGATATTGG) and HCO2198 (TAAACTTCAGGGT GACCAAAAAATCA) (Folmer *et al.* 1994) in 25-μL volumes containing 4 mM $MgCl_2$, 0.20 mm dNTPs, PCR buffer (Applied Biosystems, Foster City, CA, USA), 6 pmol of each primer and 0.5 U of Ampli-*Taq* Gold (Applied Biosystems). PCR amplification was performed under the following conditions: 94°C for 9 min, followed by 34 cycles of 94°C for 45 s, annealing at 48°C for 45 s, and 72°C for 60 s, with a final elongation step at 72°C for 6 min. PCR products were purified with a PCR Clean-up DNA purification kit (MoBio Laboratories Inc., Carlsbad, CA, USA) and sequencing was performed using the ABI prism Big Dye Terminator Cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA). Sequencing was carried out on an ABI 3700 DNA analyser and sequences were edited and manually aligned in SEQED version 1.0.3 (Applied Biosystems, Carlsbad, CA, USA).

The COI data were aligned by eye and neighbour-joining (NJ) trees were constructed with the computer program PAUP* version 4.0b10 (Swofford 2002), using HKY85 distances, and *P. macrosturtensis*, *P. mesosturtensis* and *P microsturtensis* sequences (GenBank Accession numbers FJ785739–FJ785750) were included in the analysis. A mid-point root was applied and the NJ tree was bootstrapped with 500 pseudo-replicates carried out using HKY85 distances (Hasegawa *et al.* 1985). Clustering of the larval sequences with *P. macrosturtensis*, *P. mesosturtensis* and *P microsturtensis* monophyletic clades was used to identify them to species level.

Group-specific primer design

Of the potential prey items available, the chiltoniid amphipods and harpacticoid copepods were chosen for the predation study because they are by far the most abundant taxa found in the study aquifer. Copepods and amphipods range in size from 1 to 2 mm and from 2 to 5 mm, respectively, and it is hypothesised that the larger beetles will consume larger prey items. Specific primers were designed to amplify small fragments (100–300 bp) of DNA, to detect the products of digestion. The aim was to be able to screen for presence/absence of taxon-specific PCR products on an agarose gel. Mitochondrial COI sequences from the three diving beetle species, two harpacticoid copepod species and the three chiltoniid amphipod species from Sturt Meadows, (GenBank Accession numbers FJ785739–FJ785750, FJ785766–FJ785772, FJ785785–FJ785789, FJ785801–FJ785805, FJ785806–FJ785808, FJ785815, FJ785816; Bradford *et al.* 2010), plus an epigean chiltoniid amphipod used in the feeding trials, were aligned in CLUSTAL X (Thompson *et al.* 1997). Group-specific primers were designed using the computer software AMPLICON (Jarman 2004) and their details are presented in Table 1. Because of the amount of divergence among species, two sets of primer pairs were designed for both the amphipod and copepod groups.

PCR conditions for amplifying COI (as above) were optimised for individual primer pairs, screening a range of temperatures for the highest possible annealing temperature (T_A) that still maximised the amplicon yield (Table 1). Increasing the PCR cycle number to 40 repeats was found to improve the sensitivity of the assay, without compromising specificity of the primers. Primers were tested for cross-amplification of DNA with other groups from the natural predation assay, including the beetles, and all were found to be specific to the species or group for which they were designed. To check whether the PCR product amplified from the three beetle species was the expected prey item only, *Y. sturtensis* PCR product was sequenced and found to match 100% with *Y. sturtensis* sequence (FJ785887).

Table 1. Details of group-specific primer sequences (F, forward primer; R, reverse primer), plus expected product size (bp) and annealing temperature (T_A, °C)

Target species	Primer name	Primer sequence (5′–3′)	Size	T_A
Yilgarniella sturtensis (Amphipod 1)	M849F	GTGACAGCTCATGCTTTTGT	124	60
	M850R	CCCAACACCACTCTCTACTA		
Scutachiltonia axfordi/ Stygochiltonia bradfordae (Amphipod 2/3)	M950F	GACTTGTTCCTCTAATATTGG	183	55
	M952R	GCACCAGCTAAATGTAAAG		
Copepod 1	M797F	AATAGAACTTGGTCAACCAGGGGGG	285	60
	M798R	GAAAATAGCAAAATCAACTGCGGG		
Copepod 2	M845F	ACAGCCTGGAGGAGGGATTATTAAT	111	60
	M846R	ACATATGTCTGGTGCTCCCAA		
Epigean amphipod	M677F	TTATTCGATCTGAGTTAAGTGCC	134	60
	M678R	ATTACCAAATCCTCCGATCATAAC		

Gut-content assays – feeding trials

Feeding trials were conducted to test the ability of the PCR assay to detect food items in whole-beetle extracts, as well as give an indication of time post-feeding that a prey item can be detected. At the same time, behavioural observations were made on beetles ($n = 30$) that were kept in plastic containers filled with rainwater and wrapped in aluminium foil to keep them in the dark, with a tissue as a substrate and at a constant temperature of 25°C, close to that of the aquifer of 26–28°C (Watts and Humphreys 2006). Observations were made when the beetles were fed every 3–4 weeks over 5 months, with 10 individuals remaining alive at this time point. There was a sufficient number for a feeding trial only for *P. mesosturtensis*, the most abundant of the diving beetles in the calcrete. No diving beetle larvae survived capture for longer than 24 h, because they are soft bodied and appear to be affected by the collecting process. Caution is needed in interpreting the results in relation to the natural-predation study, as the rate of digestion may differ among beetle species and life stages.

Prior to feeding trials, adult beetles were starved for at least 24 h. The beetles were fed on live epigean amphipods (Chiltoniidae; 4–8 mm long) from the Adelaide Hills in South Australia that had been squeezed with forceps to slow them down and encourage feeding. Diving beetles observed to feed were transferred to separate containers filled with rainwater and these were harvested at 0, 6, 24, 48, 72, 96 h post-ingestion, with up to 10 animals per time point. DNA extracts were prepared as above, and all amplified successfully with COI universal primers before being amplified with the amphipod-specific primer pair (M677F–M678R). Epigean amphipod extract was included as a positive control, and *P. mesosturtensis* starved for at least 14 days, with calcrete water and PCR water included as negative controls. PCR products were run on a 1.5% agarose gel, stained with ethidium bromide and visualised under UV light. Each extract was scored for presence/absence of a band at 134 bp.

Gut-content assays – natural-predation study

Forty-eight *P. macrosturtensis* adults and 22 larvae, 45 *P. mesosturtensis* adults and 10 larvae and 53 *P. microsturtensis* adults and four larvae were collected for the field study. DNA extracts were amplified successfully with COI universal primers, then screened with group-specific primers (Amphipod 1, Amphipod 2/3, Copepod 1, Copepod 2; Table 1). Prey target extract (10–100 ng) was included as a positive control, and specific beetle extract (from a leg; 10–100 ng) was included in the negative controls. Presence of target DNA in the gut was identified by a clear band on an agarose gel of the size noted in Table 1.

Carbon and nitrogen isotope analysis

Stable isotope ratio analysis (SIRA) was conducted on 5-mg samples of mulga tree roots from within the aquifer ($n = 4$), chenopod shrub leaves ($n = 3$), and up to 1.5-mg samples of *P. macrosturtensis* ($n = 20$), *P. mesosturtensis* ($n = 8$) and *P. microsturtensis* ($n = 5$) and chiltoniid amphipods ($n = 9$) from Sturt Meadows, snap-frozen in liquid nitrogen. Copepods were excluded because they were too small for the analyser, and amphipods were not separated into species, because at the time when these analyses were carried out, only one species was thought to exist in the calcrete. Owing to the small size of the specimens, the samples were not acid fumed to remove inorganic carbonates that could affect $\delta^{13}C$ readings in crustaceans; however, this is expected to have an insignificant effect on the carbon signature. Cuticle thinning is a common adaptation of subterranean animals (Christiansen 2004) and mineralisation of the cuticle is minimal in highly adapted cave animals such as those studied at Sturt Meadows. Total carbon and nitrogen and the fractionation of $^{15}N/^{14}N$ and $^{13}C/^{12}C$ determinations were made on a modified Europa Roboprep CN Elemental Analyser attached to a Finnigan Mat Conflo III and Finnigan 252 (Environmental Isotopes Pty Ltd, Sydney, Australia). Samples were analysed relative to internal gas standards calibrated using International Atomic Energy Association standards for nitrogen, IAEA-N1 ($\delta^{15}N = 0.43‰$ atmospheric air, AIR) and IAEA-N2 ($\delta^{15}N = 20.41‰$ AIR) (Bohlke and Coplen 1995), and carbon, (NBS-22 ($\delta^{13}C = -30.03‰$ Vienna Pee Dee Belemnite, VPDB) and ANU SUCROSE ($\delta^{13}C = -10.3‰$ VPDB) (Coplen *et al.* 2006). Carbon isotope data are expressed using the δ notation where

$$\delta^{13}C = [(({}^{13}C/{}^{12}C)_{sample}/({}^{13}C/{}^{12}C)_{standard}) - 1] \times 1000$$

Values are reported as per mil (‰) relative to the international standard VPDB, which is defined by the International Atomic Energy Agency standard, IAEA-NBS19 ($\delta^{13}C = +1.95‰$; Coplen 1995). Nitrogen isotope data are expressed in a similar fashion, as follows:

$$\delta^{15}N = [(({}^{15}N/{}^{14}N)_{sample}/({}^{15}N/{}^{14}N)_{standard}) - 1] \times 1000$$

and reported as per mil relative to the AIR scale defined by IAEA standard IAEA-N-1 (0.43‰; Bohlke and Coplen 1995). Errors were estimated using laboratory standards run regularly and standard deviations of better than ±0.15‰ were achieved for C and N isotope values.

Data analyses

Diving beetles with detectable gut content from the natural-predation assay were used to compare whether each of the three species had an equal proportion of amphipod or copepod amplicons. Numbers of each species that had Amphipod 1, Amphipod 2/3, Copepod 1 or Copepod 2 prey types identified were used to create a contingency table. The significance of any differences in proportions was tested with the Fisher exact probability test (Fisher 1954) in the R statistical package (http://www.R-project.org). The Fisher exact probability test is suitable for small sample sizes and where the data are unequally distributed among the cells of the table. The *P*-value is the probability of all frequency tables that is equal or less than the probability of the observed table.

To compare the presence or absence of the four prey PCR amplicons in each individual beetle, Bray–Curtis similarities (Bray and Curtis 1957) were calculated among the samples, and non-metric multi-dimensional scaling (MDS) was used for their ordination in the computer program PRIMER v6 (Clarke and Gorley 2006). Multi-variate data were analysed using the permutational analysis of similarity (ANOSIM; Clarke and

Green 1988) in PRIMER, which is the non-parametric analysis of variance on the basis of the rank similarities. Any differences in trophic niche among species were tested by comparing the observed ANOSIM test statistic, R, which is a measure of separation between groups scaled from 0 to 1, with the simulated distribution (999 permutations) of R under the null hypothesis of no group differences.

Carbon and nitrogen stable isotope ratios of the diving beetle, plant and amphipod samples were normalised, and then a resemblance matrix based on Euclidian distances was calculated in PRIMER. The multivariate data were compared for group differences by ANOSIM in PRIMER. Carbon and nitrogen stable isotope ratios for the invertebrates were also analysed separately using the permutational analysis of variance (PERMANOVA+ for PRIMER; Anderson *et al.* 2008). *P*-values obtained from 9999 unrestricted permutations of the data are a test of the null hypothesis of no group differences. The advantage of the permutation test is that there is no restriction to a balanced number of replicates and no assumptions of constant variance across groups are made.

Results

Feeding trials

Detection times of the amphipod prey were long, with two of four animals positive 96 h after the initial predation event by *P. mesosturtensis*. Number of positives (and number of surviving beetles) varied at each time point; with two of four at 72 h, four of eight at 48 h, four of five at 24 h, five of five at 6 h and six of eight animals detected as positive immediately after feeding was observed. No amphipod DNA was detected in the calcrete water, PCR water or *P. mesosturtensis* specimens that had not been fed, suggesting that the positive PCRs for amphipod prey were unlikely to be the result of contamination.

Field-caught beetle adults and larvae

Amphipod and copepod DNA were PCR-amplified from extracts of each of the three diving beetle species, suggesting that each species feeds on these two groups. There was a greater number of positive samples for *P. macrosturtensis* (83% adults and 82% larvae) than for *P. mesosturtensis* (51% adults and 70% larvae) and *P. microsturtensis* (50% adults and larvae). A test of the hypothesis that Amphipod 1, Amphipod 2/3, Copepod 1 and Copepod 2 amplicons occurred in equal proportions for adults of each species was rejected at the 5% level ($P = 0.03$; Table 2). The hypothesis of equal proportions could not be rejected for the beetle larvae ($P = 0.32$; Table 2), however, they were collected in low numbers and, hence, it is likely that there was insufficient power to reject this hypothesis. The small beetles had a greater proportion of copepod Species 2 in their diet than did the large beetles, which had a greater proportion of amphipods.

Adults of the three beetle species all overlapped in multivariate prey space, when a MDS was carried out on Bray–Curtis similarities (Fig. 2). Detected prey-amplicons differed significantly among species (ANOSIM, $R = 0.1$, $P = 0.009$). Pair-wise comparison showed a significant difference in the detected prey composition of the large species compared with that of the medium species (ANOSIM, $R = 0.09$, $P = 0.032$). The greatest separation was between the large and small beetles (ANOSIM, $R = 0.15$, $P = 0.002$). However, values of the ANOSIM test statistic R close to zero imply that the segregation among species is not large.

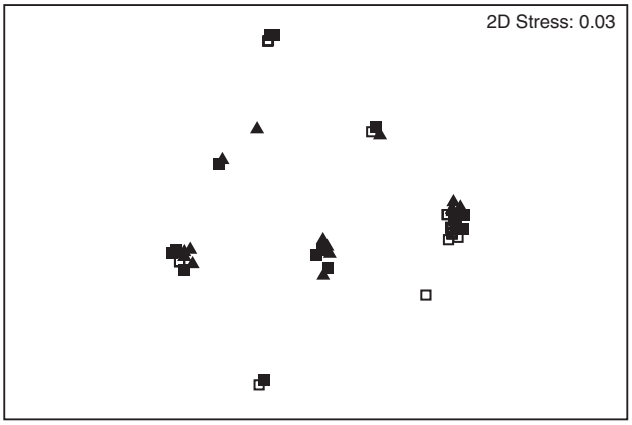

Fig. 2. Diets of the three species of diving beetle. Multi-dimensional scaling plot representing high dimensional prey space. *Paroster macrosturtensis* (solid triangle), *P. mesosturtensis* (open square) and *P. microsturtensis* (solid square).

Table 2. Natural predation study

Proportions of each prey item for the three diving-beetle species. Significance of Fisher exact probability test of independence is shown

Beetle species	Amphipod 1	Amphipod 2/3	Copepod 1	Copepod 2	Total
Adults ($P = 0.03$)					
P. macrosturtensis	33	20	2	3	58
P. mesosturtensis	16	3	2	4	25
P. microsturtensis	15	8	2	9	34
Total	64	31	6	16	117
Larvae ($P = 0.32$)					
P. macrosturtensis	15	10	2	6	33
P. mesosturtensis	4	3	0	1	8
P. microsturtensis	0	0	1	1	2
Total	19	13	3	8	43

Behavioural observations of the diving beetles in captivity

All three diving beetle species were observed coming to the water surface, although they were not observed hanging head-down from the surface film as seen in epigean species that store air under their elytra. Beetles preferred to crawl on the substrate, rather than to swim, but will swim to ascend to the surface and drop down (negatively buoyant) to descend. Only the large beetles were observed completely leaving and re-entering the water by crawling on the container surface, with the smaller species apparently unable to break through the surface tension of water. Loss of ability to control their negative buoyancy during collection and sorting, possibly because bubbles of air were frequently trapped around their abdomen, appeared to be the main cause of death in captive beetles.

Large beetles could catch and feed on the epigean amphipods, which were slightly larger than themselves, whereas medium and small beetle species required these large amphipods to be injured to slow them down, before they could feed. Group feeding of multiple species occurred commonly, with presence of one beetle on a prey item attracting others in the same container. No scavenging on dead amphipods was observed for captive beetles and they did not prey on each other. However, beetles were observed feeding on dead animals during the collection process, when they were in close contact in 5-mL collection tubes. Beetle larvae were observed to prey on live amphipods, copepods and adult beetles in collection tubes.

Stable isotope analysis

There was no clear trophic trajectory (hypothetical amplification of 4 units of $\delta^{15}N$ to two units of $\delta^{13}C$) from plants to crustaceans to the diving beetles (Fig. 3), and no significant separation among groups (ANOSIM, $R = 0.084$, $P = 0.128$). When the stable isotope ratios of only the beetle species and amphipods were compared, the null hypothesis of no group differences could also not be rejected (ANOSIM, $R = 0.041$, $P = 0.291$).

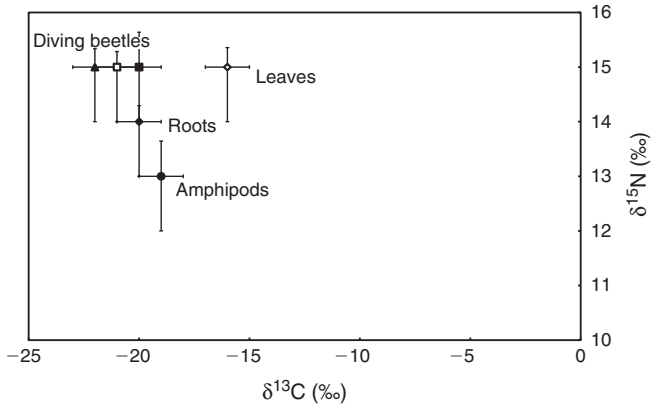

Fig. 3. Stable isotope biplot ($\delta^{13}C$ against $\delta^{15}N$) for stygofauna and plants of the Sturt Meadows calcrete. Leaves were collected from terrestrial vegetation and roots sampled from within the bore holes. Mean (± s.e.) values for amphipods and three species of diving beetle, namely, *Paroster macrosturtensis* (solid triangle), *P. mesosturtensis* (open square) and *P. microsturtensis* (solid square), are also shown.

Plants sampled for leaves from the surface and roots from the bore had very high average values for $\delta^{15}N$ of 15‰ and 14‰, respectively. Amphipods were lighter by 1‰ in average nitrogen isotope ratio than were the plant roots recovered from the bores. The three beetle species had the same average value of $\delta^{15}N$ (15‰), but were shifted towards more a positive $\delta^{15}N$ by 2‰ when compared with the amphipods, which was statistically significant (PERMANOVA, $F = 3.207$, $P = 0.034$).

The $\delta^{13}C$ of plant leaves (−16‰) was less negative than that of unidentified plant roots from the bores ($\delta^{13}C = -20$‰). Complete overlap in carbon ratios was found between amphipods and plants ($\delta^{13}C$‰ = −15 to −21‰). Values of $\delta^{13}C$ were not significantly different among *P. macrosturtensis*, *P. mesosturtensis* and *P microsturtensis*, with the mean $\delta^{13}C$ being −20‰, −21‰ and −22‰, respectively (PERMANOVA, $F = 2.553$, $P = 0.094$). Carbon isotope ratios were significantly more negative for the diving beetles than for the amphipods by 1–2‰ (PERMANOVA, $F = 4.831$, $P = 0.006$).

Discussion

Trophic niches of the Sturt Meadow's diving beetles

Molecular methods have been successfully used in the present study to identify harpacticoid copepods and chiltoniid amphipods as a major component of the prey of diving beetles in the Sturt Meadows calcrete. These species represent the majority of macro-invertebrates present in the calcrete (Allford *et al.* 2008), and behavioural observations suggest that the beetle species do not prey on each other. However, it cannot be ruled out that there are further prey items for the diving beetles, because cylopoid copepods have been observed in collections, but have not amplified with COI primers, and oligochaete worms were not included in the analyses.

Although all three beetle species preyed on the same taxa, there was some evidence for partial trophic-niche partitioning, with trends towards preferential feeding by *P. macrosturtensis* (large) on amphipods, and *P. microsturtensis* (small) on copepod Species 2. These patterns make sense in regard to the size of these predators, with *P. macrosturtensis* being almost twice the size of *P. microsturtensis*. Trends in preferential feeding are of interest because there may be times when copepods (or amphipods) are very abundant, giving beetles that can catch them a selective advantage at these times. There is considerable evidence now for ecological speciation, resulting from divergent natural selection associated with food resources, or adaptation to different ecological niches (Schluter 2001; Rundle and Nosil 2005; Schluter 2009). It is, therefore, possible that the partial trophic differences detected here may have been a driver of diversification into different body sizes, and for cases where calcretes contain sister beetle species, it may have contributed to speciation underground. Further investigation of this hypothesis, however, requires additional analyses of the diet in a wider range of independently evolved subterranean dytiscid species.

We did not discriminate between the two amphipod species, *Sc. axfordii* and *St. bradfordae*, so there is a possibility of additional dietary differences between beetle species. The two amphipod species differ markedly in morphology, with *Sc. axfordii* being comparably larger than and robust compared

with *St. bradfordae*, thus being suitable for larger spaces within the calcrete, whereas *St. bradfordae* may have a preference for interstitial spaces (King *et al.* 2012). It is therefore possible that they themselves occupy separate environmental niches, as has been found in other epigean amphipod species, even when they are similar in morphology (Wellborn and Cothran 2007), and that this segregation could influence predation by the different species of beetle. Because feeding could not be directly observed in this subterranean environment, we could also not distinguish size of the amphipod prey, which vary considerably among life stages.

It is possible that any differences found in trophic niche may reflect the sensitivity of the PCR assay, because the feeding trials suggested that there is the potential for false negatives. Ideally, feeding trials should be conducted on all three beetle species, both adults and larvae, using each of the prey groups tested in the natural predation study (King *et al.* 2008). However, this approach was not possible in the current study because of the difficulty in keeping these stygobiont species alive in captivity. The recent availability of next-generation sequencing technologies means that any future dietary studies can utilise whole extracts of beetles and their gut contents without the need for prey-specific PCR amplifications (Pompanon *et al.* 2012).

The beetle larvae are of particular interest in studying trophic-niche partitioning because they are highly predaceous and the most active feeding stage (Larson *et al.* 2000). Amphipods, and particularly copepods, are highly mobile, and species differences in larval morphology may be adaptive for catching different prey. The *P. macrosturtensis* larvae examined were all larger than the adults and both copepod and amphipod groups were detected as prey items for the larvae as well as the adults. Examination of larval morphology of the Sturt Meadows dytiscids by Alarie *et al.* (2009) found greater broadening of the head capsule than in epigean species, and that the three species differed in relative enlargement of the head capsule with *P. macrosturtensis* > *P. microsturtensis* > *P. mesosturtensis*. Diet of the diving species cannot be directly related to the proportional enlargement of the head capsule, because sufficient numbers of *P. mesosturtensis and P. microsturtensis* larvae were not available for comparison. However, these stygobiont diving beetles are eyeless, and enlargement and broadening of the head capsule are thought to increase the ability to catch prey in a non-visual manner (Alarie *et al.* 2009).

Not all individual dytiscids tested in the natural-predation assay showed evidence for feeding on copepods or amphipods. As stated above, there may be other food items preyed on by these dystiscids, such as cyclopoid copepods or oligochaete worms, which were not detected by our assay. There also may be technical limitations of the assay because of DNA degradation over time. In addition, it is also likely that animals in their natural environment would frequently have an empty gut because of difficulties locating and catching prey items, particularly at times when food is limited. Indeed, there is heterogeneity in species distributions and stygobionts occur at very low densities in some regions of the calcrete (Guzik *et al.* 2009; Bradford *et al.* 2013). Digestion times appear to be long in the diving beetles (>96 h). In other studies, detection times were found to vary from a few hours up to a week in epigean invertebrate predators (Symondson 2002; Sheppard and Harwood 2005). Compared with surface relatives, subterranean species typically have reduced metabolism as an adaptation to the energy-poor subsurface environment (Culver *et al.* 1995), a premise supported by the observation that subterranean diving beetles can live for up to 8 months without food (U'eno 1957).

Trophic ecology of the calcrete

An understanding of the trophic ecology in an enclosed aquifer can be gained by stable isotope analysis, with consumers thought to be slightly enriched in $\delta^{13}C$ (1–2‰) and $\delta^{15}N$ (2–4‰) relative to their food source (Ehleringer *et al.* 1986). However, potential trajectories of trophic increments in stable isotope ratios could not be predicted from our sampling, indicating the presence of other sources of nitrogen and carbon in the calcrete. Stable isotope analysis of the three beetle species found them to be similar in their trophic level. There was a small separation in carbon isotope ratio of 1‰ among the species, although probably not enough to indicate utilisation of different food sources among them, taking into account the spread of the data and differences in sample sizes.

High nitrogen isotope ratios for amphipods and beetles are similar to those seen for other subterranean animals, such as predatory fish ($\delta^{15}N = 12$–14‰; Humphreys 1999, 2001), amphipods ($\delta^{15}N = 13$‰; Pohlman 1997) and atyid shrimps at specific sites ($\delta^{15}N = 10$‰; Humphreys 2001). Amphipods do not appear to be the only food source for these diving beetles because they have less negative carbon isotope ratios, implying that the beetles are heavily utilising another food source, such as copepods.

Chenopod leaves and mulga roots from the Sturt Meadows calcrete had very high nitrogen isotope ratios, potentially as a result of contamination of the groundwater with faeces (Kendall 1998) from the cattle kept on the Sturt Meadows pastoral property. Because there is no indication of amplification of $\delta^{15}N$ from the vegetation through the amphipods and beetles, then the source may lie elsewhere and could result from materials imported with the groundwater flow down the palaeochannel. The carbon isotope ratios of approximately −20‰ for the beetles and amphipods are what would be expected if they were utilising a mix of C3 ($\delta^{13}C = -20$‰ to −35‰, Ehleringer *et al.* 1986) and C4 ($\delta^{13}C = -7$‰ to −15‰, Ehleringer *et al.* 1986) plants as an energy source, and further sampling of the vegetation over a range of time periods is needed in support of this hypothesis.

Increasing the sensitivity of our analyses to include stable isotope determinations of the copepods and any biofilm and detritis within the calcrete would be of great importance in understanding the food web in this system. Subterranean aquatic ecosystems are increasingly being recognised as the complex interaction of hydrogeochemistry and biology (Hancock *et al.* 2009). The nature, origin and processing of energy in aquifer ecosystems is important in understanding how such systems function and how they might respond to perturbations, such as, for example, that an increase in organic matter may permit invasion of surface species into previously oligotrophic groundwater (Malard *et al.* 1996). Because groundwater ecosystems

are perpetually dark, they are dependent for their energy on imported organic matter. Energy in such systems may be derived from chemolithoautotrophy or from photosynthetically derived particulate organic carbon, but is derived predominantly as dissolved organic carbon (DOC) (Simon *et al.* 2003; Culver and Pipan 2009) that reached the water table by downward percolation through the overlying matrix, or transported laterally within the groundwater flow (Culver and Pipan 2009). The potential contribution of chemoautotrophy in the trophic dynamics of the calcrete aquifers has been hypothesised owing to high sulphate and nitrate concentrations and the chemically stratified water column (Humphreys *et al.* 2009). Whatever the energy source, microorganisms (mostly bacteria and fungi) capture the energy, utilising either heterotrophic or chemotrophic pathways and the resulting biofilms, which form the foundation of the subterranean ecosystem, and are grazed by aquatic animals (Burns and Walker 2000; Fenwick *et al.* 2004). Because many amphipods are grazers on biofilms and diving beetles are predaceous, our working hypothesis is that the energy transfer within the calcrete aquifer will be from plants via DOC or roots, to crustaceans to beetles.

Diving beetle behavioural observations

The nature of the closed environment in which subterranean diving beetles live means that there is very little information on their behaviour that could be used to understand how co-existing species interact. Observations on Sturt Meadows diving beetles kept in captivity indicated that they are opportunistic group feeders, as well as scavengers and active predators, as has been found for stygobiontic diving beetles from the northern hemisphere (U'eno 1957; Castro and Delgado 2001). Record of this behaviour is important, because the presence of copepod and/or amphipod DNA in gut-content assays does not distinguish whether these items were alive or dead when ingested (Juen and Traugott 2005). Stygobiont copepods are very fast moving and it remains to be tested whether the beetle species can catch them alive in their subterranean environment.

These diving beetles were seen surfacing, but did not hang at the surface to take on air under the elytra, as seen in epigean diving beetles (Larson *et al.* 2000). It is possible that they can exchange oxygen through the cuticle (Castro and Delgado 2001) or that they are capturing bubbles of air at the surface as well as from their calcrete substrate. Any need to surface will restrict the depths at which diving beetles at Sturt Meadows can live and whereas the medium beetle has been collected in bore holes at depths of >1 m, the large beetle appears to be restricted to the upper 500 mm (Allford *et al.* 2008; S. J. B. Cooper, unpubl. data). It would be informative to see how often the different-sized beetles need to surface and whether this limits the depths at which they can live. As indicated above, the calcrete waters are highly stratified with strong physico-chemical gradients (Humphreys *et al.* 2009) which, combined with increasing anoxia with depth, may be another ecological partitioning mechanism for these species. There is strong evidence from modelling the size distributions of within-aquifer species, that the subterranean beetle communities are organised through the interaction among species rather than fitting into a series of pre-existing environmental niches (Vergnon *et al.* 2013). Evidence for common ecological-niche partitioning, either because of food resource or spatial partitioning/oxygen requirements, within a calcrete is required to demonstrate the importance of ecological-niche partitioning as a driving force in speciation. The resolution of the data on niche partitioning collected so far is limited, owing to the constraints on sampling of a closed habitat that can be accessed only via bore holes. Future work on ecological-niche partitioning should aim for sampling over a vertical as well as a horizontal gradient and an approach that combines multiple molecular methods, behavioural observations and collects information on biophysical properties of the habitat.

Conclusion

There is limited evidence for partial trophic-niche partitioning of the dytiscid beetle species within the Sturt Meadows calcrete, with adult beetles observed to be scavengers and group feeders, as well as active predators. The presence of discrete trophic niches, with different beetle species feeding only on specific prey items in the calcrete, appears unlikely because of the size differences in juvenile and mature amphipods that are preyed on and the observation of multiple species feeding on a single prey item. Energy transfer to amphipods and diving beetles at Sturt Meadows is proposed to be from photosynthetically derived organic carbon, because there was no evidence for chemotrophic activity by microorganisms in the aquifer from the stable isotope ratios of the macro-invertebrates investigated. Studies of trophic ecology are important for investigating any common environmental-niche partitioning in the calcretes that may have led to diversification of the diving beetles underground (Leys *et al.* 2003; Leijs *et al.* 2012). It would be of interest to repeat the trophic study for beetle species in different calcretes, using next-generation sequencing technologies, so as to investigate partial trophic partitioning in the whole diet of the beetles and larvae. Partitioning of species at different depths in the calcrete as a result of differences in oxygen requirement, which may further influence abilities of species to prey on certain food groups, is potentially an additional isolating mechanism related to their biology that could have influenced the size differentiation of beetle species, and is certainly worth further investigation.

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