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Epigean and hypogean *Palaemonetes* sp. (Decapoda, Palaemonidae) from Edwards Aquifer: An examination of trophic structure and metabolism

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Abstract

This study addresses the causes of the metabolic depression observed when examining the metabolism of hypogean versus epigean organisms. We examined the two current hypotheses regarding the cause of metabolic cave adaptation, a paucity of food and low oxygen availability, both necessary for ATP production, by first determining if the hypogean environment examined, Edwards Aquifer, was resource limited. Stable isotope analyses indicate that there is extensive microbial chemolithoautotrophic production providing resources for the hypogean organisms. $\delta^{13}C$ values (≤30‰) were well below that of terrestrial biome indicating that C in the aquifer originates from chemolithoautotrophic inorganic carbon fixation, not photosynthetically derived material resulting from terrigenous sources. Data suggest the artesian system is a complex geochemical ecosystem providing inorganic energy sources from both methane and sulfates. Metabolism, examined via key aerobic and anaerobic proxies, and organismal proximate composition indicated there was no difference between metabolic rates and energy storage of *Palaemonetes antrorum* (stygobitic) and *Palaemonetes kadiakensis* (epigean). This indicates that resources within the oxic aquifer are not limited. We demonstrate that it is necessary for one, or both, of these selective pressures to be present for metabolic cave adaptation to occur.

Keywords
Palaemonetes antrorum, *Palaemontetes kadiakensis*, Edwards Aquifer, stable isotopes, $\delta^{13}C$, $\delta^{15}N$, metabolism, citrate synthase, lactate dehydrogenase

Introduction

Scientists have long been intrigued by the adaptations of subterranean organisms to their stygian environment. Like the deep sea, the subterranean environment remains in total darkness and generally has low energy to sustain life (Poulson 2001). Subterranean environments have been perceived as extreme environments to which it is difficult for organisms to adapt, a view that is not unchallenged (Seibel and Drazen 2007). Research on subterranean life has progressed a great deal over the last several decades, and the research focus has shifted from species adaptation to an ecosystem approach (Gibert et al. 1994). It is now recognized that subterranean ecosystems support substantial diversity of both prokaryotes and eukaryotes (Culver and Sket 2000, Holmes et al. 2001, Humphreys 2008, Guzik et al. 2011) much of which may become imperiled (Van Beynen and Townsend 2005) before they are recognized taxonomically (Niemiller et al. 2013).

Subterranean aquatic environments are considered extreme on several accounts. Such habitats are in continuous total darkness and so lack direct photosynthetic energy input being mostly dependent on a slow flux of allochthonous energy input in the form of organic carbon derived from the surface, else, as is increasingly being reported, in the form of inorganic molecules derived by chemoautotrophy. Additionally, subterranean aquatic systems may be dysoxic or anoxic with regions of toxic hydrogen sulfide. Some systems, such as anchialine systems and the saline parts of the Edwards aquifer, have distinct haloclines which mark the clines conducive to microbial chemolithoautotrophic production (Hutchins et al. 2011). The structural diversity of such systems (Pohlman 2011) and the contained microbial and metazoan communities (Sarbu 2000, Seymour et al. 2007) are still poorly understood although, as noted by Humphreys (1999), they may be sought by analogy with adaptations to anoxic sediments (Fenchel and Finlay 1995).

It is widely recognized that a low metabolic rate is one of the adaptations to the low energy subterranean milieu, one that occurs in both air living and aquatic members of the subterranean fauna. However, Seibel and Drazen (2007) posit that the metabolic rates found in deep sea (and by analogy in subterranean) species are the 'normal' rates but that the higher metabolic rates found in surface species are actually elevated rates, being an adaptation to the faster pace of predator threatened epigean life. This argument cannot be sustained on first principles for subterranean species owing to the polarity in the argument; comprehensive phylogenetic studies routinely support that subterranean species are derived from epigean species (e.g., Leys et al. 2003) with few tentative claims of reversals to surface living (Domes et al. 2007, Kornicker et al. 2010, Prendini et al. 2010).

Regardless of the mechanism that resulted in colonization of the subterranean environment, stygobites tend to have convergent physical and physiological characteristics, termed troglomorphies. These include reduced body size, regressed or absent eyes, enhanced sensilla, loss of pigmentation and reduced metabolic rates when compared to their closest phylogenetic epigean counterparts. Metabolic rates were first measured on troglobitic amphipods (Gal 1903) and numerous studies followed (Poulson 1963, Vandel 1965, Barr 1968, Caine 1978, Culver 1982, Hüppop 1986, Hervant and Mathieu 1995, Gannon et al. 1999, Hervant and Renault 2002, Bishop et al. 2004), yet few researchers reported having found no reduction in metabolic rates in cave populations (Culver and Poulson 1971). The cause of the metabolic depression reported in stygobitic crustaceans and fishes has been a subject of debate for decades. One hypothesis is that depleted environmental oxygen levels limit the rate of ATP production in cave organisms. A comparison of oxygen consumption rates of congener amphipods from both an oxic environment and an anoxic to dysoxic system, resulted in a significantly greater organism mass and metabolic rate in the *Spelaeonicippe buchi* (Andres, 1975) from the oxic conditions of Túnel de la Atlántida in Lanzarote compared to *S. provo* Stock & Vermeulen, 1982 from Bahamian stygobitic systems (Bishop and Iliffe 2009). Additionally, anchialine shrimp, *Barbouria cubensis* (von Martens, 1872), collected from dysoxic to oxic cave systems in the Bahamas demonstrated significantly greater metabolic rates measured as oxygen consumption and activities of key enzymes in the intermediary metabolic pathways than *B. cubensis* collected from an anoxic environment in the Yucatan (Bishop and Iliffe 2012).

Theoretically, low environmental oxygen levels in the cave or subterranean habitat reduces the rate at which food is converted to energy, making the impacts of oxygen availability on the physiology of aquatic organisms extensive but, as mentioned above, metabolic depression can be observed even in oxynormal atmosphere. So, oxygen partial pressure cannot be the only factor leading to reduced metabolic rates in cave organisms. Gannon et al. (1999) found cave crayfish of the genera *Procambarus* Ortmann, 1905 and *Troglocambarus* Hobbs, 1942 had significantly reduced oxygen consumption yet the cave environment ranged from dysoxic to oxic and dissolved oxygen content of the cave did not vary significantly from that of the surface pools outside the cave. In addition, terrestrial subterranean animals also have lower metabolic rates and reduced respiratory surfaces than their epigean relatives although cave atmospheres are rarely very depleted in oxygen (Kuntner et al. 1999).

A second theory is that low food availability in the cave environment favors organisms with lower metabolic requirements. However, Culver and Poulson (1971) found no reduction in metabolic rate in cave populations of *Gammarus minus* Say, 1818 and speculated that energy was not limiting for the population studied and thus would not have impacted the metabolism. It is notable that this species is also widely present in epigean environments.

The hypotheses outlined above have not changed for decades yet our knowledge of groundwater fauna has increased profoundly over the same time period (Gibert 1994, Wilkens et al. 2000, White and Culver 2012). Although some subterranean environments are characterized by a reduced chemical environment and some re-

gions are dysoxic or even anoxic, not all cave systems are resource limited or oxygen depleted. Here, we investigate whether a species of stygobiont shrimp inhabiting a cave that contains abundant dissolved oxygen and adequate energy has a metabolic rate which differs from that found in its epigean relatives, both associated with the Edwards Aquifer, Texas. This study examines the existence of metabolic cave adaptations in stygobitic organisms when the organisms reside in an oxic environment. We examine metabolism, and proximate compositional differences between epigean and stygobiotic *Palaemonetes* sp. residing in Edwards aquifer. Additionally, we examine potential food sources in the subterranean environment, primarily microbial chemolithoautotrophic production with the intent to address the assumption of resource limitation in the stygobitic environment. The unique environment provided by the sample site, Edwards Aquifer, allows us to examine stygobitic and epigean congeners from an oxygenated environment. We posit that if food energy resources are adequate and the oxygen needed for ATP production is not limiting, the metabolism of hypogen organisms should not differ significantly from their epigean relatives. Additionally, we anticipate that stable isotope analyses will indicate that the two closely related species are consuming very different resources.

Edwards Aquifer and *Palaemonetes*

The Edwards Aquifer is formed in marine carbonates of Cretaceous age, ranging from 100 – 230 m in thickness, which were subsequently exposed, eroded by solution (kartsification) and overlaid by further sediments in places forming an artesian aquifer. Extensive faulting in the Edwards Aquifer region resulted in the formation of the Balcones fault zone and subsequent limestone dissolution increased porosity, resulting in large caverns and creating new subterranean habitat. The faulting also altered the ground water movement creating new entry points for freshwater organisms (Longley 1986). During the Pleistocene ice ages and at times of severe drought, the subterranean environment of the aquifer provided constant temperature and suitable environment to sustain subterranean organisms (Holsinger 1992).

Like the extensive thermomineral Movile system in Romania (Sarbu et al. 1996), the Frasassi Caves in Italy (Porter et al. 2009), and more recently, in the Ayyalon cave in Israel (Por 2007), there are indications that Edwards Aquifer may provide chemosynthetic energy sources. A study by Birdwell and Engel (2009) observed persistent signatures of microbial CDOM in the aquifer and questioned the assumed dependence of karst aquifer ecosystems on terrigenous carbon.

The oxic environment (>3 mg L^{-1} O_2) of the Edwards Aquifer supports one of the richest subterranean communities explored to date, with approximately 91 animal species of which 44 species are endemic stygobionts (Longley 1981). An abundant resident of the artesian part of the Edwards aquifer is the Balcones cave shrimp, *Palaemonetes antrorum* Benedict, 1896, whereas the Mississippi grass shrimp, *P. kadiakensis* Rathburn, 1902, is found in the surface pools of the same karstic region.

Palaemonetes Heller,1869 (Decapoda: Palaemonidae) comprise an important part of the temperate and tropical aquatic food webs (Lowe and Provenzano 1990) and about 14 species are known from North America. There has long been poor resolution of the systematic relationships of the group on account of morphological homogeneity and poor character definition (Strenth 1976), but recent molecular analysis indicates that it is paraphyletic with *Palaemon* Weber, 1795 but that those North American species included in the analysis formed a monophyletic clade (Cuesta et al. 2012). From a detailed morphological study, Collins (1998) concluded that the genus is primarily marine and that *P. kadiakensis* is not a recent invader of freshwater from the marine realm. He established that *P. antrorum* is a proper member of the genus *Palaemonetes* but is highly derived from a surface freshwater clade that includes *P. kadiakensis* and is possibly even the sister species of *P. kadiakensis* (Collins 1998, Fig 3.19).

Materials and methods

Specimen collection and sample preparation

Specimens of two species were collected at the Edwards Aquifer Research and Data Center (EARDC), San Marcos, Texas. *Palaemonetes antrorum* were collected from a well discharge pipe (ID 30.4 cm) in a 500 μm mesh net. *Palaemonetes kadiakensis* were collected from the surface pool at the EARDC, using a hand held net. Specimens were stored in a -80°C freezer until they were shipped on dry ice to Penn State University. At the time of collection, the dissolved oxygen ranged from 6.1–6.3 mgL^{-1} in the aquifer and was 8.3 mgL^{-1} in the surface pool. The temperature for both the aquifer and surface pool was 21°C.

Individual specimens were weighed to the nearest milligram (WM). Specimen size permitting, two subsamples of abdominal muscle approximately 0.1 g each were introduced frozen into the homogenizing medium, ice-cold dionized water, at dilutions of 1:10 mass: volume. For small specimens where size did not permit the removal of two subsamples, one set of samples was used for stable isotope analyses and the other was reserved for enzyme activity and proximate compositional analyses. Best efforts were made to obtain a complete suite of sizes for each assay. Tissue samples were homogenized at 0–4°C using a sonic dismembrator.

Stable isotope analyses

Following homogenization, the samples were placed in vials and dried for 72 hours at 60°C. The samples were acid fumed to remove any carbonate and then analyzed to derive $\delta^{13}C$, $\delta^{15}N$, C and N by the Stable Isotope Facility at University of California at Davis following their standard protocols for analysis of solids by a PDZ Europa ANCA-GSL elemental analyzer interfaced to a continuous flow PDZ Europa 20-20isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Standards were interspersed with

the sample runs and give a long term standard deviation of 0.2 ‰ (permil) for ^{13}C and 0.3‰ for ^{15}N. Sulfur and $\delta\ ^{34}S$ were derived by the same facility using a SerCon elemental analyzer and custom cryo-focussing system interfaced to a SerCon 20-22 IRMS (Sercon Ltd., Cheshire, UK). Standards were interspersed with the sample runs and give a long term standard deviation of 0.2 ‰ for $\delta\ ^{34}S$. Laboratory standards were directly calibrated against IAEA S-1, S-2 and S-3 and are reported on the VCDT scale.

Stable isotope data are reported as permil (‰) deviation from a standard:

$$\delta\ (‰) = (Rsa/Rstd\ -1) \times 1000,$$

where R is expressed as the ratio of the heavy to the light isotope, namely, in our case, ^{13}C / ^{12}C, ^{15}N / ^{14}N and ^{34}S / ^{32}S, with the primary standards being respectively Pee Dee Belemnite, atmospheric air, and Canyon Diablo meteorite reported on the VCDT scale.

Metabolism and proximate composition

One issue that arises when measuring the metabolism of organisms is the distinct possibility that laboratory confinement may lead to over estimation of metabolism (Quetin et al. 1994, Ritz 2000). This is of particular concern when the experiments involve the comparison of physiological parameters of organisms from disparate environmental conditions, such as well-lit surface pools and the crevicular subterranean habitat. To reduce the possible artificial introduction of variability between the two species resulting from confinement, we chose to examine the metabolic potential of both species of *Palaemonetes* by measuring the activities of key enzymes of the intermediary metabolism. The activities of these enzymes have been shown to correlate with oxygen consumption rates (Childress and Somero 1979, Hochachka et al. 1988, Thuesen and Childress 1993, Lemos et al. 2003, Seibel 2007). Citrate synthase [CS, EC 4.1.3.7; citrate: oxaloacetate-lyase (CoA-acetylating)] activity was assayed following the methods of Torres and Somero (1988). L-Lactate dehydrogenase (LDH, EC 1.1.1.27; lactate: NAD+ oxidoreductase) activity was assayed in the pyruvate reductase direction also using methods described by Torres and Somero (1988). All enzyme activities were assayed in triplicate on a spectrophotometer at 20 ± 0.1°C. Means of the replicates are reported in µmol of substrate converted to product per minute.

Specimens were also assayed for protein and lipid content following the methods described in Donnelly et al. (1993). Proximate composition is reported as concentration, a percent of wet mass, and is the component's proportion of the organism's total mass.

Statistical analyses

All analyses were conducted with significance at $p< 0.05$. F-tests were used to determine equality of variances. As a result of heteroscedasticity, all statistics on were performed on

log transformed enzyme data but means and standard errors are reported on back-transformed data. Two sample t-tests (two-tailed) were conducted to determine if differences in CS and LDH activities, as well as protein and lipid concentrations, existed between the epigean and hypogean species. All regressions were generated using the least-squares method. A two-tailed Student's t test was used to test for differences between the mass-specific enzyme activities of the two species using log of wet mass and log of the mass-specific enzyme activities. Stable isotope and elemental data were tested for differences using factorial analysis of variance with species as factors (StatView 512+).

Results

Epigean *Palaemonetes kadiakensis* were significantly larger (P =<.001, $\bar{x}$ = 0.261 ± 0.0282 g WM, n = 20) than *Palaemonetes antrorum* collected from with in the aquifer ($\bar{x}$= 0.098± 0.0067 g WM, n = 25), accordant with previous studies in which hypogean and epigean organisms were compared (Caine 1978, Issartel et al. 2005, Bishop and Iliffe 2009).

Stable isotopes

Ten samples each of *P. kadiakensis* and *P. antrorum* were assayed for the various elemental and stable isotope variables. Sampled weights assayed did not differ significantly in any analysis (Table 1). Compared to *P. kadiakensis*, the stygobitic *P. antrorum* had less N and S per sample but a greater ratio of C:N. In addition, the stygobitic species had highly significant lighter values of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ (Table 1) and the scatter and magnitude of these differences is shown in Figure 1.The two outlying $\delta^{13}C$ values denote a different energy source for these individuals and their exclusion emphasizes the magnitude of the differences between the two species' trophic biology. The 16‰ difference in ^{34}S between *P. kadiakensis* and *P. antrorum* (cf 4.8‰ for ^{13}C and 3.4‰ for ^{15}N) provides a high signal-to-noise ratio of the sources for ^{34}S and so this measure will be an especially useful addition to any multiple isotope study of the Edwards Aquifer system, and probably others, as it has also proved useful at the producer level (Connolly et al. 2004).

Enzyme activity and proximate composition

No significant difference was observed between the individual activities of the enzymes of the two *Palaemonetes* species (CS, P = 0.304; LDH, P = 0.076) (Figures 2a and b). Citrate synthase activity for *P. antrorum* ranged from 0.742–6.363 (mean 3.171±0.3517 μmol min^{-1}, n = 23) while the larger *P. kadiakensis* CS activities ranged 1.428–4.264 (mean 2.359±0.1560 μmol min^{-1}, n = 20) (Figure 2a). Stygobitic

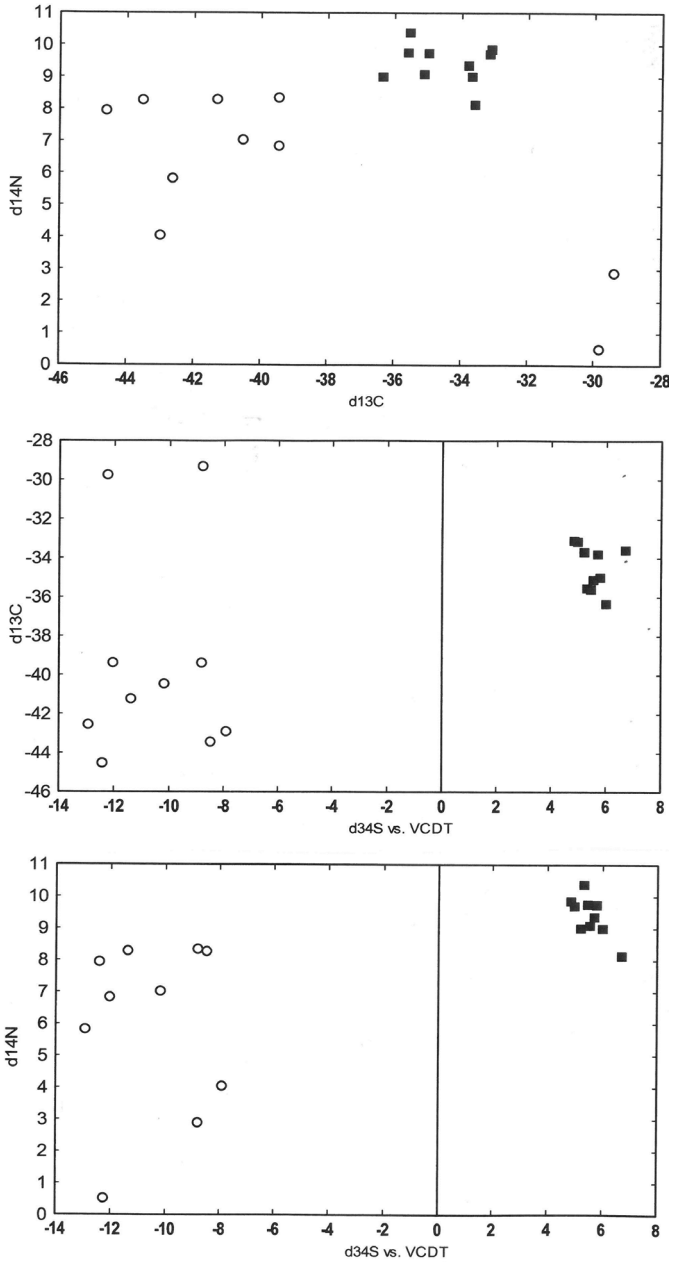

Figure 1. Stable isotope ratio plots, upper to lower: a)$\delta^{15}N$ on $\delta^{13}C$; b) $\delta^{13}C$ on $\delta^{34}S$; c) $\delta^{15}N$ on $\delta^{34}S$. Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).

organisms demonstrated only a slightly greater range of LDH activity than observed in the epigean organisms (Figure 2b). Mean LDH activities were 0.642±0.0392 μmol min $^{-1}$ and 0.632±0.0390 μmol min $^{-1}$ for *P. antrorum* and *P. kadiakensis*, respectively.

Table 1. Mean and variation in stable isotope and elemental statistics for *Palaemonetes kadiakensis* (epigean) and *P. antrorum* (stygobiont). N=10 in each case.

	$\delta^{13}C$	C (µg)	$\delta^{15}N$	N (µg)	Sample (µg)	C : N	$\delta^{34}S$ vs. VCDT	S (µg)	Sample (µg)	%S
P. kadiakensis										
Mean	-34.49	248.7	9.41	73.30	0.60	3.41	5.56	7.29	720.8	1.009
St. dev	1.16	34.4	0.63	11.37	0.02	0.12	0.54	1.56	118.8	0.117
P. antrorum										
Mean	-39.33	245.7	5.97	42.12	0.60	5.96	-10.48	4.30	582.5	0.805
St. dev.	5.42	40.2	2.69	5.00	0.01	1.46	1.90	1.91	264.7	0.199
P. kadiakensis vs *P. antrorum*										
$F_{s\ 1,18}$	7.658	0.033	15.463	62.982	0.038	30.473	661.9	14.686	2.976	7.768
P	0.0127	>0.05	0.001	0.0001	>0.05	0.0001	0.0001	0.0012	>0.05	0.0122

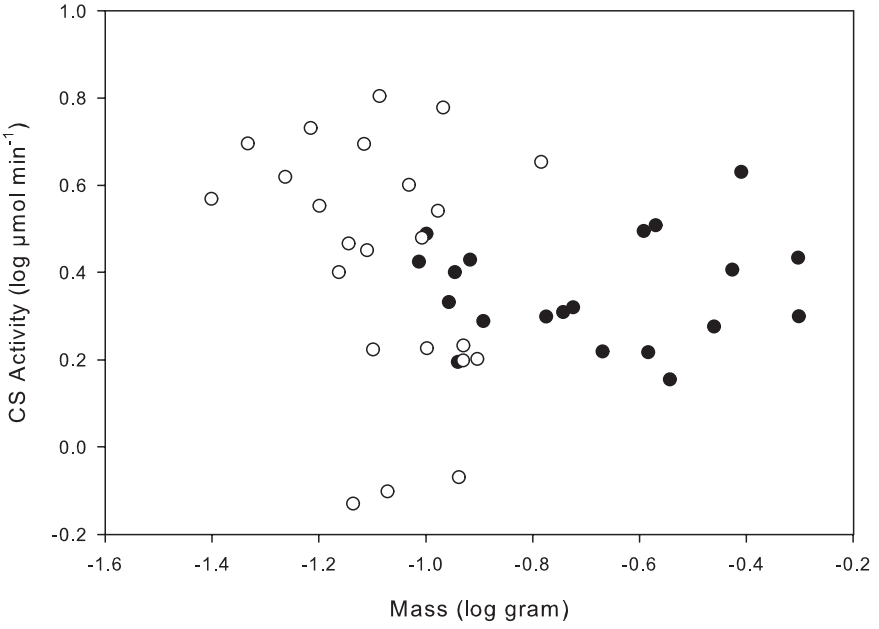

Figure 2a. Individual CS activities (log µmol min^{-1}) on Mass (log gram). Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).

As can be observed in Figure 2, neither *P. antrorum* nor *P. kadiakensis*, demonstrated a significant increase in enzyme activity (µmol substrate converted to product min^{-1}) with increasing individual mass (CS, P = 0.304; LDH, P = 0.076. However, when mass- specific (µmol min^{-1} g WM^{-1}) enzyme activities were examined, there was a significant difference in the slopes of the least squares regressions. The slope of

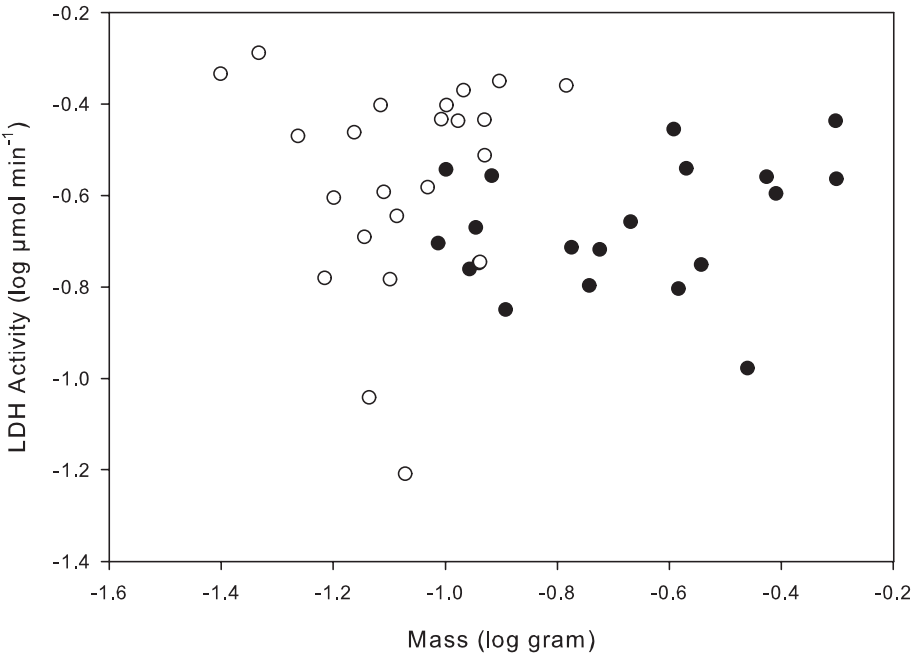

Figure 2b. Individual LDH activities (log µmol min^{-1}) on Mass (log gram). Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).

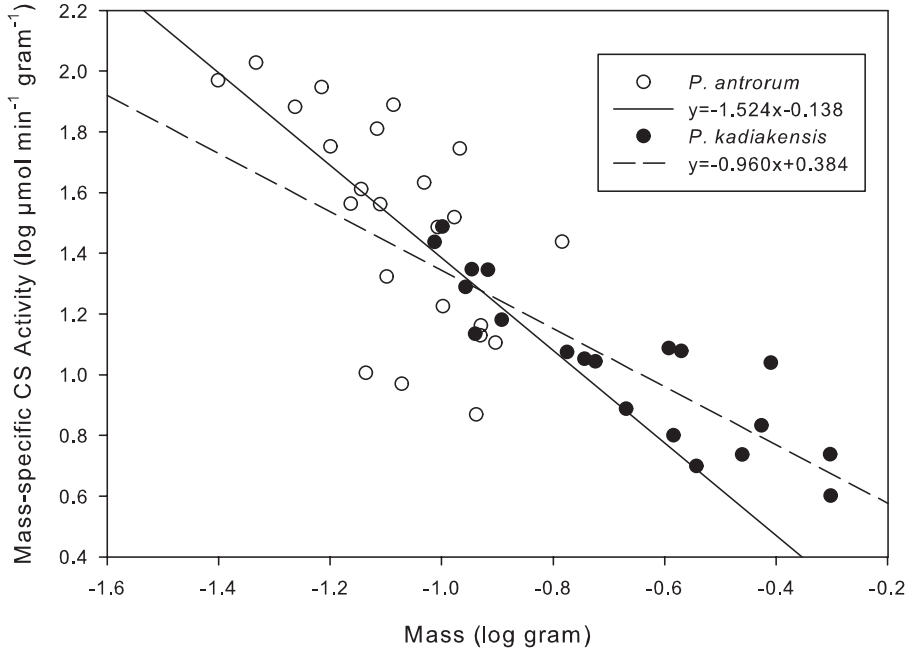

Figure 3a. Mass-specific CS activities (µmol min^{-1} g^{-1}) on Mass (log gram). Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).

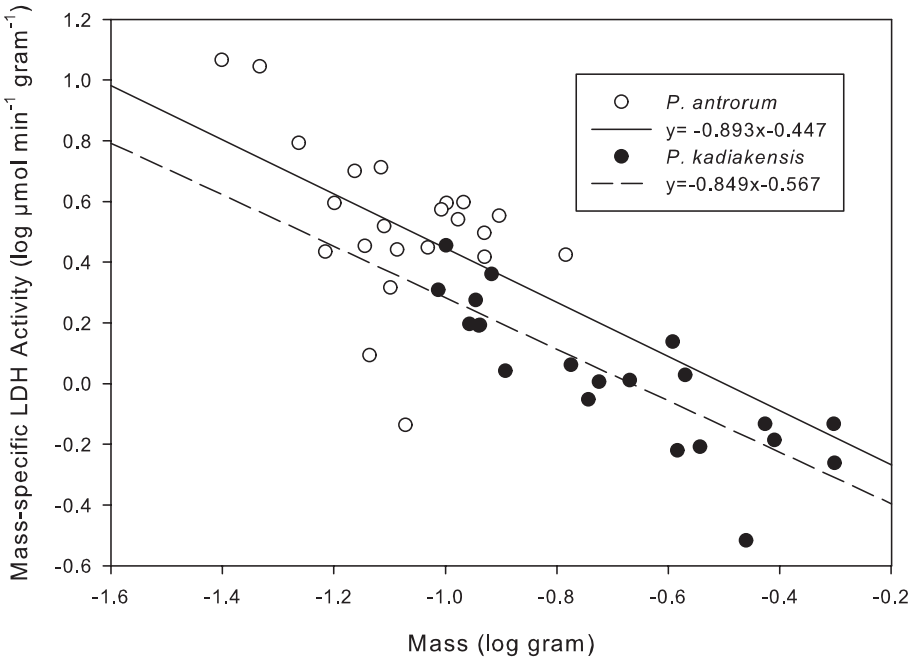

Figure 3b. Mass-specific LDH activities (log µmol min^{-1} g^{-1}) on Mass (log gram). Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).

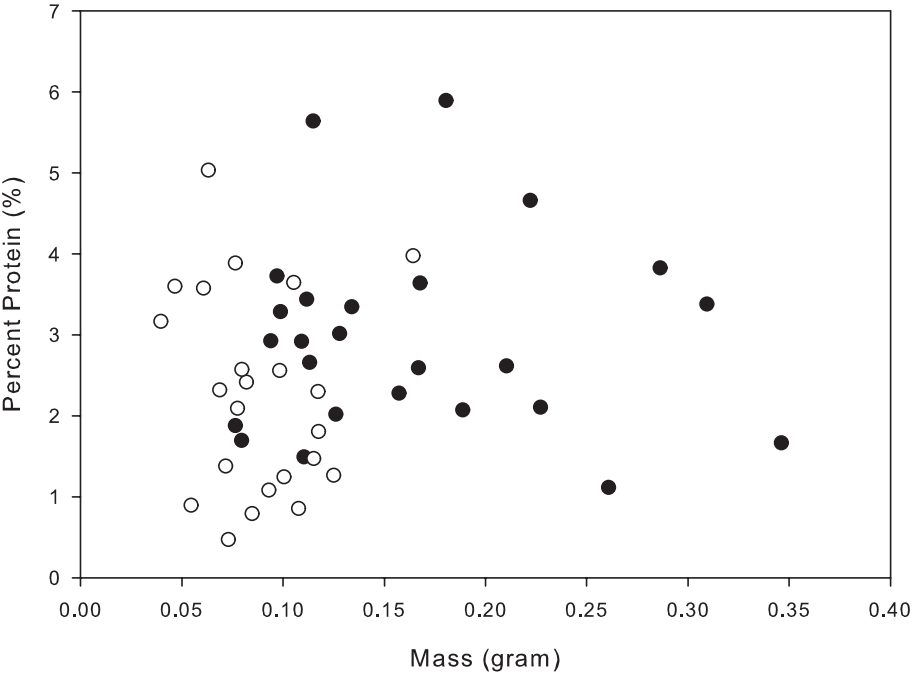

Figure 4a. Percent protein (%) on Mass (gram). Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).

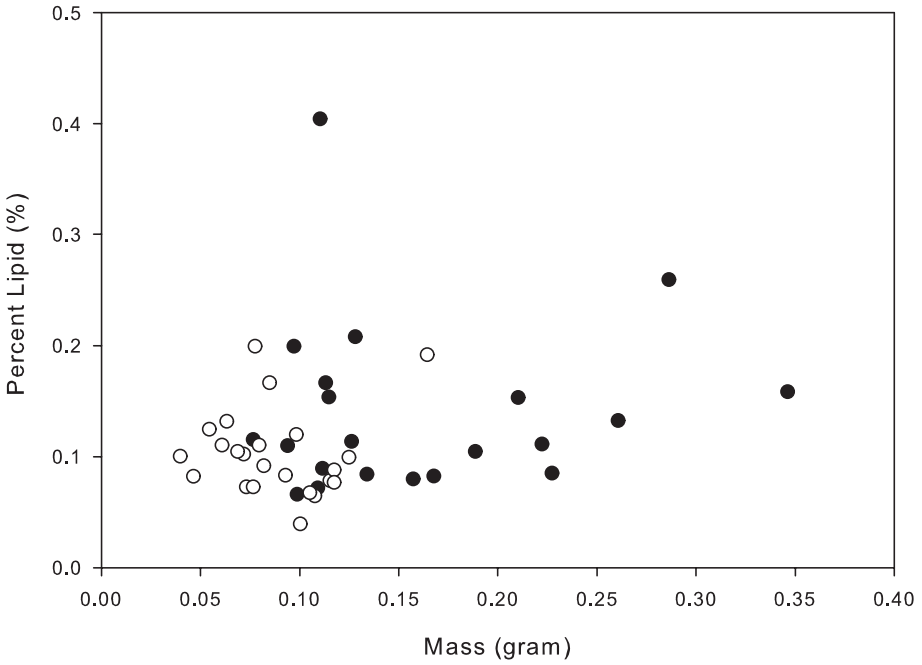

Figure 4b. Percent lipid (%) on Mass (gram). Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).

mass-specific CS activity on mass for *P. antrorum* (y = -1.524x - 0.138, n = 20, R^2 = 0.41) was significantly greater (Student's t, $t_{0.05(2),\ 39}$ = 2.023, |t| = 7.054) than for *P. kadiakensis* (y = -0.960x + 0.384, n = 23, R^2 = 0.78). Figures 3a and b show the log transformed data with regression data. A corresponding difference was not observed in LDH activities, where the slope and intercept of the two mass-specific regressions did not differ significantly between the two species (*P. antrorum*: y = -0.893x–0.447, n = 20, R^2 = 0.25; *P. kadiakensis*: y = -0.849x - 0.567, n = 20, R^2 = 0.70).

As with individual enzyme activities, no significant difference was observed between the epigean and hypogean protein or lipid concentrations (Figure 4a and b) although both were slightly higher in the epigean species. Mean protein concentration for *P. kadiakensis* was 2.953%±0.1403% and for *P. antrorum* was 2.276%±0.2591; P = 0.061. Mean lipid concentration was 0.14%±0.0171% and 0.103±0.0082%; P = 0.052 for *P. kadiakensis* and *P. antrorum*, respectively.

Discussion

The $\delta^{13}C$ and $\delta^{15}N$ values for *Palaemonetes kadiakensis* (epigean) were well grouped whereas the values for *P. antrorum* (stygobitic) has a tight group plus two outliers indicating two distinct food sources. The outliers denote a principally terrestrial input of C from C3 photosynthetic source with $\delta^{13}C$ values similar to surface aquatic amphipods

reported in the vicinity of Movile Cave (Sarbu 2000) but with much lower $\delta^{15}N$ values (ca 5 vs 10‰). The main cluster of *P. antrorum* data showed exceptionally light $\delta^{13}C$ values, mean $\delta^{13}C$ -41.78‰; as $\delta^{13}C$ values ≤30‰ are well below that of terrestrial biome, demonstrating that photosynthetically derived material is not generally important in the artesian ecosystem and that C in the ecosystem originates from chemolithoautotrophic inorganic carbon fixation (Engel et al. 2004). Values for *P. antrorum* are lighter than those recorded in Lower Kane Cave, Wyoming, a cave formed by sulfuric acid speleogenesis (Engel et al. 2004), or in the sulphidic based system in Frasassi Cave, Italy (Sarbu et al. 2000).The values are also lighter than those reported for any fauna by Pohlman et al. (2000) for Mayan Blue, an anchialine cave where the fauna were utilizing particulate organic matter derived from nitrifying bacteria which is expected to have a $\delta^{13}C$ = -45 to -35‰. They argued this would account for the unusually light $\delta^{13}C$ values measured in *Tulumella unidens* Bowman & Iliffe, 1988 (Thermosbaenacea) and *Typhlatya mitchelli* Hobbs & Hobbs, 1976 (Atyidae), mean $\delta^{13}C$ =-34.4 and -36.1 respectively, both biofilm grazers.

Only in Movile Cave, where methanotrophic and chemoautotrophic bacteria provide the basis for cave life, did the cave fauna show $\delta^{13}C$ values (mean $\delta^{13}C$ ca -42‰: Fig 17.10: Sarbu 2000) comparable to those of *P. antrorum* ($\delta^{13}C$ = -41.78, s.d. = 1.92‰). This suggests that the energy source for the artesian population in the Edwards Aquifer is also based on methane which has a $\delta^{13}C$ -60‰ as indicated in Fig. 5 which shows the $\delta^{13}C$ and $\delta^{15}N$ stable isotope data from the Edwards Aquifer together with the range of $\delta^{13}C$ values typical obtained from different energy sources. Figures depicting a succinct synopsis of the stable isotope structure of the fauna in the various chemoautotrophic cave ecosystems are provided by Pohlman (2011).

Opsahl and Chanton (2006) examined the ^{13}C, ^{14}C and ^{15}N isotopic composition of *Cambarus cryptodytes* Hobbs, 1941 from caves and bores in the Upper Floridan aquifer and concluded that in the deep aquifer—those parts of the aquifer remote from photosynthetic carbon sources—the cave crayfish lived at least partly on a methane based bacterial chemosynthetic pathway with the methane being derived from surface wetlands, but they found no difference in $\delta^{15}N$ between spring and bore living individuals. Note, however, that they found wide variation between samples and that the mean $\delta^{13}C$ value for the bore samples was about the same as for our spring samples for *P. kadiakensis* ($\delta^{13}C$ 34.7 vs 34.5‰), a value similar to that for the atyd shrimp *Typhlatya mitchelli* found in anchialine chemotrophic systems (Pohlman et al. 2000). The much lighter $\delta^{13}C$ values for *P. antrorum* and the large difference in $\delta^{15}N$ between spring (*P. kadiakensis*) and bore (*P. antrorum*) samples indicates that there is not a close coupling of surface and artesian systems as would be expected as the presence of an artesian system indicates the presence of an aquitard, which would intercept downward movement of surface derived carbon.

The $\delta^{15}N$ values for both *Palaemonetes* species are positive in marked contrast to the light (negative) values seen in samples taken from sulphidic caves in Romania and Italy (Sarbu 2000, Sarbu et al. 2000). C:N ratios for *P. kadiakensis* are comparable to those seen in marine crabs (Harms et al. 1994) whereas the ratio in *P. antrorum* (C:N

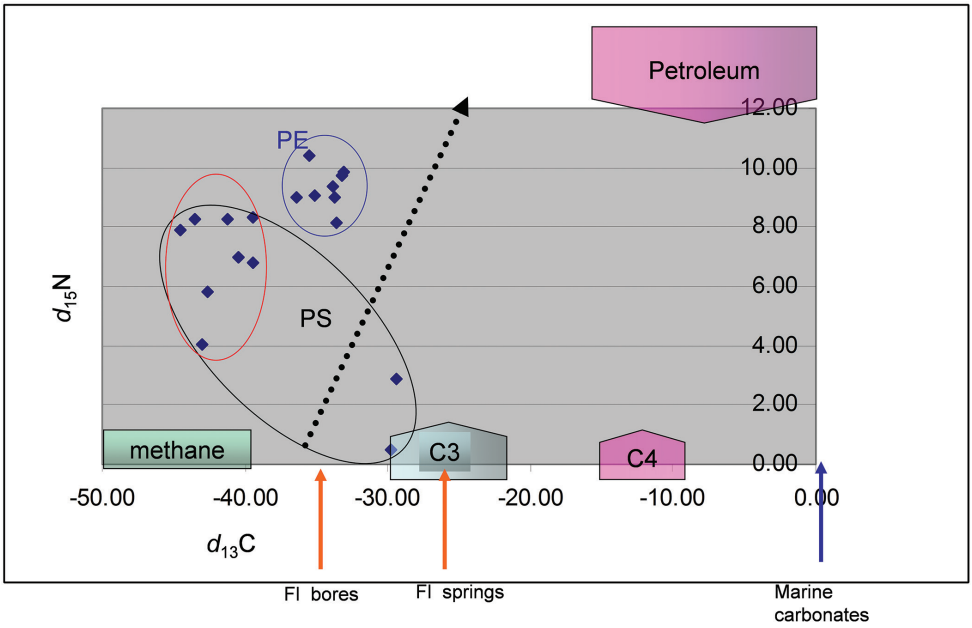

Figure 5. Plot of $\delta^{15}N$ on $\delta^{13}C$ values for the stygobiont *Palaemonetes antrorum* (PS, red oval and black oval respectively excluding and including two outliers, see text) and the epigean *P. kadiakensis* (PE, small blue oval). The range of values of $\delta^{13}C$ derived from different energy sources (see text) is indicated (methane, and photosynthesis from C3 and C4 plants) as well as values typical of marine carbonates and petroleum. The diagonal dotted line denotes a typical trajectory (not the value) of amplification of $\delta^{15}N$ values with progression through trophic levels (Vanderklift and Ponsard 2003).

5.96) is comparable to that found in the fauna of the sulphidic Movile Cave (C:N 5.7. Sarbu 2000).

The clearest isotopic separation between *P. kadiakensis* and *P. antrorum* is seen in $\delta^{34}S$ respectively +5.56 and -10.48. The strongly negative $\delta^{34}S$ values for *P. antrorum* is similar to the bivalve *Pillucina pisidium* (Dunker, 1860) in a *Zostera marina* community which harbours chemoautotrophic bacterial symbionts (Kharlamenko et al. 2001) but it is much more depleted in $\delta^{13}C$ (about -39 vs -28‰). However, the significance of this marked difference is unclear because the $\delta^{34}S$ values of microbial mats (biofilm) will reflect the $\delta^{34}S$ values of the waters from the two sources because if sulfur-oxidizing bacteria are involved they exhibit negligible sulfur isotope fractionation during the transformation of sulfide to elemental sulfur and elemental sulfur to sulfate (Toran and Harris 1989). Sulfur isotope compositions confirmed that sulfur content and sulfur speciation may not correlate to microbial metabolic processes in natural samples, thereby complicating the interpretation of sulfur records both modern and ancient (Engel et al. 2007). Kinkle and Kane's (2000) call for increased attention to be paid to the microbiology of subterranean systems has been amply justified by the subsequent investigations.

Marine waters currently have values $\delta^{34}S$ about +21‰ although they were substantially lower (17.5‰) in the Paleocene (Paytan et al. 1998). Biologically driven sulfate reduction depletes the value by -18‰ and repeated metabolic cycles can deplete $\delta^{34}S$ to -50‰ (Schlesinger 1997).

The two species of *Palaemonetes* we studied have light $\delta^{34}S$ values but those for *P. antrorum* at $\delta^{34}S$ -10.48‰ are exceptionally light compared with values reported for hydrothermal vent and seep species compiled by Conway et al. (1994) which mostly lie between $\delta^{34}S$ -5 and 0‰. Even the iconic hydrothermal vent vestimentiferan, *Riftia pachyptila* Jones, 1981, was in this category ($\delta^{34}S$ -4.7 to +4.7‰: Fry et al. 1983a) and the lightest $\delta^{34}S$ for any vent arthropod was -0.8 to -0.1‰ in a Galapagos hydrothermal vent crab *Bythograea thermydon* Williams, 1990 (Decapoda) (Fry et al. 1983b). The only values comparable to those we report for *P. antrorum* were from the lucinid clam *Pseudomiltha* sp. (Mollusca) from a Louisiana hydrocarbon seep with $\delta^{34}S$ values between -11.5 to +1.3‰ (Brooks et al. 1987) and which contain endosymbiotic chemosynthetic bacteria (Schweinemanns and Felback 1985).

The consumer assimilation effects as organic matter moves through the food chain are small for $\delta^{34}S$ in contrast to the effects on $\delta^{13}C$ and $\delta^{15}N$. The predominant biological process affecting $\delta^{34}S$ of sulphur containing compounds is dissimilatory sulfate reduction by bacteria (Conway et al. 1989, 1994).

Vetter and Fry (1998) found that thiotrophic animals relying on microbially derived reduced sulfur compounds in anoxic sediments had $\delta^{34}S$ from -30 to -20‰, whereas those relying on sulphides from seeps or hydrothermal vents had higher values of $\delta^{34}S$ -10 to +7, which nearly encompasses the values for both species of *Palaemonetes*. Animals relying on methane oxidation for their energy production may also have had low $\delta^{34}S$ from +8 to +10‰, indicating some reliance on assimilatory uptake of inorganic sulfur from seep fluids for the biosynthesis of compounds containing organic sulfur (Vetter and Fry 1998).

For *P. antrorum,* the exceptionally low $\delta^{13}C$ values coupled with low $\delta^{34}S$ suggest the artesian system is a complex geochemical system providing inorganic energy sources from both methane and sulfates, as is to be expected in a petroleum driven system. Natural oil seeps do occur along the Balcones fault zone and the hydrocarbons and oil-field brines have a major influence on the geochemistry of the 'bad water' zone in the central part of the Edwards Aquifer (Sharp and Banner 1997).

The objective of this research was to explore trophic structure, metabolism, and proximate compositional differences between the epigean *P. kadiakensis* and stygobiotic, *P. antrorum,* to address the hypotheses as to the presence and causes of metabolic adaptation to the subterranean environment. We discovered that metabolic depression was not evident in key enzymes involved in the aerobic and anaerobic metabolism of the two species, although previous studies examining oxygen consumption rates and enzyme activities in stygobitic organisms did observe a reduction in metabolic rates both as enzyme activities and respiration rates (Bishop et al. 2004, Bishop and Iliffe 2009, 2012). Although unexpected, our results are not novel. Previous studies comparing epigean and hypogean metabolic measurements, as discussed below, have

resulted in comparable rates between species, rates with no correlation with mass and even, individual rates that decline with increasing mass.

Members of the genera *Cambarus*, *Procambarus* and *Troglocambarus* are benthic dwelling crayfish from epigean and hypogean habitats. Caine (1978) examined the oxygen consumption of four species of epigean and three species of hypogean crayfish. Two of the four epigean species examined did not have significantly higher oxygen consumption rates than those observed in the hypogean *Procambarus* spp. Gannon et al. (1999), also examined cave crayfish of the genera *Procambarus* and *Troglocambarus* using Wheatly's (1989) data on the epigean crayfish *Pacifastacus leniusculus* (Dana, 1852) for comparison, and found the individual oxygen uptake rate (O_2 μmol min^{-1}) was indeed greater for the much larger epigean specimens (mean 0.291+0.407 μmol min^{-1}, range 0.002-1.309 μmol min^{-1} for specimens ranging from 0.03 – 105.4 g) but that respiration rates for the smaller hypogean species (0.18 – 3.29 g) remained within the range of the rates determined for the epigean organisms.

Crustaceans have been shown to reduce their metabolism while overwintering, potentially as a mechanism to function in a food poor environment (Quetin and Ross 1991, Meyer et al. 2002), therefore resulting in a lack of correlation of metabolism with increasing mass. Since specimens were collected in late spring early summer when food is abundant, it is unlikely that the epigean shrimp we used were conserving energy by reducing their metabolism.

A negative relationship between respiration rate and body mass was observed in *Amblyopsis rosae* (Eigenmann, 1898), the Ozark cavefish, by Adams and Johnson (2001). When examining oxygen consumption throughout the year, a positive correlation between oxygen consumption and mass was observed in spring and summer but during fall and winter months there was a negative relationship. The authors attributed this result to changes in environmental conditions within the cave, particularly as a result of food availability and the presence of a bat colony in the cave from April to October.

Lack of correlation of enzyme activity with increasing mass has also been observed in crustaceans. Wilhelm et al. (2006) when examining oxygen consumption rate as a function of mass, found that the stygobitic amphipod *Gammarus acherondytes* Hubricht & Mackins, 1940 showed no increase in individual oxygen consumption with increasing mass. The authors speculated that the higher rates observed in the smaller, and therefore possibly younger amphipods was an ontogenic adaptation, providing the young amphipods with a greater ability to convert available food to energy. Activities then did not increase with increasing mass as a mechanism for the larger amphipods to conserve energy during times of low food availability. This conclusion is supported by the mass-specific enzyme activity data presented in this study. Mass-specific aerobic enzyme activities of the very small *P. antrorum* were greater than observed in small *P. kadiakensis* and then decreased to levels below the epigean species at greater sizes. The mass-specific glycolytic enzyme activities for both species were not significantly different since neither species was exposed to an oxygen limiting environment.

It is possible that metabolic potential may be uncoupled from oxygen consumption during function at normoxic conditions. The strategy of maintaining high metabolic

potential while reducing oxygen consumption would provide the hypogean organism the metabolic machinery necessary to best utilize resources when available. Selective pressures would favor a reduced oxygen consumption rate while maintaining a high metabolic potential. This is the situation observed by Simčič et al. (2005) when examining electron transport system (ETS) activity and oxygen consumption of hypogean and epigean amphipods between caves and two surface locations. ETS activities were found not to differ between animals from different locations, or between epigean and hypogean amphipods yet oxygen consumption was lower in the hypogean organisms.

Examination of the ratio of an organism's maximum aerobic potential to anaerobic potential (CS:LDH) can indicate the degree of evolutionary adaptation to environmental conditions (Childress and Somero 1990). If the ratio is >1, the organism is considered to be aerobically poised while values <1 can indicate an organism that is exposed to anaerobic conditions and must rely heavily on glycolysis. *Barbouria cubensis* collected from anchialine cave systems in the Bahamas and the Yucatan (Bishop and Iliffe 2012) reveals that metabolic potential may vary somewhat according to environmental parameters. *Barbouria cubensis* were anaerobically poised while the *Palaemonetes* in our study were all aerobically poised as anticipated since both the surface pools and the aquifer at the site of collection were both oxic.

Hervant and Renault (2002) and Ritar et al. (2003) examined prolonged fasting and utilization of energy reserves in crustaceans. Both studies showed that protein and lipids were utilized in a series of three successive phases but there was disagreement on the order of protein versus lipid utilization. Hervant and Renault (2002) state the order of utilization was 1) depletion of arginine phosphate and glycogen, 2) use of triglycerides, and 3) depletion of proteins and lipids. This strategy selects for the ability to prolong survival and resulting competitive abilities by depleting protein, i.e. muscle, as the final resort (Sánchez-Paz et al. 2006). Ritar et al. (2003) also propose three phases but the first stage of starvation is the utilization of energy rich lipid reserves, followed by protein and the final stages of starvation results in the utilization of structural lipids. Either strategy would provide energy rich reserves to be utilized should resources become limiting.

Proximate composition, in the form of lipid and protein, was not significantly different between the hypogen and epigean *Palaemonetes* sp. In fact, when compared to other epigean crustaceans, the protein and lipid concentrations for both *Palaemonetes* were within published ranges for crustaceans (Childress and Nygaard 1974, Torres et al. 1994, Bishop et al. 2004), albeit on the lower end of the ranges for both species. Previous studies comparing proximate composition of stygobitic organisms, *Barbouria cubensis,* from environments with differing oxygen concentrations showed no significant difference between two populations collected from oxic and dysoxic environments (Bishop and Iliffe 2012). Although our stable isotope analyses indicated that there was a significant difference in the food being consumed by the epigean and hypogean species of *Palaemonetes*, that difference was not translated into significant proximate compositional differences despite the large differences in C:N ratio and S content, the latter

being lower in *P. antrorum* and C:N greater owing to significantly lower N values in *P. antrorum*. We interpret this result to indicate that food resources within the aquifer were not limited. It is possible that the storage of lipids in crustaceans is a constructive feature enabling the survival of the population, but which is not necessary if resources are constantly available, as is the case with the *Palaemonetes* studied here, and therefore not selected for in the population of either the epigean or stygobitic species.

We shall now return to the initial question posed. In a cave environment containing abundant dissolved oxygen and adequate energy, will the metabolic rate of cave adapted species differ from their epigean relatives? We have demonstrated through stable isotope analyses that the aquifer is not resource limited and by comparing the proximate composition of the hypogean organisms to the epigean, we can see that the protein and lipid levels are not different, further supporting that resources are not limiting. Based upon enzyme activities, the maximum aerobic potential, or the greatest rate at which an organism can convert food into energy, and the maximum glycolytic potential, providing information on an organism's ability to function in an anaerobic environment, there was no difference in the metabolism of the two species of *Palaemonetes*. We did not observe a depressed metabolism in the stygobitic organisms indicating metabolic cave adaptation. But why were the metabolic rates of the stygobitic *Palaemonetes* not lower than in the epigean species? Why was the characteristic metabolic depression found in many cave organisms not observed in this situation? We have addressed two of the current hypotheses regarding the cause of lower metabolism in cave organisms, namely, the limitation of either/or oxygen and food. Metabolic cave adaptation is a constructive feature affected by selection; therefore, the intensity of selection may be responsible for the level of reduction of a characteristic (Hüppop 1986). Mitchell (1969) pointed out that in caves with a relatively high energy input, such as in tropical systems, where the biomass in the tropical epigean is great and production is uninterrupted, the selection pressures leading to cave adaptation could be expected to not be as strong. Because both food and oxygen are abundant in the Edwards Aquifer, the selection for a reduced metabolism is depressed. For metabolic cave adaptation to occur, selective pressures, either in the form of reduced food supply or low oxygen availability, or as in some stygobiont environments, both, must be present to exert directional selection to lower the metabolic rate in subterranean organisms.

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