

ABSTRACT

Title of Document: HOW THE AVAILABILITY OF NUTRIENTS
AND ENERGY INFLUENCE THE
BIODIVERSITY OF CAVE ECOSYSTEMS

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Resource constraints can affect species on multiple levels. In this dissertation, I combine laboratory experiments, an ecosystem-level manipulation experiment and statistical modeling to examine how resources maintain and constrain cave biodiversity and structure cave communities.

Chapter I examines how N-limitation may drive morphological adaptations of cave arthropods. By analyzing free amino acid contents, I show that, in comparison to cave-transient millipedes, cave-obligates have decreased concentrations of essential, nonessential and N-rich amino acids, and amino acids associated with pigmentation and cuticular development. **Chapter II** tests the hypothesis that stoichiometric mismatches impose growth constraints on cave animals. Although results show that cave resource quality is similar to surface leaves, I do show that millipedes experience a strong mismatch to their food. Also, cave-obligate millipedes have lower %P and RNA/DNA (protein synthetic capacity) compared to cave-

transient millipedes. Results from these chapters suggest that cave adaptations may reflect stoichiometric challenges of caves.

Chapter III describes the manipulation experiment, wherein I removed all organic material from 12 caves, and, while excluding all natural subsidies, I added standardized quantities of leaf packs or rodent carcasses. For 23 months, I monitored the recipient communities to see how subsidies influence species abundance, diversity, and community dynamics. I observed 19,866 arthropods representing 102 morphospecies. Rat treatments supported greater abundances, but the treatments did not differ in richness. Multiple community-level analyses demonstrated that community composition differed drastically depending on treatment. Lastly, the communities changed directionally over time, diverging faster in caves receiving leaves.

Chapter IV uses annual bioinventories of 65 caves to investigate occupancy patterns of terrestrial invertebrates. I estimated richness using classical estimators in concert with estimators that incorporate detection. I also used multispecies occupancy models to examine relationships between estimated richness and physical cave characteristics; demonstrating the importance of cave length, entrance geometry (a surrogate for energy input), and connectivity. The results show how inventory data, even if incomplete, can provide valuable information about the distribution of rare species.

Resource availability can affect cave ecosystems on multiple levels. Here I illustrate how the biochemical composition, community dynamics, and occupancy patterns of cave species are influenced by resource constraints.

HOW THE AVAILABILITY OF NUTRIENTS AND ENERGY INFLUENCE THE
BIODIVERSITY OF CAVE ECOSYSTEMS

By

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Dr. Barbara Thorne

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Introduction

Spatial resource subsidies can greatly affect the composition and dynamics of recipient communities. While aquatic subsidies to terrestrial habitats, terrestrial subsidies to aquatic habitats and aquatic subsidies to aquatic habitats have received previous attention, little is known about direct terrestrial subsidies to terrestrial habitats where primary productivity is absent. Caves represent one such habitat. Due to the absence of light, there is no primary productivity within caves. Thus, the animals that reside within these ecosystems are dependent upon on allochthonous resources (i.e. food derived from the surface), such as decomposing plant material, wood, or eggs, feces, and decaying bodies of animal visitors. Through my research, I found that although there were many skeletal remains, most of the 1.5 tons (wet weight) of material that was removed from 12 caves consisted of nutrient-poor plant material. The stress of nutrient limitation may affect cave species on multiple levels. My dissertation is comprised of four chapters that investigate this stress using biochemical, empirical, and statistical methods to test hypotheses regarding how nutrient and energy availability influence the biodiversity of cave ecosystems.

In the first two chapters, I investigate how cave-adapted morphological characteristics and life-history strategies may have evolved in response to nutrient limitation. In comparison to surface-dwelling animals, cave invertebrates are depigmented, possess thin cuticles, and have slow growth rates. Chapters 1 and 2 examine how these adaptations may be linked to the limitation of nitrogen (N) and phosphorus (P) in this detritus-based system, respectively. First, recent stoichiometric analyses suggest that long-term nitrogen (N) deficiency may select for preferential

reliance on specific classes of amino acids within an organism (e.g., N-poor vs. N-rich amino acids). Via constraints on the availability of N-rich amino acids, long-term N-limitation may drive aspects of protein evolution, which may have morphological consequences. In Chapter 1, I obtained the free amino acid content of cave millipedes and amphipods (in association with Dr. David Renault). Using these data, I compared the free amino acid pools of cave and surface dwelling animals, testing the hypotheses that cave animals would have reduced concentrations of N-rich amino acids, essential amino acids, and amino acids associated with the production of pigmentation and cuticle.

While the first chapter investigates the effects of nitrogen deprivation, the second chapter examines the role of phosphorus limitation in this system. Based on the growth rate hypothesis (Elser et al. 1996), I propose that the slow growth rates employed by cave species may be in response to the nutritional constraints imposed in this system; I specifically investigate if there are stoichiometric imbalances (mismatches between elemental ratios of consumers and their food) in cave ecosystems that may impose severe growth constraints. To test this hypothesis, I quantified the C: P of organic material collected from caves and compared the quality of cave resources to surface detritus (both field-collected samples and previously published values). To quantify the phosphorus content of cave animals, I set up and performed in-house laboratory analyses. I complemented these results with the quantification of the RNA and DNA content of cave millipedes (through collaboration with Dr. Adam Kay). I predicted that RNA/DNA, an index of protein

synthetic capacity, would be lower in the cave millipedes, reflecting the slowed growth rate of these organisms.

The findings of both of these chapters bring great insight into the rising field of ecological stoichiometry, specifically examining detritus-based terrestrial ecosystems, where little is currently known despite the importance of detritus (Moe et al. 2005). Both chapters compare the chemical composition of terrestrial cave arthropods to highlight the potential for the stoichiometric challenges of cave environments to drive the morphological and physiological adaptations of cave species.

The type of food that comes into caves varies in terms of regularity, duration, and usability. The most prevalent source of food is that of dead and decaying leaf and wood debris that has fallen, blown, or washed into caves (Barr 1967, Culver 1982, Poulson 2005). Another major source of energy input into these temperate caves is the carcasses of animals that fall down shafts or otherwise get lost within a cave (Barr 1967, Culver 1982, Poulson 2005). In Chapter 3, I present the results of an ecosystem-level resource manipulation experiment designed to investigate how different resource subsidies can influence arthropod community dynamics in caves. For this rigorous manipulation, I experimentally removed all natural food from 12 caves, constructed exclusion boxes to prohibit natural resources from entering, and introduced standardized amounts of the two major subsidies to caves: leaves (in the form of leaf packs) and carcasses (in the form of commercially supplied rodents). Monthly (for 2 years), I rappelled into each cave and measured the colonization and utilization of these resources. In this chapter I use this rich data set to examine how

resource subsidies influence richness, abundance and arthropod community structure. I also examine how long-term resource manipulation can influence a detritus-based terrestrial community (e.g. directionally change or stabilize a system). The findings of this chapter show how allochthonous resources can drive the community dynamics of terrestrial invertebrates in cave ecosystems and highlight the need for the surface environment to be considered when managing and protecting these unique habitats.

Lastly, nutrient limitation may affect the spatial distribution of invertebrates across caves. Building upon the work of my master's thesis, I assisted with (2004) and lead (2007) field surveys to bioinventory all (65) caves in a small karst area of West Virginia. Previous work indicates that not all cave species are collected based on a single bioinventory (Schneider and Culver 2004). Through the use of recent advances in statistical methods, I take advantage of the temporal and spatial replication of our data set to examine occupancy patterns of cave species, while including information about detection. First, I use classical and novel methods to estimate the number of species that are likely to be present in this area (but undetected in all surveys). While the classical methods are based primarily on binary presence/absence data, the more recent method incorporates information from the temporal replication so that detection can be integrated into the model. Detection is also important when considering the factors that are likely to influence the spatial distribution of cave species. In the fourth chapter I also use occupancy modeling techniques to investigate the relationship between estimated richness and the physical characteristics of caves (cave length, connectivity, and the size of the entrance (a surrogate for energy input)). One main focus of this chapter is to show that

information from biological surveys, even if incomplete, can still provide insights into the spatial occupancy patterns of rare species. The results suggest where terrestrial obligate cave species are likely to occur, which can inform conservation and management of these unique ecosystems.

Resource constraints (e.g., food energy, nutrients, and available habitat) can affect species on multiple levels. Through recent advances in ecological stoichiometry, it has been shown how the availability of N and P can influence the morphology and physiology of consumers (Moe et al. 2005). Through investigations of habitats such as streams, tree-holes, desert islands and lakes, it has been shown how spatial subsidies can influence the dynamics of recipient communities (Anderson et al. 2008). Lastly, through many biogeographical studies, it has been shown how resource constraints can influence the spatial distribution of animals (Brown 1995). Cave ecosystems afford unique opportunities to explore these issues of resource constraints and constitute a system where conventional ideas about the interrelationships of productivity, diversity, and food web structure do not apply (Culver 2001, Gibert and Deharveng 2002). The chapters that follow describe laboratory experiments, an ecosystem-level manipulation experiment, and annual bioinventories across 65 caves which examine the role of energy in maintaining and constraining cave biodiversity and the structure of cave communities.

Chapter I: So pale and so thin: Investigating the evolutionary consequences of nitrogen deficiencies on invertebrate free amino acid content.

Co-authored with: D. Renault and W.F. Fagan

Abstract

Recent stoichioproteomic analyses suggest that long-term nitrogen (N) deficiency may select for preferential reliance on specific classes of amino acids within an organism (e.g., N-poor vs. N-rich amino acids). Via constraints on the availability of N-rich amino acids, long-term N-limitation may drive aspects of protein evolution, which may have morphological consequences. Here we develop and test specific hypotheses directed at whether morphological characteristics of obligate cave invertebrates (depigmented, thin cuticles) are associated with free amino acid (FAA) pools suggestive of dietary N-limitation and nutritional constraints. Specifically, we examined the FAA content of two pairs of cave and surface species (millipedes, aquatic crustaceans), where, in each pair, the cave species shows morphological adaptations. Compared to the non-cave species, we found that the cave millipede has 1) decreased amounts of N-rich FAAs, 2) decreased amounts of essential and nonessential amino acids, and 3) decreased concentrations of amino acids associated with pigmentation and cuticle development. In contrast, nearly all of our hypotheses were rejected when examining amphipods, which is likely attributed to differences in

N-limitation and biochemical constraints in terrestrial vs. aquatic environments. Reanalysis of previously published experimental data on the link between diet and cuticle structure in ants supports our findings from the millipedes. Our results suggest that dietary protein quality can immediately influence acquisition of AAs and that, over evolutionary time, these constraints result in selection bias against N-rich AAs. Our findings help understand the evolutionary ecology of terrestrial cave species, suggesting that resource quality may drive the morphological adaptations of these animals.

Introduction

Background and purpose of study

In several natural environments, both the physiology and life-history of arthropods are affected by mismatches between the elemental demands of the consumer and the elements present in its resources (Cross et al. 2003; Denno and Fagan 2003; Elser et al. 2000b; Markow et al. 1999; Moe et al. 2005 [and references therein]). Advances in this research area, recently coined as “ecological stoichiometry” (Sterner and Elser 2002), make clear that nutrient limitation triggers metabolic trade-offs constraining the evolution of essential life traits (e.g. somatic maintenance, development, growth and fitness) (Boggs 2009) as well as the morphological characteristics of consumers (e.g. size, shape and color). Because amino acids represent a substantial fraction of non-protein nitrogen within an animal (Awapara 1962), and are essential resources in the manufacture of proteins and hormones, studying the impact of amino acid (nitrogen) limitation within an organism’s diet is of particular interest. Nitrogen deprivation alters the exoskeletal

composition of beetles (Rees 1986) and leads to decreased cuticular investment by canopy ants (Davidson 2005). Similarly, several mechanisms link the availability of nitrogen to arthropod biochemistry: the availability of dietary nitrogen *per se* might select for specific classes of amino acids for use in building proteins (Baudouin-Cornu et al. 2001; Elser et al. 2006; Fagan et al. 2002).

Though all amino acids contain at least one N-atom in their amine group, some also contain between one and three additional N-atoms in their side chain (Lehninger et al. 1993). Reliance upon these nitrogen-rich amino acids may be selected against when organisms are faced with nutritional constraints over evolutionary time. This phenomenon has been seen in yeast and bacteria, where the proteins and enzymes that form the N-uptake pathways are significantly N-poor relative to the rest of the organisms' respective proteomes (Baudouin-Cornu et al. 2001). Over shorter time frames, the concentration of individual amino acids was found to change within an individual as they are metabolized or synthesized in the face of a range of environmental stress (Day et al. 1990 [mussels]; Goto et al. 2001 [corn borers]; Issartel et al. 2005 [crustaceans]; Lalouette et al. 2007 [beetles]; Michaud et al. 2008 [midges]; Renault et al. 2006 [tenebrionid beetles]; Yi and Adams 2000 [Colorado potato beetles]). Particularly significant impacts on the allocation dynamics of FAA are those involving nutrient limitation. It may result in alteration in the proportion of N-rich amino acids in the free amino acid (FAA) pool of an organism, as well as in the balance of essential amino acids (EAA) *vs.* non essential ones (NEAA). Indeed, EAA are well known to be limiting compounds in several arthropod species because they must be acquired through feeding. In some

nectar-feeding moths, O'Brien et al. (2002) demonstrated that nonessential amino acids may also constrain life traits, because they are synthesized within the animal from endogenous nitrogen sources and therefore may entail a significant metabolic cost.

Because all organisms' life traits depend on the allocation dynamics of nutrients (Boggs 2009), we hypothesize in the present study that there may also be long term evolutionary effects of stress that may create changes in the FAA pool across species. Specifically, we expect that organisms that must consistently face nutritional constraints will have decreased concentrations of N-rich amino acids as well as decreased concentrations of both essential and nonessential amino acids. To address these hypotheses, we examined two pairs of cave-obligate versus cave-transient arthropods (terrestrial millipedes, aquatic amphipods). Due to an absence of primary productivity underground, the majority of cave systems are subsidized primarily by allochthonous resources (resources derived from the surface), which are often nutrient-poor (Barr 1967; Culver 1982; Poulson & Lavoie 2000). Obligate cave animals, that spend their entire lives underground, have evolved morphological and physiological adaptations, such as thin and depigmented cuticles (Culver 1982), to adapt to the subterranean environment. Because there is a correlation between FAA concentration in the tissues and in insect hemolymph (Bailey 1975), and more generally in the whole body (Liadouze et al. 1995), we speculated that any relative dearth of N-rich or nonessential amino acids that we might find in the FAA pool could be indicative of an overall lack of these amino acids body-wide, and might thus be associated with physical changes in response to stress that have already been

documented in pigmentation (Benassi et al. 1961) and cuticular structure (Neville 1975). Our specific predictions for these two physical manifestations are outlined below and in Table 1.

Pigmentation

Nitrogen-deprived organisms are often at least partially depigmented (nonmelanized). Examples include butterfly larvae fed on drought stressed plants (Talloon et al. 2004), mosquito larvae deprived of tyrosine (TYR) and phenylalanine (PHE) (Chapman 1982), and phytoplankton starved of N (Latasa and Berdalet 1994). Recently, Lee et al. (2008) demonstrated how dietary quality, not quantity, influences the degree of insect melanization, with noctuid caterpillars fed low quality food having significantly less melanin than the same caterpillars fed high quality food. In that study, the lack of N in the poor quality diet may have limited the production of melanin.

Four amino acids are directly involved in the best-known arthropod pigmentation pathways: TYR, PHE, tryptophan (TRP) and β -alanine (β -ALA). TYR is a primary component in the biosynthetic pathway of melanin in insects (True 2003). Oxidation of TYR by tyrosinase produces a reddish pigment, which, upon heating, turns black and pigmented (Raper and Wormall 1925). The final product, melanin, contains proportionally more nitrogen than TYR (8.4 vs. 7.73 % N, Raper and Wormall 1925). Depigmented or partially pigmented cuticle may be due to a lack of tyrosine itself (Hartwell 1923), or a lack of its precursor PHE, an essential amino acid from which TYR is synthesized (Brunet 1963). Beyond melanin, ommochromes (N-rich compounds synthesized from TRP) can be responsible for arthropod

coloration, and are the primary components producing coloration in various insects (Linzen 1974), including odonates (Chapman 1982) and locusts (in the form of insectorubin [Goodwin and Srisukh 1950]). Lastly, β -ALA plays a major role in the tanning of insects cuticle, and insects deprived of β -ALA exhibit unusually dark cuticles (Brunet 1963, Hodgetts and Konopa 1973).

Although other pathways can lead to pigmentation in some taxa (e.g., the presence of carotenoids, or cross-linking between cuticular proteins [Chapman 1982]), we test here for changes in the concentration of the four amino acids mentioned above (two essential: PHE and TRP, and two nonessential: TYR and β -ALA). We contrast depigmented species that must routinely contend with nutritional constraints and related pigmented animals that do not face such constraints.

Cuticular structure

The arthropod cuticle is a nitrogen-rich structure, containing protein (17 % N by mass) and chitin (7% N by mass) (Chown and Nicolson 2004). Chemically, the cuticle contains proteins, peptides, and amino acids – specifically, large quantities of proline (PRO), alanine (ALA), valine (VAL), arginine (ARG), and glycine (GLY) (Neville 1975; Stankiewicz et al. 1996). Aromatic amino acids are important in the exoskeleton: for example, TYR (Behmer and Joern 1993) which is associated with sclerotization (Trim 1941) and its derivatives also influence exoskeleton hardness when they interact with proteins (Brunet 1963). The other aromatic amino acids, PHE and TRP, are also important for cuticle development and sclerotization. For example, PHE has been shown to be selectively favored in the diets of immature grasshoppers, presumably to maximize growth and cuticle production (Behmer and Joern 1993).

Food scarcity can cause cuticular growth layer deposition to cease in water bugs (Cullen 1969) and other hemipterans (Zwicky and Wigglesworth 1956). Here, we hypothesize that cave obligate animals faced with constant nutritional constraints will show decreased concentrations of both essential (VAL, ARG, PHE and TRP) and nonessential (PRO, ALA, and TYR) amino acids in their FAA pool compared to related cave-transient organisms that do not face similar constraints.

Overall goals

In this paper, our expectation was that obligate cave animals, restricted to the subterranean environment, face strong nutritional constraints that would be reflected in their biochemical composition. In comparison, we expected that transient/surface animals experience some release from these nutritional constraints due to their competitive and dispersive abilities, which may enhance their access to nutrient rich resources both inside and outside caves. For millipedes and crustaceans, we developed datasets of the FAA content of cave-obligate and transient/surface species. The transient millipedes are pigmented (purple), whereas the cave-obligate (nutrient-limited) millipedes have thin cuticles and are white. The same generalities are true for the crustaceans; where the surface species is pigmented when compared to the unpigmented subterranean species. We complement our comparison of cave-obligate and cave-transient species with a reanalysis of experimental data from Williams et al. (1987) that examined the amino acid content of nutritionally-deprived (and depigmented) ants. We expected that the physical and biochemical manifestations of nutrient deprivation would be parallel between the ants and the two cave species, and allow us to focus on the immediate (ants) and evolutionary (cave species)

consequences of dietary quality on the FAA pool of these organisms. Specifically, we predicted that the FAA pools of nutritionally-deprived arthropods (whether deprived within the laboratory or naturally within the caves) should display (1) lowered concentrations of N-rich amino acids, (2) decreased amounts of nonessential amino acids (synthesized from endogenous nitrogen stores) and essential amino acids (acquired from diet), (3) decreased concentrations of the four amino acids associated with pigmentation (TYR, TRP, PHE and β -ALA), and (4) decreased concentrations of the seven amino acids associated with cuticle structure and development (VAL, ARG, PHE, TRP, PRO, ALA and TYR).

Methods

Analyzing the FAA content of millipedes and amphipods

Cave transient millipedes (Diplopoda: Chordeumatida: *Pseudotremia hobbsi* Hoffman 1950) and obligate cave millipedes (*P. fulgida* Loomis 1943) were collected from Buckeye Creek Cave (located north of Lewisburg, Greenbrier County, West Virginia, USA), in September 2007. *Pseudotremia hobbsi* is a pigmented, large-eyed species that occurs both inside and outside caves (Shear 2008). The range of *P. hobbsi* extends between WV and VA (USA). *Pseudotremia fulgida*, in contrast, is highly cave-adapted; it is blind and white, possesses a thin cuticle, and is restricted to caves in just two WV counties (Shear 2008). Both millipedes are detritivores.

We also compared an obligate cave amphipod (Crustacea: Decapoda: Amphipoda: Niphargidae: *N. rhenorhodanensis* Schellenberg 1937), to a closely-related surface-dwelling species *Gammarus pulex* (Amphipoda: Gammaridae). Both amphipods are widespread throughout Europe, but *N. rhenorhodanensis* is completely

restricted to the subterranean habitat. Although these two species are not in the same genus, species from these genera are frequently used for physiological comparisons (Canivet et al. 2001; Hervant & Mathieu 1995; Hervant et al. 1995, Hervant et al. 1997; Issartel et al. 2005; Issartel et al. 2006). *Niphargus* individuals were collected in May 2006 in the Jura Mountains (France). Animals were caught directly using baited traps in small pools within the cave or by filtering water of the resurgence spring. *Gammarus* individuals were collected in April 2008 from Amous River (Gard, France) with a net. Because these amphipods were collected at different times, we compared the general results to published data from Issartel et al. (2005), where the FAA content of *N. rhenorhodanensis* and *Gammarus fossarum* were analyzed.

Both millipedes and amphipods were collected in the field and brought home alive in a cooler prior to being stored in a -80°C freezer until prepared for analysis. For FAA quantification, samples were lyophilized for 48h before being weighed. Two tungsten beads (3 mm diameter) and 900 µl of methanol-chloroform (2:1) were added to each sample. The samples were then blended in a Bead-Beater (Retsch™ MM301 bead-beating) for 2 minutes. 600 µl of ultra pure water were added to each sample (methanol-chloroform-water 2:1:2), and the samples were vortexed for 15 s. Samples were then centrifuged (4000G, 4°C) for 10 minutes. A two-phase mixture was obtained, with polar metabolites (sugars, polyols, amino acids) in the aqueous phase, and non-polar metabolites (lipids) in the organic phase. 700 µl of the aqueous phase were collected and dried using a speedvac (Speed Vac Concentrator, Savant™), also allowing spinning. Ultra pure water was then added to each dried sample. The samples were then used for amino acid derivatization (Bouchereau et al. 1999), using

Waters Corporation protocol, and analyzed in the UPLC (Ultra Performance Liquid Chromatography, Waters Corporation, Milford, USA).

Ant data

To complement our comparison of cave-obligate and cave-transient animals, we used data from Table 1 of Williams et al. (1987), who studied FAA content of the red imported fire ant (Hymenoptera: Formicidae: *Solenopsis invicta*). In that study, the authors found that when larvae of worker ants were deprived of insects in their diet and fed a lower quality diet supplied with honey water, ground beef and chicken eggs (without insects), newly emerged mature adults were depigmented (non-melanized) and possessed thin cuticles. On the other hand, diets supplied with insects resulted in “normal” and fully pigmented ants. With the expectation that the two morphs would differ in their TYR concentration (because of the aforementioned role of TYR in the melanin production pathway), Williams et al. (1987) analyzed the FAA pool of fourth instar larvae of both normal and depigmented ants but did not find an explanation for the depigmented, thin cuticles in the nutritionally deprived individuals. Here we reanalyze their data in light of new hypotheses posed by ecological stoichiometry and new advances in understanding biosynthetic pathways. Unfortunately, Williams et al. (1987) did not report sample sizes nor the variability associated with amino acid concentrations so, unlike our own studies, we can only discuss mean differences.

Assessing the relative abundance of N-rich amino acids

For each of the three FAA content comparisons – transient *vs.* obligate cave millipedes surface *vs.* cave amphipods (our datasets), and normal *vs.* depigmented

ants (Williams et al. 1987), we examined how the interspecific variation in the relative concentration of each amino acid changed as functions of 1) the number of N atoms per side chain (the number of additional N atoms not including the 1 N in the base amine group) and 2) the % N of the molecular mass for that amino acid (the number of additional N atoms times 14.007 [the molecular weight of N] divided by the molecular mass of the amino acid [Lehninger et al. 1993]). Using this calculation, seven AA qualified as N-rich (Lysine [LYS], Glutamine [GLN], Asparagine [ASN], Histidine [HIS], Ornithine [ORN], ARG and TRP), because they have at least 1 N atom in their side chains. The % N by mass that we calculated for each amino acid ranged from 0 (if the amino acid did not contain extra N in the side chain) to 42 % (in the case of ARG). For each amino acid, we then plotted the relative ratios of the “normal” to the depigmented, and determined whether the N-rich amino acids were proportionally higher in the “normal” species.

Assessing interspecific differences in concentrations of essential and nonessential amino acids

In arthropods, the ten essential amino acids are ARG, HIS, isoleucine [ISO], leucine [LEU], LYS, methionine [MET], PHE, threonine [THR], TRP and VAL (Dooley et al. 2000). These essential amino acids (EAAs) are acquired directly from diet; and we hypothesized that the “normal” counterpart, relaxed from dietary constraints, would have greater concentrations of essential amino acids. Nonessential amino acids (NEAAs), on the other hand, can be synthesized within the organism using endogenous nitrogen stores. Because we hypothesized that the “normal” counterpart would have overall greater % N, we hypothesized that it would also have

greater total quantity of NEAAs compared to the depigmented counterpart. For each species pair (millipedes, amphipods, ants), we performed one-tailed paired t-tests on the difference in the average concentrations ($\mu\text{mol/g}$) of essential and nonessential amino acids.

Assessing interspecific differences in amino acids associated with pigment and cuticle

We then tested our predictions for the differences in specific amino acid concentrations ($\mu\text{mol/g}$ dry mass) associated with cuticular structure and pigmentation (outlined in Table 1). First, we used ANCOVA to test for differences in amino acid concentrations between the transient and obligate cave millipedes and between the surface and cave amphipods using body size (dry mass) as a covariate. Data were log transformed to homogenize variances as appropriate, and for amphipods, there were a few cases where between two and four outliers (of the $N = 37$ data points) needed to be removed to account for nonnormal data. In cases where there was no significant effect of size (as was the case for all analyses involving millipedes, and all but three of the amino acids for amphipods), we instead performed one-tailed t-tests with the direction specified by our *a priori* hypotheses for each amino acid (Table 1). The published data set for ants did not include body size or variability in the individual amino acid concentrations, thus we could only examine differences in means and no statistical analyses could be performed.

All statistical analyses were completed in R (version 2.7.0; R Development Core Team 2008).

Results

FAA pools: size and composition

The size and composition of the free amino acid pools varied across the species (raw data for all results are presented in Appendix A). A total of 24 amino acids were present in the millipede FAA pool. The total average FAA concentration was higher in transient (178.95 $\mu\text{mol/g}$ dry mass) than in obligate cave millipedes (139.94 $\mu\text{mol/g}$ dry mass). Alpha-ALA was the most abundant amino acid in both species of millipede, representing 14 % and 16 % of the total FAA pool for transient and obligate species, respectively. AABA and β -ALA were the least abundant amino acids detected, with trace quantities present (each representing less than 2 % of the FAA of either millipede species).

The same 24 FAA that were reported in the millipedes were also present in the surface amphipod: but only 20 FAA were detected in the cave species. The four present in the surface, but not the subterranean species, were AABA, β -ALA, GABA and homoserine. Despite having fewer amino acids, the cave species still had a larger total FAA pool than the surface species (365.91 vs. 105.72 $\mu\text{mol/g}$, respectively). GLN was the most prevalent AA in the subterranean species, representing 20% of the total FAA pool for that species. ARG and GLN were the most prevalent in the surface species, both representing 16 % of the total FAA pool for that species.

In ants, 15 FAA were reported (Williams et al. 1987). The total average FAA concentration was lower in normal (77.15 $\mu\text{mol/g}$) compared to depigmented ants (103.44 $\mu\text{mol/g}$). In both morphs, PRO was the most abundant amino acid, representing nearly 17 % of the depigmented ant and 25% of the “normal” ant.

Overall tests of our predictions

Our *a priori* hypotheses and our overall findings regarding the representation of amino acids based on their biochemical composition and function are outlined in Table 1. For the comparison between millipede species, 12 of our 13 results were in agreement with our predictions. Of these 12 successes, nine exhibited significant interspecific differences at a one-tailed p value of < 0.09 , and the remaining three exhibited nonsignificant trends in the direction predicted (Table 1). Unlike the millipedes, results for the amphipods were contrary to 11 of our 12 hypotheses. Only concentrations of ASN were found to differ between species in the direction that we predicted. Lastly, for the ants, data trended in the direction to support our hypotheses for five out of eight amino acids. Support was especially strong for the suite of explanations concerning the dominance of N-rich amino acids in normal ants, where published results supported our hypotheses in all three cases where data were available. All of these results are reported in more detail below.

Assessing the relative abundance of N-rich amino acids

When calculating (on a per amino acid basis) the relative ratio of amino acid concentrations between transient and obligate millipedes, transient millipedes had higher concentrations of six of the seven N-rich FAA (Fig 1a, Table 1). The concentrations of the N-rich amino acids (TRP, LYS, GLN, ASN, HIS, ORN and ARG) were typically 20 to 50 % higher in transient millipedes. Glutamine, which has 19.43 % N in its side chain, was nearly double the concentration in transient millipedes (85.4% higher). Only one amino acid, ORN, exhibited a (non-significant)

trend opposite to our predictions (Table 1), and was slightly higher in the cave-obligate millipede (0.58 $\mu\text{mol/g}$) than the transient millipede (0.49 $\mu\text{mol/g}$).

Amphipods, however, did not show the same pattern. In fact, six of the seven N-rich amino acids were found in higher concentrations in the subterranean amphipod (Fig 1b). Two of these six amino acids were 50 - 60 % higher in subterranean amphipods (HIS and ARG, Appendix A). The other four (GLN, LYS, TRP, and ORN) were 332%, 895%, 840% and 1430% higher in subterranean animals. In accordance with our prediction, ASN was significantly higher in surface amphipods (12.45 $\mu\text{mol/g}$ vs. 8.84 $\mu\text{mol/g}$ in subterranean amphipods, Table 1, Fig 1b).

Williams et al. (1987) reported FAA data for 15 amino acids. Only three amino acids that they detected have at least one N in their side chain; all of which were found in greater concentrations in the normal ants (Fig 1c). Concentrations of HIS, which has 34.55 % N in its side chain, and ARG, which has 41.96 % N in its side chain, are roughly 75 – 80 % higher in normal ants (Appendix A). The third N-rich amino acid, LYS, is 186% higher in normal ants.

Assessing interspecific differences in concentrations of essential and nonessential amino acids

Here, we predicted that quantities of essential amino acids (EAAs) would be lower in the depigmented species because they must be acquired through feeding. We also predicted that the nonessential amino acids (NEAAs) would be lower in the depigmented species because these amino acids are manufactured within the animal using endogenous nitrogen stores, which we assume to be limited in these animals resulting from dietary constraints.

For every pair of EAAs and NEAAs, the quantity in the cave millipede was either equal to, or lower than, the quantity in the transient species (Fig 2a). In agreement with our predictions, compared to the obligate cave millipedes, the transient millipedes had significantly higher average concentrations of EAAs (7.86 vs. 5.99 $\mu\text{mol/g}$, respectively; $t = 3.83$, $df = 9$, $p < 0.010$). In addition, the transient millipedes also had significantly higher average concentrations of NEAAs (7.16 vs. 5.72 $\mu\text{mol/g}$, respectively; $t = 3.18$, $df = 13$, $p < 0.010$).

For each EAA, the quantity in the subterranean amphipod was higher than the quantity in the surface amphipod (Fig 2b). The same pattern is true for the NEAAs; and with the exception of one NEAA (ASN), the cave species had higher concentrations of all NEAAs. It is not surprising, therefore, that contrary to our predictions, the surface amphipod did not have higher concentrations of either EAAs ($t = 4.25$, $df = 9$, $p = 0.999$) or nonessential amino acids ($t = 2.31$, $df = 9$, $p = 0.977$) compared to the subterranean amphipod species.

For ants, we found no consistent pattern of the concentration of individual EAAs (Fig 2c). Some EAAs, such as VAL and LEU are much higher in the depigmented ants, whereas others, such as LYS and HIS are higher in the normal ants (Table 1). In total, the depigmented ants had a higher average concentration of NEAAs (9.82 $\mu\text{mol/g}$ compared to 6.20 $\mu\text{mol/g}$), and with the exception of one (ALA), each NEAA was found in higher concentrations in the depigmented morph (Fig 2C). Overall, the two ants did not differ in the concentrations of either EAAs ($t = -0.08$, $df = 7$, $p = 0.531$) or NEAAs ($t = -1.79$, $df = 6$, $p = 0.938$).

Assessing interspecific differences in amino acids associated with pigment and cuticle

The four amino acids hypothesized to differ between pigmented and depigmented animals are TYR, PHE, TRP, and β -ALA. With the exception of β -ALA, we predicted that each of these amino acids would be found in higher quantities in the pigmented animal. The statistics for these comparisons are presented in Table 1. We found that when compared to the cave species, quantities of TYR were indeed significantly higher in surface millipedes, but were not higher in surface amphipods (Fig 3a). Similarly, PHE was higher in surface vs. cave millipedes, but not in the surface vs. subterranean amphipods (Fig 3b). Unlike the surface amphipods, the surface millipedes were also higher in their average concentration of TRP (Fig 3c), though this difference was not statistically significant. Lastly, as we predicted, the cave millipede had significantly higher levels of β -ALA than did the transient species (Fig 3d). No β -ALA was found in the subterranean amphipods.

In Williams et al. (1987), data for only two of the four pigment amino acids were available. In line with our predictions, the average concentration of PHE was higher in the normal ants (4.85 vs. 4.52 $\mu\text{mol/g}$). The average concentration of TYR, on the other hand, was much higher in the depigmented ants (6.16 vs. 2.19 $\mu\text{mol/g}$).

The seven amino acids hypothesized to differ between animals as a consequence of cuticular changes are ARG, TRP, TYR, PHE, PRO, ALA and VAL. In millipedes, all seven of our hypotheses were supported; four of which were supported with statistical significance (Table 1). In contrast, none of these hypotheses were supported in the amphipods. As differences in TRP, TYR and PHE were

discussed above (due to their role in pigmentation), we will focus on the remaining four amino acids (ARG, PRO, ALA and VAL).

For the millipedes, surface species trended to have higher concentrations of ALA (Fig 4a) and PRO (Fig 4b), whereas again, cave amphipods were surprisingly higher in both (Table 1). Similarly, the surface millipedes had significantly higher concentrations of valine (Fig 4c) and arginine (Fig 4d), whereas the cave amphipods were higher in both compared to their surface counterparts (Table 1).

In Williams et al. (1987), data were available for six of the seven amino acids associated with cuticular development; no data was available for TRP (Table 1). Like phenylalanine (described above in *pigment*), both alanine and arginine were found in higher concentrations in normal compared to depigmented ants. In contrast, like tyrosine (also described above), valine and proline were found in lower concentrations in the normal ants (Table 1).

Discussion

Organisms that regularly endure bouts of food limitation have to make compensatory adjustments in their metabolism. Of particular significance are diets deficient in proteins or EAAs that may result in a reduced protein turnover within an organism and an enhanced reutilization of the EAAs. As a result, the size and composition of the FAA pool reflects the constraints on the ability of an organism to metabolize nitrogen and manufacture proteins (Liadouze et al. 1995), and often reflects an organism's physiological demands (Tillinghast and Townley 2008). Because all organisms' traits depend on the allocation dynamics of nutrients (Boggs 2009), nutritional deficits can constrain life traits (O'Brien et al. 2002). Thus,

limitations that influence amino acid acquisition and manufacture may help shape life history evolution.

In general, we found substantially more support for our hypotheses regarding FAA pools for the terrestrial animals (millipedes, ants) than for the amphipods (Table 1). Specifically, with the exception of ORN, the nutritionally deprived terrestrial animals had decreased concentrations of all N-rich amino acids, supporting our prediction that nutrient limitation can have both immediate and evolutionary consequences that are reflected in the FAA pool of an organism. These consequences, which are likely to be manifested in physical attributes of these organisms, are discussed in greater detail below.

Our findings for the aquatic animals, in contrast to the terrestrial animals, did not meet our predictions. As predicted, subterranean amphipods have decreased concentrations of the nonessential N-rich amino acid ASN, but for the remaining N-rich amino acids, subterranean amphipods, unlike the cave millipedes, had concentrations of N-rich amino acids that were nearly equal to or greater than their surface-dwelling counterparts (Fig 1b). Subterranean amphipods also had greater concentrations of both NEAAs and EAAs in comparison to the surface species (Fig 2b). Lastly, none of our hypotheses concerning pigmentation or cuticular amino acids were met when examining these amphipods (Table 1).

In order to test if our results were attributed to seasonal differences when these animals were collected, we compared our findings to data from Issartel et al. (2005). We examined their raw data, comparing *N. rhenorhodanensis* and a *Gammarus* species closely related to *G. pulex* (*G. fossarum*) that were maintained

under laboratory conditions. We found that at the control temperatures, the subterranean species still had higher concentrations of most of the reported amino acids. Surface species were slightly higher in proline (0.986 vs. 0.831 $\mu\text{mol/g}$, respectively) and glutamine (2.938 vs. 2.844 $\mu\text{mol/g}$), but these differences were not significant. When explaining why the cave animals had elevated amino acid concentrations, Issartel et al. (2005) attributed increases in proline, alanine, and glycine to cold acclimation. In addition to limited nutrient supplies, aquatic subterranean species also have to cope with alternate periods of hypoxic and normoxic conditions that together have resulted in the selection for energy efficiency. Hervant (1996) previously suggested that subterranean amphipods periodically rely upon fermentation during periods of anaerobiosis, and that amino acids play a significant role in this process.

Because the subterranean amphipods had extremely high levels of all of the amino acids, we also cannot rule out differences resulting from phylogeny. More research is needed to see if these results would hold when comparing congeneric subterranean amphipods, collected from the same region at the same time. However, it is also possible that our hypotheses were not appropriate for the aquatic environment because aquatic animals do not face the same physical constraints as terrestrial animals. In particular, compared to terrestrial habitats, N is much less limiting in aquatic environments: groundwater, specifically, is often quite rich in N due to fertilization and minerals leached from bedrock (Langmuir 1971; Simon and Benfield 2001; White 1988). In addition, the biochemical demand for a cuticle is very different in the aquatic system, and generally, only terrestrial cave arthropods are

described as having thin cuticles (Christiansen 2005; Culver et al. 1995). Because of these differences between habitats, we will limit the rest of our discussion to the findings of terrestrial species (millipedes and ants) and focus on the immediate (ants) and evolutionary (millipedes) consequences of dietary quality on the FAA pool of these organisms.

Assessing the relative abundance of N-rich amino acids

Many studies have shown that nitrogen limitation can have immediate effects on the morphology of terrestrial invertebrates (Greene 1996, Karowe and Martin 1989 [and references therein]). The ants that we reanalyzed showed morphological changes, combined with decreased concentrations of N-rich amino acids (Fig 1c), over just a single generation (Williams et al. 1987). Over evolutionary time, nitrogen-deprivation can also have severe consequences. In plants, for example, prolonged nutrient deprivation imposes selection pressures that result in genome-wide changes in protein composition (Elser et al. 2006). Here, we suggest that these evolutionary consequences of N-limitation are also evident in the FAA pool of arthropods. Specifically, we found that obligate cave millipedes had decreased concentrations of six N-rich amino acids when compared to congeneric surface-dwellers (Fig 1a). The only N-rich amino acid that was found in higher concentration in the cave millipede is ORN, which may not be reliably quantified with the analytical method we used. This finding has implications for arthropod evolutionary ecology in that the effects of dietary nitrogen deprivation are expressed biochemically within an organism through the production and acquisition of N-rich amino acids.

Assessing interspecific differences in concentrations of essential and nonessential amino acids

Obligate cave millipedes had decreased concentrations of both EAAs and NEAAs when compared to transient millipedes (Fig 2a). EAAs, which must be acquired through diet, are likely restricted in the cave environment, where cave animals are completely dependent on detrital resource subsidies from the surface. In Lepidoptera, authors found that EAAs in adults are actually stored during larval development (O'Brien et al. 2002) whereas NEAAs are manufactured during adulthood. In contrast to EAAs, the carbon skeletons of NEAAs can be synthesized by most arthropods. The other key component of NEAAs manufacture requires a source of endogenous N, which is most likely supplied by transamination from other existing amino acids. It is therefore not surprising that NEAAs are also found in lower concentrations in the cave-obligate millipedes, which face strong metabolic constraints and lowered concentrations on N-rich amino acids (discussed above). The nutritionally deprived ants, on the other hand, were still receiving EAAs during development (from the other protein sources in their diet) and retained the potential to manufacture NEAAs. Because the essentiality of amino acids did not differ between ants, this supports the N-related hypotheses in driving the pattern of depigmentation for these animals.

Assessing interspecific differences in amino acids associated with pigment and cuticle

It is a well-established fact that many obligate cave invertebrates lack pigment, but what mechanisms drive this pigment loss are unknown (though pigment loss is rather well understood in cave vertebrates [Jeffery 2006, Felice et al. 2008]).

Many authors propose that, due to relaxed selection pressures underground, the genes that control for luxuries such as pigmentation are often disregarded in favor of more important pathways (e.g. metabolic efficiency) (Cloudsley-Thompson 1988). Here, we propose that nutrient-limitation, specifically N-limitation, may be responsible for the lack of pigment in most cave-adapted invertebrates.

Certain amino acids are often implicated in differences between pigmented and depigmented organisms. The absence of β -alanine in arthropods, for example, often results in a darker color. While we saw very large, significant differences between the two millipede species in their concentration of β -alanine, we must approach these results with caution: as the trace amounts recorded are subject to experimental error. Similar to previous work, we saw more conclusive results in the other three amino acids associated with pigmentation. For example, in their study of albino locusts, Benassi et al. (1961) conclude that the FAA concentrations of tyrosine, phenylalanine, and tryptophan are higher in pigmented *Schistocerca* when compared to the albino form. Here, we saw that depigmented millipedes did indeed have less tyrosine, phenylalanine and tryptophan in their FAA pool when compared to a pigmented, surface dwelling congener.

We also found that depigmented ants had less phenylalanine than normal ones. Williams et al. (1987) were surprised that they did not observe the expected differences in tyrosine between the ants, and in fact, the depigmented morph had more than double the concentration of tyrosine in its FAA pool. This is interesting because tyrosine, like all aromatics, is quite metabolically expensive to produce. Why might the depigmented ants have higher values of tyrosine than their pigmented

counterparts? One possibility is that the reported values are not representative of the species but of the life stage. Tyrosine values can change dramatically within the life span of an organism (Brunet 1963). In their recent paper, Tillinghast and Townley (2008) discuss factors, such as recent activity or life stage, which may influence FAA measurements at a given point in time. For example, tyrosine decreases after molting, because it is allocated towards tanning (Chapman 1982), and needed for sclerotization after molting (Urich 1994). In addition, tyrosine may increase because of its role in the synthesis of hormones such as tyramine, dopamine, and octopamine, the production of which are increased during stress. However, any of these possible explanations must be treated with caution because Williams et al. (1987) did not report sample sizes and we cannot estimate the error associated with their measurements.

Our results suggest that terrestrial cave millipedes have decreased quantities of the amino acids associated with pigmentation, especially the nonessential amino acid TYR, which may be because of a lack of endogenous N stores. It is interesting to note that not all cave species are without pigment. Predatory cave taxa, such as carabid beetles and pseudoscorpions, often exhibit a reddish pigment. Likewise, the lack of pigment is also seen outside of cave habitats, for example in forest-dwelling detritivores and cryptozoic species (Cloudsley-Thompson 1988). It may be that cave predators, at a higher trophic level, may have access to greater N-rich food sources (prey) than detritivores, and thus may be able to obtain the components necessary to retain pigmentation.

An additional explanation for why some cave animals retain pigment may be the presence of symbionts. Many detritivores, including pill millipedes (Rawlins et al. 2006), rely upon symbionts for pigmentation. For example, cockroaches depend on symbionts to synthesize precursors of pigment (Chapman 1982; Henry and Cook 1964), and the grain weevil *Calandra* is lighter in color and smaller in size without symbionts (Chapman 1982). This is also of note because in Williams et al. (1987), once the worker ants were fed cockroaches, they regained pigmentation. Though the authors fail to mention if the cockroaches are alive or dead, previous research has shown that symbionts from cockroaches can still be transferred whether or not the host is living (Thorne 1990 and references therein).

The thin cuticle of terrestrial cave arthropods is typically attributed to the adaptation to the humid subterranean environment. Here, we showed that the cave animals also have lower concentrations of all of the amino acids associated with cuticular development, and in 57 % of the cases these trends were supported statistically (Table 1). In the ants, half of our hypothesized results were supported. It may be possible that other factors that contribute to cuticle formation, such as lipids, may play more of a role in the cuticular differences between the species pairs.

Overall conclusions

In their recent paper, Lee et al. (2008) demonstrated the link between dietary protein quality and insect melanization. Our goal in this paper was to suggest that diet quality can immediately influence acquisition of amino acids, and that over evolutionary time, these constraints result in selection bias against N-rich amino acids. We found that cave millipedes, obligately dependent on allochthonous detritus

from the surface, have decreased concentrations of N-rich amino acids compared to their surface counterparts, which is likely a result of their dietary constraints. We also found that cave millipedes have decreased concentrations of amino acids involved in the production of pigmentation and cuticle. Our results help understand the evolutionary ecology of terrestrial cave species, suggesting that resource quality may be a driving force behind the morphological adaptations of these animals.

Acknowledgements

This idea was generated, in part, during discussions of the “Spatial Stoichiometry” working group at the National Center for Ecological Analysis and Synthesis (Santa Barbara, California). The authors would like to thank Bill Jeffery and Sergei Sukharev for helpful discussion early on in this project, and J. Gilbert for constructive comments on an earlier form of this manuscript. The authors also thank Pr. A. Bouchereau of the University of Rennes 1, UMR INRA APBV, for the use of the analytical equipment. This work was supported by grants from the Cave Research Foundation, the Cave Conservancy Foundation, the National Speleological Society, the West Virginia Association of Cave Studies, and the University of Maryland Hockmeyer Graduate Fellowship to KS. Collecting permits for cave millipedes were obtained from the WV DNR to KS (permit number: 2007.018).

Tables

Table 1: Hypothesized and observed results for amino acid contents in normal (N) and depigmented (D) arthropods. Key: * $p < 0.09$; ** $p < 0.05$; *** $p < 0.01$ by one sided t-test or ANCOVA (see text); gray boxes concordant with predictions. Ant results are based on pattern, whereas the results for millipedes and amphipods include trends and statistically significant results.

AA	Prediction	Explanation	Our Datasets		Literature data
			Millipedes	Amphipods	Ants
ALA	N > D	Cuticle	$N > D$ t = -0.790, df = 9.259, p = 0.225	$N < D$ t = 20.591, df = 19.434, p = 1	$N > D$
PRO	N > D	Cuticle	$N > D$ t = -0.811, df = 8.657, p = 0.220	spp : $F_{1,33} = 29.861$, $p < 0.001$ size : $F_{1,33} = 26.987$, $p < 0.001$	N < D
VAL ^c	N > D	Cuticle	$N > D$ ** t = -1.968, df = 11.779, p = 0.037	$N < D$ t = 16.023, df = 17.218, p = 1	N < D
PHE ^c	N > D	Pigmentation & Cuticle	$N > D$ * t = -1.743, df = 11.911, p = 0.054	$N < D$ t = 14.272, df = 15.206, p = 1	$N > D$
TYR	N > D	Pigmentation & Cuticle	$N > D$ ** t = -2.038, df = 7.612, p = 0.039	$N < D$ t = 17.260, df = 26.962, p = 1	N < D
TRP ^c	N > D	Pigmentation Cuticle & N-rich	$N > D$ t = -1.071, df = 11.518, p = 0.153	$N < D$ t = 15.160, df = 27.138, p = 1	No data
β ALA	N < D	Pigmentation	$N > D$ *** t = 4.053, df = 6.168, p = 0.003	No data	No data
LYS ^c	N > D	N-Rich	$N > D$ * t = -1.790, df = 6.593, p = 0.059	$N < D$ t = 15.314, df = 31.845, p-value = 1	$N > D$
GLN	N > D	N-Rich	$N > D$ *** t = -3.409, df = 10.997, p = 0.003	$N < D$ t = 26.672, df = 12.959, p = 1	No data
ASN	N > D	N-Rich	$N > D$ * t = -1.475, df = 11.999, p = 0.083	$N > D$ ** sp*size: $F_{1,32} = 4.73$, p = 0.037, spp: $F_{1,32} = 20.601$, $p < 0.001$	No data
HIS ^c	N > D	N-Rich	$N > D$ * t = -1.663, df = 11.996, p = 0.061	$N < D$ (log) spp : $F_{1,33} = 36.179$, $p < 0.001$ size : $F_{1,33} = 32.916$, $p < 0.001$	$N > D$
ORN	N > D	N-Rich	$N < D$ t = 0.885, df = 10.599, p = 0.8021	$N < D$ (log) t = 35.598, df = 32.990, p = 1	No data
ARG ^c	N > D	N-Rich & Cuticle	$N > D$ ** t = -2.187, df = 9.692, p = 0.027	$N < D$ (log) t = 4.327, df = 21.331, p = 0.999	$N > D$

^c: essential amino acids

Figure Legends

Figure 1. The ratio of the concentration of each amino acid content in normal and depigmented animals plotted against the % N in the side chain for that amino acid (see text for calculation). a) transient vs. cave millipedes, b) surface vs. cave amphipods, c) normal vs. nonmelanized ants.

Figure 2. Paired concentrations of essential and nonessential amino acids for millipedes (a), amphipods (b) and ants (c). For all comparisons, the “normal” species is on the x axis and the depigmented species on the y. The line represents a 1:1 line where the concentrations are equal.

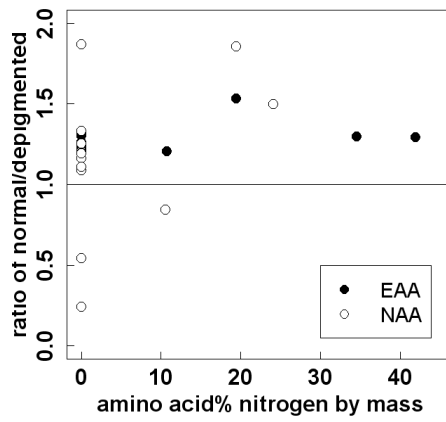
Figure 3. Differences between cave species and surface species in the amino acids associated with known pigmentation pathways (see Table 1 for statistics). For each graph, millipedes are represented with open circles and amphipods are filled circles.

Figure 4. Differences between cave species and surface species in the amino acids associated with cuticular development (see Table 1 for statistics). For each graph, millipedes are represented with open circles and amphipods are filled circles.

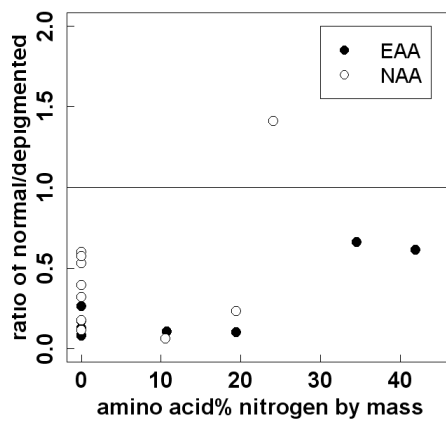
Figures

Figure 1

a) Millipedes



b) Amphipods



c) Ants

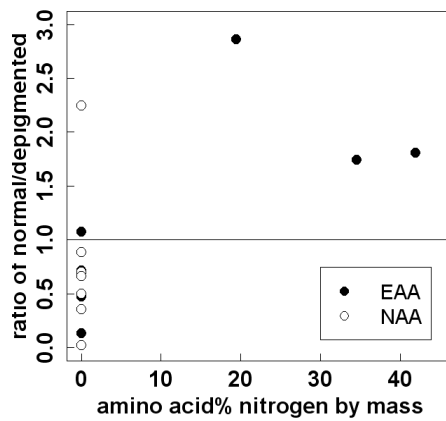
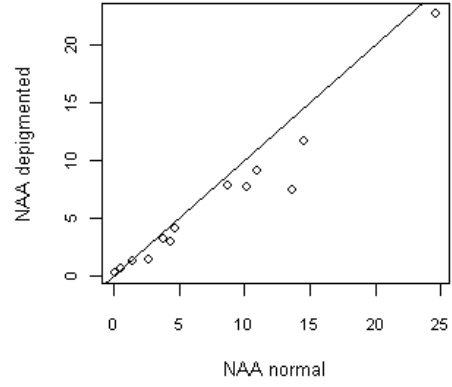
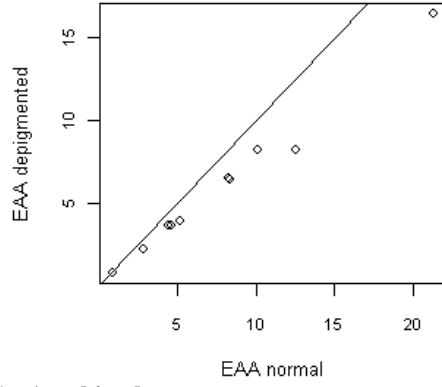
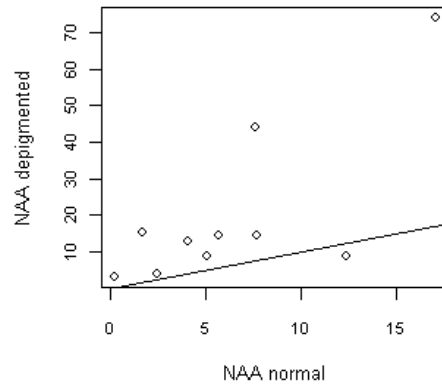
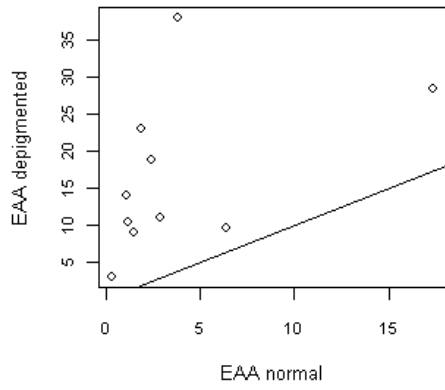


Figure 2

a) Millipedes



b) Amphipods



c) Ants

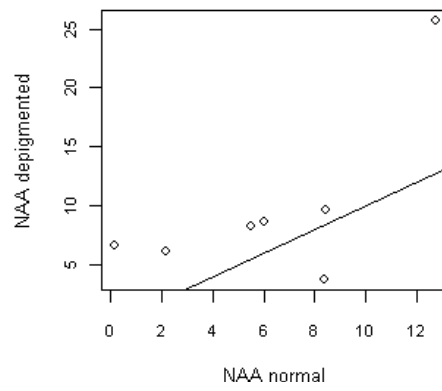
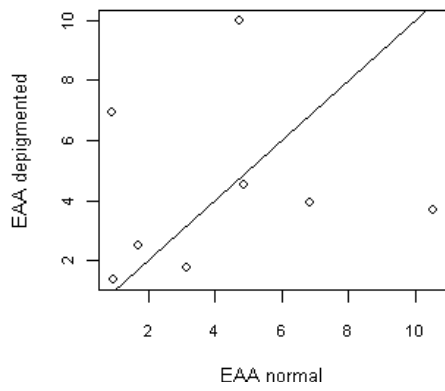


Figure 3

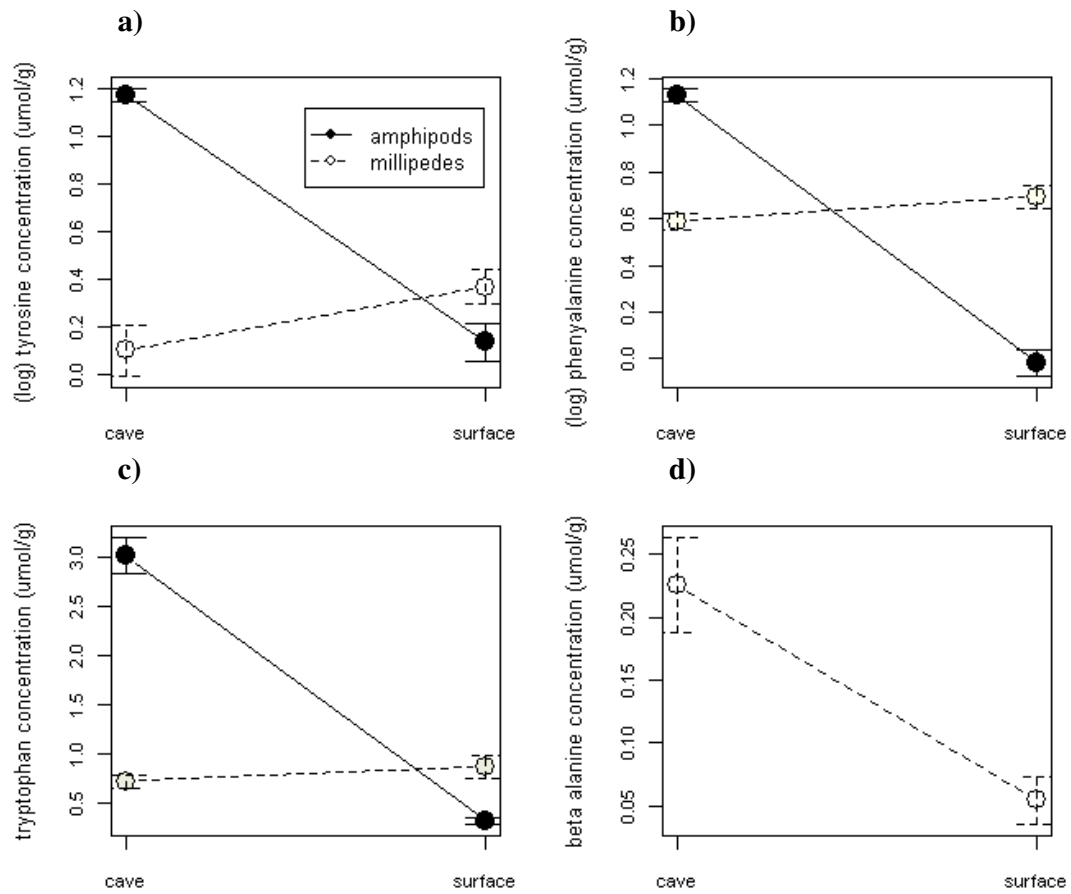
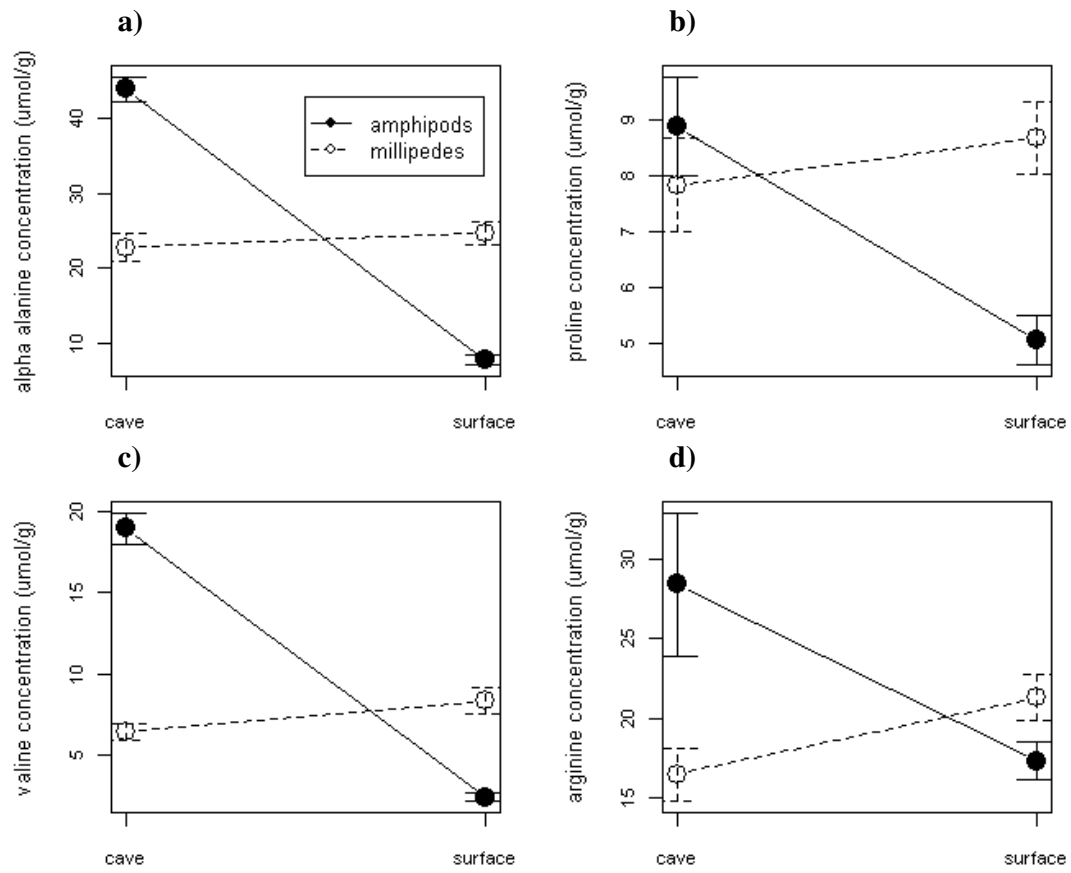


Figure 4



Chapter II: Adaptation to a limiting environment: The phosphorus content of terrestrial cave arthropods

Co-authored with: A.D. Kay and W.F. Fagan

Abstract

1. Stoichiometric imbalances (mismatches between elemental ratios of consumers and their food) are expected to be especially important in detritus-based systems, because poor resource quality may impose severe growth constraints. Such imbalances have been highlighted in producer-based food webs and detritus-based aquatic systems, but similar investigations of detritus-based terrestrial ecosystems are absent from the literature.
2. Cave animals are dependent on detrital subsidies from the surface, and classic studies of cave invertebrates have focused on the consequences of low resource *quantity* for species growth and performance. Here we examine the extent to which nutrient quality, not resource scarcity, may constrain consumer strategies. Specifically, we report the phosphorus (P) content of detrital resources and 17 arthropod morphospecies from a cave food web. We predicted that cave food webs would have large stoichiometric imbalances compared to surface webs due to poorer resource quality in caves.
3. We also predicted, based on the growth rate hypothesis, that cave animals would have a low P content and RNA/DNA ratio relative to counterparts on the surface.

4. We found that cave resources had high carbon (C): P ratio compared to surface litter during the same season, suggesting that cave animals face stronger nutritional constraints than surface detritivores, at least for a portion of the year. Such constraints may be especially important for millipedes, whose C: P was particularly low (i.e. nutrient demanding) relative to cave detritus and relative to other arthropods.
5. Consistent with stoichiometric theory, we found significant negative % P allometry across major phylogenetic groupings and among conspecific cave carabid beetles. We did not, however, find allometric scaling of %P with body size in two millipede species, which may be due to a high P threshold needed for the millipedes' unique cuticular structure. This result is consistent with studies that found % P allometry for predators, but not detritivores.
6. Consistent with our hypotheses, a cave-obligate millipede species that possesses a wide variety of adaptations for cave life had less % P and a lower RNA/DNA ratio than a congeneric cave-transient species that is not adapted for cave life.
7. Our results highlight the potential nutritional constraints of terrestrial cave animals and suggest that their morphological and physiological adaptations may, at least in part, reflect the stoichiometric challenges of cave environments. This study introduces and explores the potential utility of a novel explanation for physiological cave adaptation and may yield insights into cave biodiversity and biogeography.

Introduction

The impact of nutrition on consumer success often hinges on imbalance between the supply and demand of nutrients (Frost et al. 2005, Schade et al. 2005). When supply of a nutrient decreases, an organism must find ways either to increase intake of that nutrient or to minimize nonessential usage. Mobile consumers facing nutrient shortages can increase intake through dispersal or migration (Denno et al. 1980, 2002, Lee et al. 2004, Huberty and Denno 2006, McGlynn et al. 2007). In addition, consumers faced with short-term resource shortages may compensate by increasing feeding rates (Simpson and Simpson 1990, Slansky 1993, Huberty and Denno 2006) or by supplementing their diet through exudate-feeding (Mira 2000, Cook and Davidson 2006) or cannibalism (Denno and Fagan 2003). Alternatively, chronic resource constraints may select for modified life history strategies that are compatible with reduced resource availability. Indeed, several authors have proposed that the nutrient content of available food resources can influence the evolutionary ecology of arthropod species (Elser et al. 2000b, Cross et al. 2003, Denno and Fagan 2003, Kay et al. 2005, Elser 2006). Specifically, in terrestrial systems, widespread phosphorus (P) limitation (Elser et al. 2000a, Elser 2006) may favor lower P requirements for physiological, morphological, and behavioral adaptations of arthropod consumers (Woods et al. 2002, Schade et al. 2003, Denno and Fagan 2003, Perkins et al. 2004). Detritus-based systems are extremely nutrient limited (high C: N, C: P) and may impose particularly severe constraints on the species that reside there (Cross et al. 2003, Tibbets and Molles 2005). Such constraints select against animals with high nutrient demands (Elser et al. 2000b, Martinson et al. 2008, Hambäck et al.

2009), and increase the importance of stoichiometric imbalances (Moe et al. 2005). Thus it is expected that consumers with low nutrient-demands, which suffer less in the face of these constraints (Schulz and Sterner 1999), may be favored in these nutrient-poor ecosystems.

A good example of a nutrient-poor, detritus-based ecosystem is a temperate cave. In the absence of photosynthetic primary productivity underground, almost all caves are detritus-based systems that are supported entirely by food resources which passively fall, wash or are blown in, or by resources that are actively deposited via animal vectors (crickets, bats, wayward animals) (Barr 1967, Culver 1982, Poulson and Lavoie 2000, Fagan et al. 2007). Although some of these resources are nutrient-rich (e.g. animal carcasses, eggs, or feces), most of the food that regularly enters cave environments is nutrient-poor leaf and wood debris. These nutrient-poor plant materials are colonized by bacteria and fungus. Detritivorous arthropods, such as millipedes, some mites and collembola, either feed directly on this leaf material or on the microbial/fungal colonists. Predatory arthropods, such as spiders, pseudoscorpions and beetles, feed on the detritivores (Barr 1967). The nutrient-poor plant materials at the base of the cave food web are likely to affect the life-history strategies of the animals that reside there. Previous cave researchers have hypothesized that these adaptations are a result of low energy (the energy economy hypothesis: Poulson 1963, Culver 1982, Hüppop 2005). Here we examine the extent to which nutrient quality, not food scarcity, may constrain consumer strategies.

Nutrient constraints on growth rate may be particularly important due to the demands for P-rich ribosomal RNA needed to meet the protein synthesis demands of

rapid growth (Elser et al. 1996, Sterner and Elser 2002). Growth rates of cave animals are known to be low relative to surface counterparts (Barr 1968, Mitchell 1969, Poulson and White 1969), but no previous study has investigated whether the unique stoichiometric challenges of cave environments may contribute to this pattern.

In this paper we draw several links between the availability of a key nutrient, P, and these characteristics of terrestrial cave invertebrates. A similar nutrient-related hypothesis has been previously tested in regards to nitrogen and mineral content of cave invertebrates (Studier 1996), which found that both cave orthopterans and their egg-predator, an obligate cave carabid, are low in both nitrogen and potassium. However, to our knowledge, no research on the P content of cave arthropods has yet been reported. We also examine the RNA content and RNA/DNA ratio of cave animals. RNA/DNA ratio, an index for protein synthetic capacity, measures the concentration of protein-making machinery per cell (Buckley 1984) and is a known correlate of growth rate (Buckley 1984, Vrede et al. 2002 (and references therein), Kyle et al. 2003, Weider et al. 2005). High food quality is known to lead to an increased RNA/DNA ratio (Vrede et al. 2002), and generally reflects elevated protein production in response to beneficial conditions (Buckley and Szmant 2004).

In general, our expectation was that variation in resource quality and interspecific stoichiometric condition would covary with previously established variation in above- versus below-ground life histories. Here, we analyze cave resources and cave invertebrates to test the predictions that: 1) Resources found in caves are low quality (low P and high C: P ratio) compared to surface resources; 2) Arthropods found in caves, especially those that are cave-obligates, will have low

nutrient demands (low body % P) compared to related species that are not restricted to caves; 3) Predatory species will have similar % P to the primary consumers (detritivores), as seen in other systems (Martinson et al. 2008); 4) Imbalances between resource C: P and consumer C: P will reflect those seen in other detritus-based systems (Cross et al. 2003); 5) Previously established allometric patterns, wherein % P content decreases with body size (Woods et al. 2004, Hambäck et al. 2009), will also be seen for cave species; and 6) Obligate cave animals will have less P and decreased RNA/DNA ratios than closely related animals (not restricted to caves), reflecting the slowed metabolic rates of cave animals (Mitchell 1969, Hüppop 2005). Investigating the stoichiometry of cave resources and the animals that inhabit these nutrient-poor environments will test these predictions of ecological stoichiometry in a novel system and may help to explain some of the well-known physiological adaptations of these unique species.

Methods

Study site

The study site was a cave-rich region located within a 20 km² area just north of Lewisburg, West Virginia, USA, within the Buckeye Creek Drainage System (USGS HUC 05050003). Pits (vertical caves) chosen for the intensive analysis of resource quality were all located on private land interspersed in a karstic area (a limestone area characterized by dissolution rather than erosion) typical for West Virginia. Some of the dominant trees in this area include elm (*Ulmus* sp.), hickory (*Carya* sp.), oak (*Quercus* sp.), and maple (*Acer* sp.), the leaves of which constitute

the major source of detritus into these caves. The majority of arthropods were collected from the largest cave in this study area (Buckeye Creek Cave), though several additional individuals were collected from four neighboring caves (located less than 1.2 km from the entrance to Buckeye).

Collection methods and sample preparation

We first compared the stoichiometric quality of surface leaves to the quality of resources removed from 12 caves in West Virginia. To provide baseline measures of in-cave resource quality, all macroscopic organic material and the top 6 cm of soil were removed from 11 pits. Vertical caves (commonly called “pits”) were chosen, as opposed to caves with horizontal entrances, because the resources that fall into pits can be quantified easily and are localized primarily within the drop zone (the area directly below the opening to the surface). The chosen pits ranged in depth from 4.5 to 19 m. Organic material (dead leaves, dead animals, fungi, fecal material, and organic-rich soil) was removed from each pit using garbage bags and a pulley system in July 2005. A total of 1.5 metric tons of material (wet-weight) was removed to create a detritus-free baseline condition for a related project that will be reported elsewhere. Representative subsets from each cave were lightly rinsed over a 250 μm sieve to separate dirt and other inert materials from organic material. This rinsing may have disrupted any bacterial films coating the decomposing organic materials and potentially removed bacteria, arthropod fecal material, and other nutrient-rich components. Additional representative subsets, which were not rinsed and thus still contained soil and other particles, were also assembled. Though these samples retained all the nutrient-rich components potentially affected by rinsing, carbon could

not be reliably quantified in these samples because of an excess of inert, inorganic material. However, taken together the two subsets from each cave allowed us to calculate both carbon and phosphorus of cave detrital resources, respectively. Both subsets were dried at 60°C for a minimum of five days, ground to a fine powder using a coffee grinder and a mortar and pestle, and prepared for chemical analysis.

To assess how the detrital resources found in caves differ from those available on the surface, detritus was collected monthly for one year via flower pots (dimensions: height 20 cm, top circumference 0.04 m) embedded at the surface next to the entrance to each pit. Surface detritus consisted of leaves shed in autumn as well as year-round materials or organisms that fell, blew or crawled into the flower pots. We emptied these flower pots monthly because we expected seasonal differences in the quality of surface detrital resources. The contents were prepared for chemical analysis as above.

To explore the sources of variation in % body P among cave invertebrate species, we hand-collected representatives of 17 morphospecies from within Buckeye Creek Cave. We supplemented these collections with additional arthropods collected over 24 h periods in empty pitfall traps smeared with Limburger cheese, which is the standard protocol for baiting cave arthropods (Schneider and Culver 2004). The collection consisted of obligate and transient cave hexapods (Collembola, Coleoptera, Orthoptera), diplopods (millipedes), and arachnids (mites, spiders, pseudoscorpions). Collections were sorted to major groupings and included a representative subset of the core terrestrial cave community. Two pairs of species (millipedes and rhagidiid mites) contained both a cave-transient and a cave-obligate member. Cave-obligate

species exhibit characteristic adaptations to cave life (e.g., absence of pigmentation, elongated appendages, loss of vision) whereas cave-transient species are essentially surface-dwelling species that occasionally wander into caves. For each morphospecies in these pairs, at least two individuals were collected; due to the rarity and conservation status of cave organisms, more individuals could not be collected. Specimens were then stored in a refrigerator for one day to clear their digestive systems and subsequently frozen until preparation for chemical analysis. We designated each morphospecies as either predatory or detritivorous based on the classifications typical for that order/family and literature on cave animals.

To examine further the long-term impacts of prolonged exposure to nutrient constraints on cave species, we compared the C and P content of paired samples of cave species and their closest available surface-dwelling relatives. First, we examined *Pseudanophthalmus* beetles (Coleoptera: Carabidae), a clade of 157 predaceous species and subspecies wholly restricted to caves (Christman and Culver 2001). Here, we focus on two obligate cave species, one of which (*P. fuscus* Valentine 1931) is smaller than the other (*P. grandis* Valentine 1931) (range of size of *P. fuscus* = 4.4-5.6 mm vs. *P. grandis* = 4.9-6.8 mm; Valentine 1932). Individuals were collected from four caves located within the study site. To compare this exclusively cave-dwelling genus to surface dwelling relatives, we searched the literature and recovered previously published P values for carabid beetles (data from Woods et al. 2004).

The next species pair we examined included two detritivorous cave millipedes, *Pseudotremia hobbsi* Hoffman 1950 and *P. fulgida* Loomis 1943 (Chordeumatida: Cleidogonidae). While *P. hobbsi* can be found in caves, it is not a

cave-obligate species, and does not show the morphological adaptations typical of cave-obligate species. *Pseudotremia fulgida*, on the other hand, is a blind, depigmented, obligate cave species. These two millipedes co-occur in many caves, and representatives of both species, (including subadult individuals) were hand-collected from Buckeye Creek Cave. Subadult individuals were not identifiable to the species level because identification is based on mature male gonopods (Shear 1969), but were known to be either of the two *Pseudotremia* species of interest. Specimens were stored in a refrigerator for one day and subsequently frozen.

To investigate the potential molecular mechanism underpinning the differences observed in P content, we measured the RNA content and RNA/DNA ratio of the millipedes. Because P is predominantly found in rRNA, and cave animals typically show reduced growth rates, we predicted that cave millipedes would have less RNA (and lower RNA/DNA) than their surface counterparts. This is a key prediction of the molecular mechanisms underlying the growth rate hypothesis of ecological stoichiometry (see Kay et al. 2005 and references therein). Animals that were set aside for RNA were collected in the field and brought home alive in a cooler prior to being stored in a -80°C freezer.

Chemical analyses

Analysis of C content was performed on dried samples of detritus (surface and cave) and prepared animal specimens using a LECO CHN analyzer. For analysis of P content, animal specimens (at least two individuals per morphospecies) were removed from the freezer and dried at 60°C for three days. Animals smaller than 2 mg were assayed whole, whereas animals greater than 2 mg were homogenized into a fine

powder, subsamples of which (0.5 - 2 mg) were then analyzed via colorimetric analysis after persulfate digestion using the ascorbate-molybdate method (APHA 1992, Woods et al. 2004). Percent recovery in P and CN assays was determined by comparison to either apple leaves or bovine muscle standards.

DNA and RNA concentrations were measured in whole organisms stored in a -80°C freezer until analysis. DNA and RNA were measured using the assay described in Kyle et al. (2003); this involves sample homogenization (with mortar and pestle) in an extraction buffer containing N-lauroylsarcosine, followed by sonication, and then staining with Ribogreen (Molecular Probes, Eugene, OR, USA). DNA and RNA content was estimated from comparisons of fluorescence in replicate subsamples that were treated with RNase, RNase and DNase, or were left untreated. DNA and RNA estimates per wet mass were quantified from comparisons to fluorescence in standards; standards were baker's yeast RNA and calf thymus DNA (Sigma-Aldrich, St. Louis, MO, USA). DNA and RNA estimates per wet mass were converted to estimates per dry mass using the parameters of the relationship between wet mass and dry mass (previously determined using separate *P. hobbsi* (n = 11) and *P. fulgida* (n = 8) individuals).

Data analyses

Surface resource quality was obtained by calculating the average % P per month using the flower pot samples. We chose to examine averages over time to account for temporal variation in input rates (e.g., leaf fall) and litter quality, and to assess the seasonality of the resources that are most likely to fall into a cave. Because chemical analyses for C and P were each independently replicated on different

resource subsets from a given cave, we calculated the average C content and average P content for each pit and used these values to calculate the average molar C: P ratio for each pit. We then compared the average C: P across all pits to the quality of the surface detritus (as reflected by monthly molar C: P). After log transforming the C: P values and removing one outlier (from n = 135 samples) to account for non-normal data, we performed a t-test with unequal variances to test if cave resources and surface litter differed in average C: P ratio. We also performed a Wilcoxon rank-sum test on the means between the cave samples and the July surface samples to investigate if cave resources differed from surface resources during the same season as when the cave resources were removed.

Designating the cave animals as either predators or detritivores, we then tested whether trophic and phylogenetic constraints could explain variation in the stoichiometry of cave arthropods. Based on the findings of Woods et al. (2004), Hambäck et al. (2009) and Martinson et al. (2008), we predicted that there would be no distinguishable difference in P between predators and detritivores. This prediction follows from Woods et al. (2004) who suggested that while herbivores eat lower quality food, they eat more of it, whereas predators eat higher quality food, but consume smaller quantities. To examine the validity of this prediction, we first averaged the body mass and % P values for all individuals within a morphospecies (excluding subadult millipedes unidentifiable to species). After log-transforming average P values and dry mass, we performed an ANCOVA, with the model: $\log(\text{body P}) \sim \text{trophic level} * \log(\text{body mass})$, with each species as an observation. To account for variation driven by phylogeny, we compared log-transformed P content

across the major groups (Diplopoda, Hexapoda, and Arachnida) with an ANCOVA model also including log(body mass) as a covariate. We tested for paired differences between groups using the same linear model with planned contrasts.

Using average % C and % P, we calculated the degree to which the (molar) C: P of resources differed from the C: P of the consumers by looking at the ratio of these two numbers (Fagan and Denno 2004). This ratio provides one measure of how mismatched the consumer is from its resources (*i.e.*, the stoichiometric constraint faced by the consumer). We examined the ratio between cave resources and one type of cave detritivore (the obligate cave millipede) as well as the ratio between one type of cave predator (the obligate cave beetle) and a potential prey species (either the obligate cave millipede or a collembola). Because we did not explicitly measure carbon content of the collembola, we used data from the literature to acquire the average carbon content of three species of entomobryid collembolans (= 47.5 % C, data from Teuben and Verhoef 1992), and used this to generate an approximate C: P of the collembola (incorporating our quantification of phosphorus (see Elser et al. 2000a for similar methods). We assume that these potential prey species are representative of the types of prey that the beetles may consume. We compare these ratios to published values provided in Table 1 of Cross et al. (2003). Though the authors in that paper used the arithmetic difference between ratios as “elemental imbalance”, we calculated the ratio of their C: P values for a more direct comparison with our results.

Because cave species are completely dependent on allochthonous detritus (detritus that originated on the surface), we predicted that they would have lower

body P content than surface-dwelling animals. Within each of the two groups of cave species (beetles, millipedes), we used linear models to test whether % P differed across species based on habitat. We constructed a model of body P content with the categorical predictor *species*, the continuous variable *size*, and their interaction. If the interaction term was not significant, that covariate was removed from the analysis. Both models required the exclusion of one outlier to correct for non-normal residuals. The same model ($y \sim \text{species} * \text{size}$) was also used to test whether % DNA, % RNA, and RNA/DNA concentration differed between congeneric cave- and surface-dwelling millipedes. To account for non-normal residuals, the models for both % DNA and % RNA each necessitated the removal of two outliers (one shared). These three points were therefore also excluded from the model examining the RNA/DNA ratio. For these models, all variables were log-transformed.

All analyses were performed in R (version 2.7.0; R Development Core Team 2008).

Results

Characterizing the elemental stoichiometry of cave resources

Across the 12 caves, the resources removed varied greatly in % P, ranging from 0.04% to 0.63 %, with an average of $0.14 \% P \pm 0.02$ (mean \pm 1 SE) in the rinsed samples, and ranging from 0.05% to 0.96%, with an average of $0.22 \% \pm 0.05$ in the unrinsed samples. The resources on the surface varied seasonally, ranging from $0.06 \% P \pm 0.01$ in November/December to $0.12 \% P \pm 0.01$ in April. Using the rinsed cave resource data, the (log) molar C: P of the cave resources was not significantly different from that of the time-averaged surface litter (t-test with unequal variances: t

= -1.34, df = 12.04, p = 0.206). However, the C: P of the unrinsed cave resources was significantly different from the annual surface litter (t-test with unequal variances: t = -2.36, df = 11.74, p = 0.036), with the cave samples having a lower average C:P than the surface samples. The average C: P of the rinsed cave resources was higher than the surface litter during the same time of year when that the caves were originally “emptied” (Cave resource C: P = 1181.5 vs. July surface detritus C: P = 698.7), and this difference was marginally significant (Wilcoxon W = 28, p = 0.069). The unrinsed cave resources, though also higher in C: P than the July surface resources were not significantly different (Cave resource C: P = 913.1 vs. July surface detritus C: P 698.7, W = 40, p = 0.3451). Surface resources from the fall (October and November/December) were of the lowest quality, with high C: P ratios (average C: P 2598 and 2697, respectively) compared to the other months (Fig. 1).

The phosphorus content of terrestrial cave invertebrates

Average values for the % P in arthropods found in Buckeye Creek Cave ranged from 0.71 % P (oribatid mites) to 3.11 % P (immature millipedes). Overall, the millipedes and collembola were the groups highest in P (Fig. 2). The species that we identified as cave-obligate (denoted with asterisks in Fig. 2) included the two carabids (*P. grandis* and *P. fuscus*), the cave millipede (*P. fulgida*), and a cave-dwelling rhagidiid mite. The cave-dwelling rhagidiid mite and chordeumatid millipede both contained less P than their respective surface-dwelling counterparts (Figure 2).

We hypothesized that there would be no difference in body % P between detritivores (including the millipedes, collembola, oribatid mites, and crickets) and

predators (rhagidiid mites, carabid beetles, spiders, and pseudoscorpions). We found that, despite the slightly higher average % P of detritivores (Fig. 2, inset), this group is highly variable in P content. Therefore, no significant difference was found between the two trophic levels ($F_{1,14} = 0.938$, $p = 0.394$). When species were classified into broad phylogenetic groupings, the interaction of body size and phylogenetic group was not significant ($F = 0.001$, $p = 0.990$) and was removed from the model. However, P content did differ with phylogenetic grouping ($F = 11.10$, $p = .002$, Fig. 3A) and body size (log transformed) ($F = 18.16$, $p = 0.001$). All phylogenetic groups differed significantly from each other (arachnids vs. diplopods: $F = 16.10$, $p = 0.007$; arachnids vs. hexapods: $F = 4.95$, $p = 0.048$; diplopods vs. hexapods: $F = 96.17$, $p < 0.001$). Interestingly, the slope of the allometric relationship between log body % P and log body size was nearly identical for all three groupings (slope estimates: arachnids = -0.151, diplopods = -0.145, hexapods = -0.156, Fig. 3B).

Calculating the mismatch between cave resources and cave species

The C: P mismatch between cave detritus and cave millipedes was twice the mismatch between that of stream detritus and shredders or between terrestrial plants and herbivores reported elsewhere, regardless of whether the rinsed or not rinsed cave samples were used (Table 1). In fact, the imbalance between cave detritus and the detritivorous millipede, which is driven primarily by the very low C: P of the millipede, is higher than any other stream resource/consumer or the terrestrial herbivore/vegetation comparison. Cave predators, on the other hand, match closely with their food source, considering either millipedes or collembola as potential prey

items. This mismatch between beetles and detritivores is negligible in comparison to the mismatch between the detritivores and detritus (Table 1).

The P content of cave carabids and millipedes

The average P content of the surface carabids compiled from the literature was $0.617 \% \pm 0.07$ ($n = 5$ species), which was lower than that of the obligate cave-dwelling carabids ($0.815 \% \pm 0.04$ P; $n = 18$ individuals). This difference in % P between habitats was significant ($F_{3,19} = 10.32$, $p < 0.001$), but there was also a significant interaction between species and size ($F = 16.35$, $p < 0.001$). There was a marginal negative allometry of body % P for the large obligate cave beetle species ($F_{1,15} = 4.104$, $r^2 = 0.2148$, $p = 0.061$), but no relationship could be observed for the smaller obligate cave beetle species (Fig. 4A). An allometric relationship with % P was observed for surface carabids taken from Woods et al. (2004) when one outlier was removed ($F_{1,2} = 27.10$, $p = 0.035$).

The average P content for the transient millipede was $1.50 \% \pm 0.04$ ($n = 19$ individuals), which was slightly higher than that of the obligate cave-dwelling millipede ($1.39 \% \pm 0.03$ P; $n = 27$). There was no effect of size or the size * species interaction when comparing adults of the two species. With size removed from the model, the species were marginally different in % P ($F = 3.59$, $p = 0.065$). The subadult millipedes, which could not be assigned to species, had exceptionally high P content, with an average of 3.11% (Fig. 2). Across all millipedes sampled, we found a negative allometric relationship ($F_{5,46} = 84.82$, $p < 0.001$, Fig. 4B), but there was a significant size * species interaction ($F = 61.87$, $p < 0.001$). When we examined each species alone, we found negative allometry in the subadults ($F = 30.85$, $p = 0.005$), but

not among adults of either species (*P. hobbsi*: $F=0.2203$, $p = 0.645$; *P. fulgida*: $F = 0.0877$, $p = 0.770$).

Biochemical content of cave millipedes

Without accounting for species' size, the only biochemical component that differed between cave and transient millipedes was the RNA/DNA ratio (Fig. 5A). To statistically analyze the biochemical content of cave millipedes, we fitted three separate linear models of biochemical content (log transformed % DNA, % RNA or RNA/DNA ratio) with the predictor variables of species, size (log transformed), and their interaction. DNA content (as a percentage of dry mass) differed between cave millipede species ($F_{3,15} = 65.10$, $p < 0.001$), with a significant interaction between species and size ($F = 6.49$, $p = 0.026$, Fig. 5B). In contrast, RNA content (% dry mass) did not differ between species ($F_{2,13} = 2.169$, $p = 0.154$, Fig. 5C). For the RNA/DNA ratio, there was no effect of size and the RNA/DNA ratio of transient millipedes was 45% higher than that of the obligate cave millipedes ($F_{1,13} = 21.60$, $p < 0.001$, Fig. 5D).

Discussion

Our goal was to examine the potential for resource quality to constrain the biochemistry of cave arthropod consumers. The C: P content of cave resources was not as different from above-ground detritus as we anticipated. Seasonal variation in the quality of allochthonous resources entering caves and the potential for bacterial enrichment of detrital resources in caves may both contribute to the overall lack of difference between surface and in-cave resources. Although our findings about the

relative nutrient content of basal resources are equivocal, we did observe that obligate cave animals have less body % P than closely-related surface-dwelling relatives. We also showed that cave millipedes have a lower RNA/DNA ratio than transient millipedes, indicating a decreased capacity for protein synthesis in the obligate species and suggesting a physiological mechanism for the previously-established reduced growth rate of cave invertebrates. Overall, our results suggest that the great nutritional mismatch between resources and cave-obligate consumers may contribute to a mechanistic explanation for known cave adapted life-history traits.

Characterizing the elemental stoichiometry of cave resources

Although on average, cave detrital material was nutrient poor (0.14 or 0.22 %P, rinsed and unrinsed samples, respectively), some of the caves had rather P-rich detrital resources (e.g. 0.95% P). This material was likely nutrient-rich fecal material, fungus, or bacterial films on these decomposing resources (Maraun and Scheu 1996, Cross et al. 2003). The 29 % difference in C: P between rinsed and unrinsed cave detrital samples supports the conclusion that soluble nutrient-rich material was present in at least some of these sample (Fig. 1). Despite the variation between caves in detrital % P, the average C: P ratio of both cave resources (rinsed: 1181.5; unrinsed: 913.1) was within the range of the C: P ratio of surface litter (July: 698.7; Nov/Dec: 2679.6). We had expected to find a substantial difference in the nutrient content of detritus in caves compared to above ground samples. For example, the bulk litter on the forest floor of a geographically and ecologically similar mature oak forest in New Jersey was much richer, with a C:P ratio of 360 (Lang and Forman 1978). It is likely that our method for sampling the surface litter quality is not comparable to

the resources removed from the caves. The surface litter that we collected consisted of leaves that had fallen or blown into the flower pots. Thus, examining only leaves on the surface in part explains why the surface resources were of poor quality compared to the cave resources, which also contained organic rich material such as feces or bacteria. This comparison results in the high carbon content of the surface leaves (annual average = 39.9 % compared to the cave resources (34.7 %)), and the higher phosphorus contents of cave samples. For a more accurate comparison, studies investigating the litter layer of the surface soil should be employed. For example, examining different forest ecosystems in Greece, Kavvadias et al. (2001) collected all litter on the forest floor at the three horizons of the humus profile (litter (L), fermentation (F), and humus (H)), and found higher quality resources in the fermentation and humus layers than in the litter layer (average C: P 658 L compared to average C: P 367 F, H). Such a sampling strategy, if applied to the surface above the caves, is likely to yield higher quality, decomposing resources, than the freshly fallen litter that we used for this comparison, and a more appropriate comparison to the cave samples.

Cave resources were of lower quality than the surface resources collected at the same time of year as the initial cave resource removal (July). Though marginally significant, the cave resource C: P (rinsed) was 69 % higher than the surface litter collected at the same time (Fig. 1). Because cave resources are of poor quality even during the summer, we suspect that cave resource quality would only degenerate during the fall and winter months, when surface resources are of poor-quality and there is the greatest potential for input of these nutrient-poor leaves into caves.

The phosphorus content of terrestrial cave invertebrates

Consistent with previous studies (Woods et al. 2004, Martinson et al. 2008, Hambäck et al. 2009), we did not find a significant difference in body % P between detritivorous (millipedes, collembola and oribatid mites) and predaceous (spiders, carabid beetles, rhagidiid mites and pseudoscorpions) species. The lack of a difference between trophic groups may be in part due to the large variation in % P content of detritivores, as some species contained very high levels of P (millipedes) compared to others (oribatid mites). Subadult millipedes, which as outliers were excluded from our analysis, were extremely rich in P. Higher P levels in juvenile individuals have also been seen in *Daphnia* and *Drosophila*, in which juvenile stages have higher growth rate and P requirements than adults (Boersma and Kreutzer 2002, Vrede et al. 2002, Cross et al. 2003, Elser et al. 2006). The P content of the adult cave millipedes was nearly twice as high as the reported average for other arthropods (Woods et al. 2004, Martinson et al. 2008), but was within the range reported for decaying millipede carcasses on the surface (ranging from 1.07 %P at death to 1.59 %P during the first month of decomposition, Seastedt and Tate 1981) and was similar to mealworms and waxworms (Barker et al. 1998). The relatively high body content of P in millipedes may result from their rigid, generally heavily calcified cuticle (Cloudsley-Thompson 1950). As in vertebrate bones, calcium (Ca) and P appear to co-occur in arthropod cuticles, where they may operate jointly to increase cuticular strength and durability. For example, analysis using electron microprobes has found Ca and P embedded in the cuticle of a ground-dwelling fly larva (Cribb et al. 2005), a burrowing species for which a strong cuticle would be especially important. Cuticular

P content may also explain the high % P in terrestrial isopods (Tibbetts and Molles 2005) and stream crustaceans (Evans-White et al. 2005). Such a situation would also explain the high P content we have observed for aquatic obligate cave isopods ($1.88\% \pm 0.16$, $n = 9$, unpublished data).

We found no allometric pattern of body % P and dry body mass in the adult millipedes. Such a lack of P allometry in detritivores has recently been reported in Martinson et al. (2008). The lack of P allometry in adult millipedes may be because P is predominantly important in the immature millipedes, which above and beyond their cuticular needs also require P for rapid growth and do not yet have the body composition of an adult. Once adulthood is reached, there may be a threshold amount of P needed for maintaining body composition (namely cuticular structure), and less required for every day maintenance (as the adults do not grow). Similar ontogenetic transitions in body composition have been found in *Drosophila* (Watts et al. 2006), *Daphnia* (DeMott 2003), and the copepod *Mixodiaptomus* (Carillo et al. 2001). We did, however, see interesting allometric patterns across large phylogenetic groupings, finding that across broad groups (arachnids, diplopods, and hexapods) there exist nearly identical relationships between log size and log % P (Fig. 3B). We also discovered an interesting allometric pattern with the cave beetles. As other studies have found for predators (Woods et al. 2004), the smaller species (*P. fuscus*) had higher % P than the larger species (*P. grandis*). *Pseudanophthalmus grandis*, the larger species, also has a very large geographic range compared to the smaller species, a phenomenon also reported for other groups of cave beetles (Barr 1967). It is possible that the less nutrient-demanding, larger species is able to survive in a

greater range of locations. Further examination of the P content of other *Pseudanophthalmus* species may yield insights into cave biogeography.

Differences between cave-dwelling and surface species

In two cases where recognizable pairs of obligate-cave and surface species existed, (chordeumatid millipedes and rhagidiid mites), the cave species were both lower in % P than their surface counterparts, supporting the growth rate hypothesis (Sturner and Elser 2002). When obligate cave carabid beetles were compared to literature data for surface carabid beetles, we found a significant interaction between species and size. However, because the literature data included many different carabids, phylogenetic and environmental variation are likely to influence this result.

Obligate cave millipedes had less % P than transient millipedes, which may be in part due to their thinner cuticle, which is widely considered an adaptation to the humid cave environment (Culver 1982). In addition, as predicted by the growth rate hypothesis, obligate cave millipedes may have had less P due to decreased allocation to P-rich rRNA for growth (Sturner and Elser 2002). While the millipede species differed in % DNA owing to a species by size interaction, they did not differ in their RNA concentration. While RNA content represents potential for growth, the RNA/DNA ratio represents the growth that is actually achieved, especially if it is constrained by nutrient deficiency (Vrede et al. 2002). As a measure of protein synthetic capacity, this ratio depends on metabolic growth and has been shown to be higher in the growing season for certain species (Buckley and Szmant 2004). In our study, cave millipedes had a significantly lower RNA/DNA ratio compared to their congeneric counterparts. Our finding supports the use of the RNA/ DNA ratio as a

surrogate for synthetic capacity in these animals and is further evidence of the slower growth rates in cave animals vs. transient congeners.

Because of their overall high body % P (1.39 %) compared to other arthropods, cave millipedes are greatly out of stoichiometric balance with their food resource. The great disparity between C: P of cave detritus and C: P of cave millipedes (around 20 for both the cave-transient and cave-obligate millipedes) is larger than any previously reported stoichiometric mismatch (Table 1). This mismatch, a magnitude higher than mismatches reported elsewhere, suggests that cave millipedes may be faced with extreme nutrient constraints. Some of this mismatch may be offset by millipedes selectively feeding on particular nutrient-rich components of the detrital resource base. For example, although millipedes have been reported to feed directly on dead wood (which is extremely nutrient-poor, Kerkhoff et al. 2006), they have also been found feeding on nutrient-rich fecal material (Shear 1969). Nevertheless, to offset the unusually large dietary stoichiometric mismatch reported here, such selectivity in millipede feeding would have to be quite extensive. To the degree that the stoichiometry of the millipedes' realized diets even remotely approximates the stoichiometry of within-cave resources, the observed mismatch between detritus quality and millipedes' needs would certainly provide a reasonable explanation for the slow growth rates (Cross et al. 2003) and reduced protein synthetic capacities (RNA/DNA; Fig. 5) of cave millipedes.

It is also possible that protein synthesis may be limited by other nutrients, such as nitrogen, whereby it is not transcription, but translation that is inhibited (Hessen et al. 2007). Limitation of energy or nitrogen has been shown to decouple the

relationship between RNA and P (Elser et al. 2006). Nitrogen limitation was not investigated here, but Studier (1996) found that cave crickets do indeed have less N than surface crickets, which may be due to the thin exoskeleton of cave species. The possibility of N-limitation in cave species and its biochemical ramifications are currently under investigation (Schneider et al. in review, see Chapter I).

Conclusions and future directions

The nutrient-poor environment of caves is an ideal system in which to investigate questions focused on the interplay between resource quality and generalized adaptations to cave life. These adaptations include morphological changes such as a lack of pigment and thin cuticles, as well as physiological characteristics, such as slow reproductive and developmental rates. Though we have focused here on the terrestrial cave environment, recent syntheses concerning aquatic cave organisms and their habitats suggest some potential routes for future research. For example, subterranean aquatic habitats are monopolized by amphipods, isopods, and copepods, yet aquatic insects (which dominate many surface habitats) are scarcely represented in caves (Gibert and Deharveng 2002). Perhaps stoichiometric theory could potentially explain what makes for a good cave colonizer and allow us to investigate which species “succeed” in different environments (Michaels 2003), such as the nutrient-limited cave habitat. Perhaps the nutritional constraints imposed by cave environments are sufficiently strong as to exclude certain types of consumers, such as those that cannot efficiently store or assimilate limiting nutrients, although more research is needed to substantiate this possibility.

Acknowledgements

We wish to thank the local landowners who allowed us onto their property to collect cave arthropods. J. Gulley, J. Hajenga, and D. Cowan greatly assisted KS in the field, painstakingly emptying caves of resources. S. Bertram and M. Kyle provided guidance on running the P analyses, and S. Seidl and H.M. Martinson helped with the lab work. This work was supported by grants from the Cave Research Foundation, the Cave Conservancy Foundation, the National Speleological Society, the West Virginia Association of Cave Studies, and the University of Maryland Hockmeyer Graduate Fellowship to KS. Collecting permits were obtained from the WV DNR to KS (permit numbers: 2005.259, 2006.122, 2007.018). We thank J.D. Gilbert, D. Gruner, H.M. Martinson, and D.C. Culver for their constructive comments on an earlier form of the manuscript.

Tables

Table 1. Comparisons of % C, % P and molar C: P among detritus removed from caves (including rinsed (R) and not rinsed samples (NR), see text) and obligate cave invertebrates. Numbers in parentheses are sample sizes for invertebrate analyses. The transient cave millipede, *P. hobbsi*, is included for comparison. Also included are previously published values of stream and terrestrial resource-consumer pairs.

	%C	% P	C: P	C: P Mismatch ¹
Detritus				
Resources removed from caves (R)	34.77	0.14	1181.50 ²	
Resources removed from caves (NR)		0.22	913.11	
Detritivores				Detritus/Detritivore
<i>P. hobbsi</i>	28.84 (7)	1.39 (27)	60.51	19.52 (NR) 15.09 (R)
<i>P. fulgida</i>	32.31 (5)	1.50 (19)	62.82	18.81 (NR) 14.53 (R)
Collembola ³	47.52 (6)	1.36 (18)	101.91	11.59 (NR) 8.96 (NR)
Predators				Detritivore/Predator
<i>P. fuscus</i>	44.54 (3)	1.21 (2)	107.28	0.59 (Millipede) 0.94 (Collembola)
<i>P. grandis</i>	50.83 (13)	0.77(17)	192.51	0.33 (Millipede) 0.53 (Collembola)
C: P Mismatches in other systems⁴				
Leaf detritus / Stream shredders				9.76
Stream epithilion / Stream scrapers				4.72
Stream prey / Stream predators				1.45
Terrestrial plants / Terrestrial herbivores				8.34
¹ C: P mismatch calculated as the ratio of C: P (resource) / C: P (consumer).				
² Average molar C: P of cave resources is the grand average across the average C: P for each of the 11 caves. For NR samples, we used the average Carbon from the rinsed samples.				
³ The average C concentration for collembola is taken from data from Tueben & Verheof (1992).				
⁴ Data for other systems taken from Table 1 in Cross et al. 2004. We used their raw numbers of C: P to calculate mismatch values (whereas they used arithmetic differences).				

Figure Legends

Figure 1. The quality (average molar C: P) of food removed from eleven caves (thatched bars) compared to the quality of the litter collected monthly on the surface. The two bars for the cave resource quality include samples that were either rinsed (R) or not rinsed (NR) (see text).

Figure 2. A community-wide comparison of the P content of 17 arthropods collected from Buckeye Creek Cave. Predatory species are shaded in gray. Asterisks denote species that are restricted to caves. The inset figure shows the average % P for detritivores compared to predators.

Figure 3. The P content (log transformed) of A) three major phylogenetic groups (arachnids, diplopods, hexapods), all of which are significantly different from each other and B) these three major groups plotted against average log (dry mass). Each point represents a species in that grouping, and the lines represent the regression of % P on body size for that group.

Figure 4. Percent P allometry of A) carabid beetles, including surface carabids (compiled from the literature) and two congeneric obligate cave beetles (*Pseudanopthalmus fuscus* and *P. grandis*) and B) chordeumatid millipedes, including two congeneric cave millipedes (obligate = *Pseudotremia fulgida*, transient = *P. hobbsi*) and subadult stages of either *P. hobbsi* or *P. fulgida*.

Figure 5. The concentrations of DNA and RNA (as percent dry mass) and the RNA/DNA ratio of two cave millipedes (A); the concentrations of B) DNA (percent dry mass), C) RNA (percent dry mass), and the ratio D) RNA/DNA for the two millipede species as a function of log body size (dry mass (mg)).

Figures

Figure 1.

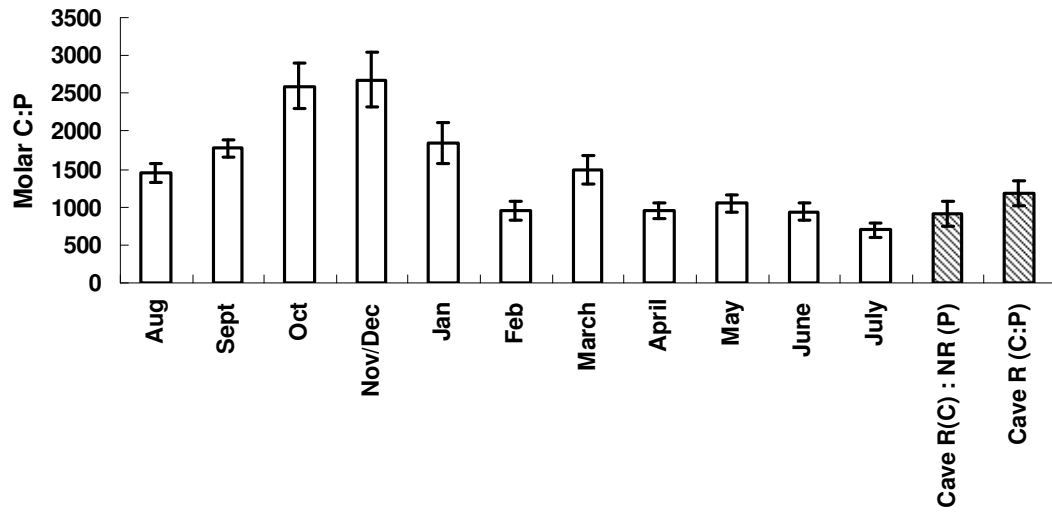


Figure 2.

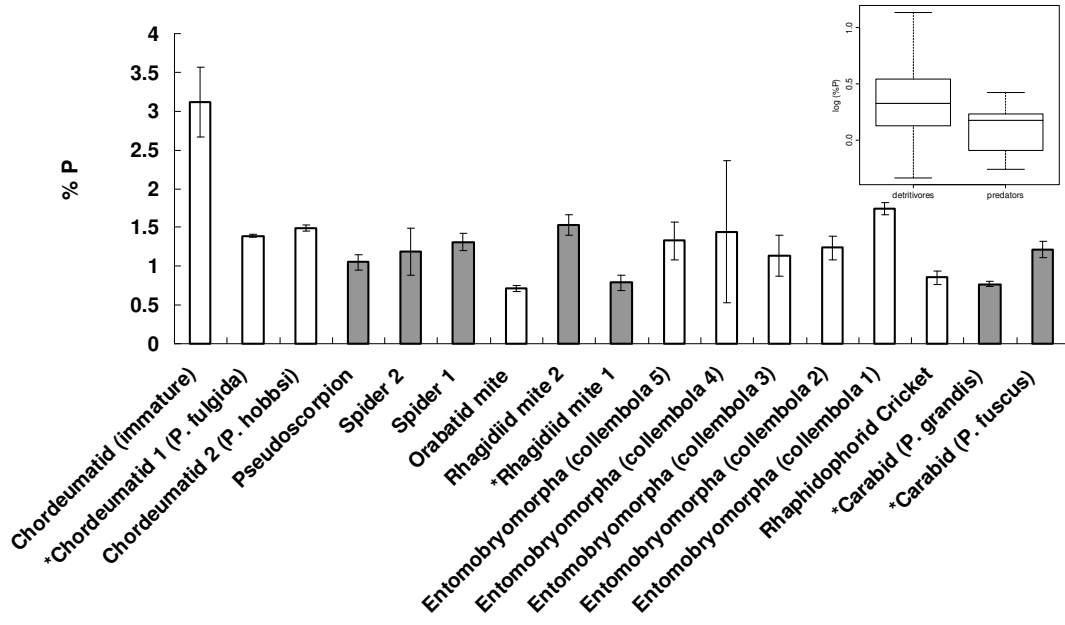


Figure 3.

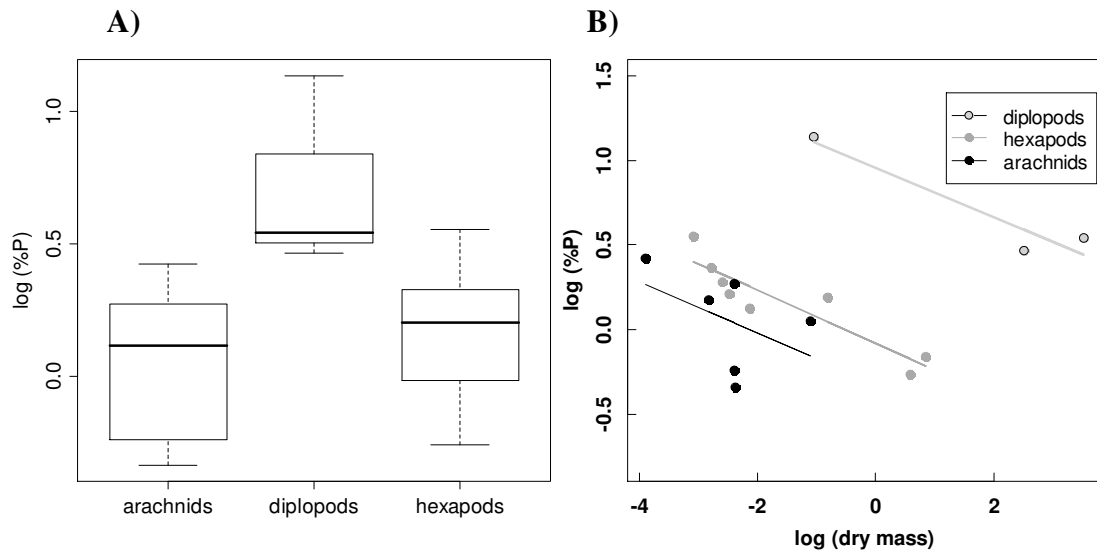
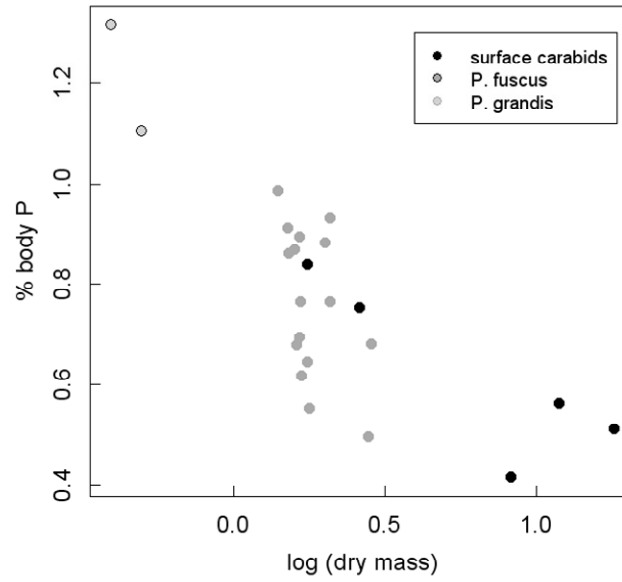


Figure 4.

A) Carabid Beetles



B) Chordeumatid Millipedes

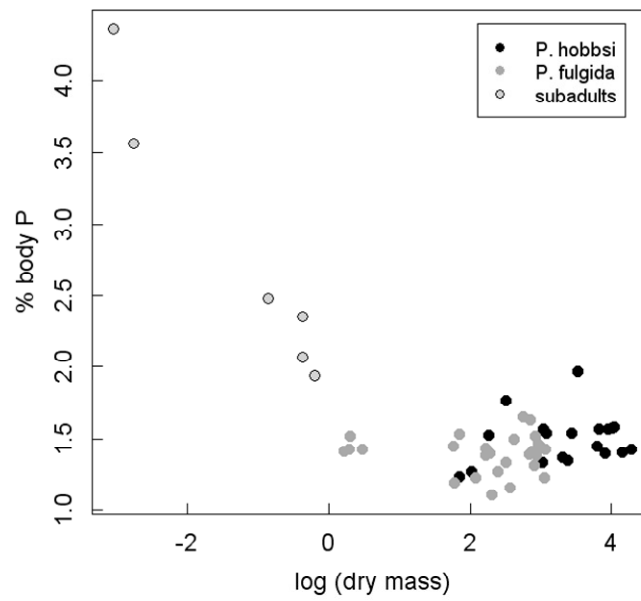
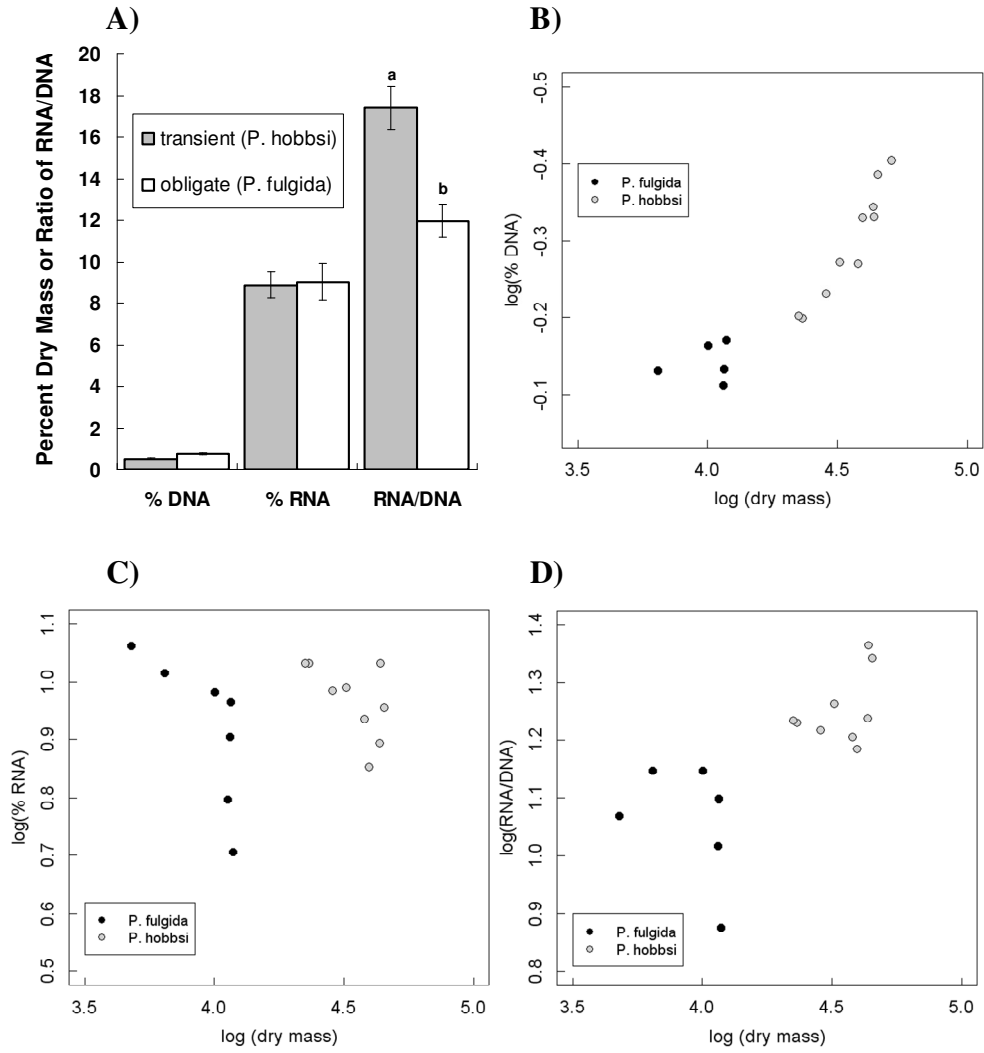


Figure 5.



Chapter III: Invertebrate succession in a completely donor controlled system: Results from an ecosystem resource manipulation experiment

Co-authored with: M.C. Christman and W.F. Fagan

Abstract

Spatial resource subsidies can greatly affect the composition and dynamics of recipient communities. While aquatic subsidies to terrestrial habitats, terrestrial subsidies to aquatic habitats and aquatic subsidies to aquatic habitats have received previous attention, little is known about direct terrestrial subsidies to terrestrial habitats where primary productivity is absent. Caves represent one such habitat. Here, we performed an ecosystem-level manipulation experiment to test the direct influence of detrital subsidies on community structure in a terrestrial system without autochthonous productivity. After performing baseline censuses of invertebrates, all organic material was removed from 12 caves and exclusion boxes were constructed to prohibit natural resources from entering. Next, each cave was stocked with standardized quantities of two major natural subsidies to the cave environment: leaves (leaf packs) and carcasses (commercially supplied rodents); these were restocked upon exhaustion. Monthly for two years, we measured the invertebrate colonization and utilization of these resources. Over the course of the experiment, 102 morphospecies were observed. Overall, detritivorous collembolans and diplopods

were the most abundant invertebrates on the leaf packs whereas dipteran larvae and collembolans were most abundant in the rat treatments. On average, caves that received rat and leaf treatments did not differ in species richness, but invertebrate abundance was significantly higher in rat caves over both the duration of the experiment and the temporal “life” of the individual resources. Post-manipulation invertebrate communities differed depending on the type of subsidy introduced, and by the end of the experiment, caves that received the same subsidy clustered together based on community composition. In addition, the invertebrate community utilizing the resource changed over the duration of the experiment, and evidence of succession (i.e. directional change) was observed. Results from this study show how allochthonous resources can drive the community dynamics of terrestrial invertebrates in cave ecosystems and highlight the need for the surface environment to be considered when managing and protecting these unique habitats.

Introduction

Resources, especially in the form of spatial subsidies, can greatly influence biodiversity patterns and community dynamics. For example, allochthonous detritus (i.e. detritus present in a location different from its place of origin), can have great effects on recipient communities (Yee et al. 2007), by invoking direct numerical responses in the resident populations (Polis and Hurd 1995) ultimately influencing species interactions, trophic structure, and community assembly and dynamics (Anderson et al. 2008). In addition, detrital subsidies often stabilize the recipient community (Moore et al. 2004), especially in unproductive systems or systems that receive regular and strong pulses of such subsidies (Polis et al. 1997).

The type of community response generated by an allochthonous detrital subsidy depends on the type of the resource (Yee et al. 2007), the trophic level that receives the input (Huxel et al. 2002) and the type of habitat studied (Polis et al. 1997). Because “detritus” encompasses all decaying and extruded matter, the quality of detritus and the temporal usability of detritus vary dramatically across resource types. For example, when compared to plant material, animal detritus is a high quality resource (Yee and Juliano 2006) that decomposes at a faster rate (Swift et al. 1979, Yee and Juliano 2006, Yee et al. 2007) and may be more directly available to consumers (Garman 1991, Hunt 1975, Mason and MacDonald 1982). Many studies have examined the consequences of heterogeneous resource subsidies, specifically in aquatic systems, such as tree-holes (Yee and Juliano 2006), lakes (Cole et al. 2006), pitcher plants (Miller and Kneitel 2005 and references therein) and streams (Kawaguchi et al. 2003, Kawaguchi and Nakano 2001). In terrestrial systems, such as desert islands and deserts themselves, the influence of detrital subsidies have also been studied, yet these systems still maintain *in situ* resource production, making it difficult to assess the direct consequences of the allochthonous resources (but see Morrison 2005). As a result, these studies must also include the indirect effects, such as the ability of detrital resources to enhance primary production in the recipient community (Sanchez-Piñero and Polis 2000).

The relative contributions of allochthonous and autochthonous resources can influence the structure and dynamics of food webs (Moore et al. 2004), and obscure the direct effects of resource subsidies on community dynamics. Unlike aquatic systems, few terrestrial systems are supported entirely by allochthonous resources

such that they would be compatible with a direct investigation of the consequences of resource subsidies. Caves represent one such terrestrial system where allochthonous inputs and local productivity are not confounded. As there is no primary productivity underground, the cave food web is completely dependent on allochthonous inputs.

The types of food resources that come into caves vary in terms of regularity, duration, and usability. The most prevalent source of food is that of dead and decaying leaf and wood debris that has fallen, blown, or washed into caves (Barr 1967, Culver 1982, Poulson 2005). Another major source of energy input into these temperate caves is the carcasses of animals that fall down shafts or otherwise get lost within a cave (Barr 1967, Culver 1982, Poulson 2005). Fecal matter, deposited by crickets and bats, represent another nutrient-rich energy source in these nutrient-deprived systems (Fagan et al. 2007, Poulson 2005). Cave invertebrates are numerous where these resources are abundant (Peck 1976, Poulson 2005, Weinstein and Slaney 1995) and cave species will respond numerically to nutrient and water supplementation (Humphreys 1991).

Because caves feature detritus-based food webs that depend solely on spatial subsidies from the surface, caves are ideal systems to examine the flux of resources from one terrestrial habitat to another and the direct consequences of spatial subsidies on the invertebrate community. In addition, caves are naturally replicated and thus allow ecosystem-level manipulation experiments to examine the link between resource availability and biodiversity in a terrestrial habitat. Here, we adopt a community level perspective and investigate the influence of subsidies on consumer-resource dynamics, specifically examining 1) how nutritional and temporal variability

in resources influence the richness and abundance of invertebrate consumers, 2) how changes in community composition depend on the type of the resource subsidy, and 3) how long-term resource manipulation can influence a detritus-based terrestrial community (e.g. directionally change or stabilize a system). Results from this study demonstrate how allochthonous resources can drive the community composition and dynamics of terrestrial invertebrates in cave ecosystems.

Methods

Study site

The caves (technically ‘pits,’ caves with vertical shafts approached from the surface [Veni 2005]) chosen for this experiment are all located within a 2 km² region on private land in Greenbrier County, West Virginia. Caves with vertical entrances (commonly called “pits”) were chosen, as opposed to caves with horizontal entrances, because the resources that fall into pits can easily be quantified and are primarily localized within the drop zone (the area directly below the opening to the surface). The pits range in depth from 4.5 to 19 m.

Experimental design

In July 2005, prior to resource removal, a baseline census of invertebrates was performed in each cave using pitfall traps baited with limburger cheese, supplemented with visual inventories (standard census techniques for sampling cave biodiversity [Schneider and Culver 2004]). In August 2005, all macroscopic organic material and the top 6 cm of soil were removed from each pit. Organic material (dead leaves, dead animals, fungi, fecal material, and organic rich soil) was removed from each pit using

garbage bags and a pulley system. A total of 1.5 metric tons of material (wet-weight) was removed. We dried the material, reweighed it, and then transferred the material to a two-ton incinerator to quantify the actual organic material that had burned off, separated from the clay, rock, and soil that remained.

After each pit was “cleaned”, we constructed exclusion boxes at the top of each pit to prohibit natural resources from entering (Fig 1). The exclusion boxes consisted of a wooden frame and a tightly pulled cover of plastic sheeting (that could withstand the weight of falling debris). The boxes were elevated above the pit, and hardware cloth lined the sides of each box, as not to disturb air flow into and out of the pit. The pits remained covered, and without allochthonous resources, for five months (August 2005 - January 2006). This five month period covered the fall; the period of the year when most allochthonous resources would naturally fall into pits (Schneider et al. *in review*).

In January 2006, each cave was stocked with standardized quantities of the two major natural subsidies to the cave environment: leaves (in the form of leaf packs) and carcasses (in the form of commercially supplied dead rodents). To make the leaf packs, we collected and combined representative leaves from the surface above every pit. After we homogenized the leaves, we rinsed them with distilled water, allowed them to dry, and placed 50 grams into unused mesh onion bags. The large white rats (120 grams) were purchased from an online supplier (The Mouse Factory, <http://www.themousefactory.com>). The wet mass of a leaf pack approximated the fresh mass of the rat carcasses, which decrease rapidly in mass as they dry (Pellett and Kaba 1972). Six of the twelve caves (3 rat caves and 3 leaf

caves) had multiple “drop zones” (flat surfaces where allochthonous subsidies would naturally accumulate). These six caves received two subsidy units (either two rats or two leaf packs) placed in different drop zones instead of just one subsidy unit.

Caves were paired based on size, and the rat treatment was assigned randomly to one member of each pair. Using techniques described below, we resampled the caves one week, and again two weeks after the addition of the first experimental subsidies. Subsequently, resampling (using the same methodology) occurred monthly for a total of 23 months through November 2007. To maintain a ‘press’ type resource manipulation (Bender et al. 1984), leaf and rat resources were restocked when depleted (i.e. when only bones remained for the rat, or when approximately half of the leaf particles were small enough to pass through the openings of the mesh bag (5mm diameter mesh). Overall, there were 25 sample dates (two early samples in January followed by 23 monthly visits) and 18 treatment sites (12 caves, six of which received two treatment subsidies), for a total of 450 site*date visits. Information about the pits, including the treatments that they received (and the number of resource subsidies) is supplied in Appendix B.

Documenting and identifying invertebrates

Every month for two years, we recorded all invertebrates found on, underneath, and within a 30cm radius of each of the rats and leaf packs. During each visit, leaf packs were emptied into a white sorting tray. The internal cavity of the rats was examined after the black putrefaction and prior to the butyric fermentation stage of decomposition (Bornemissza 1957). Animals were identified to morphospecies based on external morphology in the field. Detailed notes and field keys were used to

keep these identifications consistent over the two year study. To avoid disturbing the experiment or minimize disruption to the community succession by removing individuals, animals were rarely collected, and then only when it was essential to obtain voucher specimens for identification. Individuals were identified to lowest possible taxonomic position in the field. While most of the identifications were made *in situ*, individuals that were observed for the first time were collected and brought back to the lab for further identification. When possible, specimens of some commonly seen species that were not familiar to one of us (KS, who has over a decade of experience working with West Virginia cave invertebrates) were sent to expert taxonomists for identification. Collected animals were preserved in 70% ethanol and remain in the collections of the taxonomists or with KS. In a few cases, juvenile cave organisms (which cannot generally be assigned to species but which are likely to play different functional roles than their adult forms) were retained as separate morphospecies in our analyses below. Though the term “cave organism” commonly refers to a cave-obligate species, the majority of the organisms investigated in this study are “troglophiles”, or cave-loving species that are not restricted to caves. Though troglobionts are of primary conservation concern, troglophiles represent an important component of the ecological cave community and are the most abundant players in this ecological study.

Data analysis: Overall trends

To evaluate statistical differences between treatments in the number and abundance of morphospecies, we performed a generalized linear mixed model (GLMM; Pinheiro and Bates 2002) using either the number of morphospecies or total

abundance as the response variable assuming a Poisson error structure (appropriate because of count data), treatment as the fixed predictor effect, and random effects of “pit” (i.e. cave ID), “trap” (i.e. resource site ID within each cave [either 1 or 2]), and “replicate” (exact identity of each rat or leaf pack, since resource packs were replenished over time).

To evaluate if the treatments differed over the course of the experiment, we performed another GLMM with the fixed effects of “treatment”, “months since beginning of experiment” (continuous variable), and “season”, and the random effects due to subsampling (again: pit/trap/replicate) as well as the random effect of the age of the resource (a potential source of error). Because many of the invertebrates observed are surface-dwelling, we chose to code the “season” variable into two categories (May – Oct vs. Nov – April) based on when invertebrates are most active on the surface (separating “warm” from “cool” months). After we discovered a significant three-way interaction of “treatment * months * season” using the whole dataset, we decided to split the data to examine the temporal effects of each treatment separately and in more detail. This is justified because of the different temporal dynamics on the resources and the unequal persistence times of the subsidies (see Results, below).

For both GLMMs, the two separate dependent variables were the number of morphospecies and total invertebrate abundance. For each dependent variable (richness and abundance) we also conducted five additional tests to explore the effects of sample composition on the experimental results and assess the generality of our findings. First, to test for the overpowering effects of extremely rare taxa, each

analysis was performed with the singletons and doubletons removed ($n = 19$). Second, to eliminate strong effects from the most common taxa, each analysis was repeated with the most dominant species removed (those species represented by > 1000 individuals, $n = 4$) (Rango 2005). Third, we removed the dominant species and the extremely rare taxa. Fourth, we tested for the effects of unidentifiable juveniles by removing them from the analyses. Lastly, we examined effects due to differential taxonomic resolution by assigning morphospecies to Order and conducting the analyses using the count of known unique Orders ($n = 28$) as an alternative measure of diversity separate from morphospecies richness. Without including the effects of time, the results of the GLMMs were the same regardless of how the data were subset. Thus, only results for the whole dataset are presented, though all results of the five additional analyses are presented in Appendix D, Table D1.

To identify those sampling periods where the rat vs. leaf treatments differed in number or abundance of morphospecies, t-tests with Bonferroni adjustments were performed for each of the 23 sample periods, and for each resource “age” (binned by 30 day intervals).

Hierarchical cluster analysis

To compare the overall invertebrate communities between treatments, we created two sets of dendrograms, one set based solely on occurrence (Presence/Absence) and the other including abundance data. For the occurrence clusters, we used data on whether each of the 102 morphospecies was ever present in a given pit. We then calculated a Jaccard index ($C_j = a / (a+b+c)$, where a is the total

number of samples present in both samples, b is the number of species present in only sample 1, and c is the number of species present in only sample 2) to calculate similarity in occupancy between sites (Legendre and Legendre 1998, Magurran 2004). For the cluster that included abundance data, we calculated the Bray-Curtis index of similarity between the sites. The Bray-Curtis index, which ranges from 0 to 1, incorporates both richness and evenness and is commonly used for ecological community comparison (McCune and Grace 2002). The Bray-Curtis index is based on the equation $C_N = 2jN / (N_a + N_b)$ where N_a is the number of individuals in site a, N_b is the number of individuals in site b and $2jN$ is the total abundance of shared species in the site with the lower sum (Magurran 2004). Jaccard and Bray-Curtis indices were calculated using EstimateS (version 8.0.0 Colwell 2006). We used hierarchical clustering to create community dendrograms, constructed from each similarity matrix using the averaging method. Clustering was performed in R (version 2.7.0; R Development Core Team 2008).

We examined four time periods using this technique. First, we examined the baseline data collected for the 12 caves: first in July 2005 (prior to resource removal) and again in January 2006 (after the caves had been empty of resources for five months, but immediately prior to the initial experimental stocking event). We then clustered the 18 resource sites (recall, six of the 12 caves each had two resource units each) using data from the last day of the experiment (November 2007), and separately using abundance data summed across the entire duration of the experiment.

Redundancy analysis

To test if the pits differed based on the treatments they received, we performed an explorative redundancy analysis (RDA; CANOCO 4.55 Ter Braak and Šmilauer 2006) on log transformed abundances. RDA is a constrained ordination technique, wherein one attempts to explain the variation in species data using environmental data. By performing multiple (and simultaneous) linear regressions for each species on the explanatory variables (while accounting for covariables), the RDA biplot depicts the main pattern of the community described by the environment variables (the weighted fitted species data), and the relationship between individual species and the environmental variables (the species data) (Ter Braak and Prentice 1988). In addition to “pit” (the environmental variable), we also included visit number, season (differentiating cool vs. warm seasons, coded as above), and trap number as covariables in the design matrix, and scaled the RDA on the intersample distances. To evaluate the RDA, we performed a Monte-Carlo permutation test, with 499 permutations, randomizing within the caves, but restricting the shuffling to fall within sampling visits.

Time lag analysis

To evaluate how the community composition changed over time, we used the community-level time lag analysis (TLA) of Collins et al. (2000), which allows for the investigation of community compositional change as a function of increasing time lags between samples. We performed the TLA independently for each treatment site ($n = 18$ sites). For each pair of time steps involving a treatment site (beginning at the last of the three sample dates in Jan 2006, and continuing until Nov 2007, yielding 23

equally spaced time steps), we calculated community dissimilarity. We then created a diagonal matrix of time lag and dissimilarity distance, and performed linear regressions of dissimilarity as a function of the square root of the time lag (Collins et al. 2000). To determine if each correlation was significant, we performed Mantel tests between the dissimilarity matrices and the separation in time matrix using 10,000 permutations. Mantel tests were performed using the program PASSAGE (version 2, Rosenberg 2008). In the TLA analysis (Collins et al. 2000), if dissimilarity increases over time (a significant positive slope), this indicates that the community is undergoing directional change, i.e., samples that are more separated in time are increasingly divergent. In contrast, if dissimilarity decreases over time (i.e., the community is becoming more similar over time), this indicates that the community is converging on a composition similar to one of the early samples. Lastly, if no change is observed over time, the community may either be stable or inundated with stochastic variation (Collins et al. 2000).

We restricted the TLA to a modified dataset including only the 92 species that comprised at least 3% of the community in one or more of the possible 450 site*date visits (Geissen and Kampichler 2004). In separate suites of TLA analyses, we tested both Euclidean and Bray-Curtis measures of community dissimilarity. In their original paper, Collins et al. (2000) suggest that other metrics (beside Euclidean) may be more appropriate for TLA, and while most studies continue to use Euclidean metrics (e.g. Collins and Smith 2006), some have also employed Bray-Curtis in TLA (Beche and Resh 2007). Because we found the same results using both metrics, we present only the results involving Bray-Curtis dissimilarity distances.

Results

Data analysis: Overall trends

In the 23 months after introduction of resources, 19,866 individual invertebrates were observed (Appendix C). The invertebrates were classified into 102 morphospecies, representing 11 Classes and 30 Orders (Table 1). Among the Orders present, Coleoptera, Collembola and Diptera were most speciose, containing 14, 18 and 19 morphospecies, respectively. Collembola and Diptera, which contained 33% and 37% of the individuals observed, were most numerically dominant. Fourteen morphospecies were only represented by one individual (singletons [(Preston 1948)]), and seven morphospecies were represented by two individuals (doubletons). Over the course of the study, the two most abundant morphospecies were Diptera in the family Calliphoridae (blow flies) and collembola in the family Entomobryidae (specifically the Entomobryid referred to as “Collembola 5”, Appendix C).

Overall, 77 % of the individuals (15344 of the 19866) were found in the 6 rat treatment caves. The most commonly observed morphospecies (Calliphorid larvae and “Collembola 5”) were also the most abundant morphospecies in the rat treatment caves, representing 34.3 and 11.1 % of the individuals, respectively. Though the Calliphorids were more abundant, the collembolans were more frequently observed over time, occurring in 116 of the 225 cave * visit samples possible, whereas the Calliphorids were only observed in 51 of the 225 samples. Twenty-one morphospecies, primarily dipterans, but also including beetles, collembola and millipedes, among others, were found exclusively in rat treatment caves (Table 2).

A total of 4,522 individuals were found in the 6 leaf caves over the 225 site*visit samples. The most abundant morphospecies in the leaf treatment caves were *Euhadoenecus* crickets, *Pseudotremia* millipedes, and Collembola 5, representing 12.5, 13.1, and 17.8 % of the invertebrates found in the leaf treatment caves. Collembola 5 and *Pseudotremia hobbsi* were also the most frequently observed; their presence was recorded on 124 and 105 of the possible 225 cave * visit samples, respectively. Twelve morphospecies, representing 11 Orders, were found exclusively in leaf treatment caves (Table 3).

Overall, the rat treatment yielded significantly more individuals (Appendix D, Table D1, $p < 0.001$). This difference is evident when examining both the patterns of abundance over the entire experiment (Figs 1 A, B) and over the period of time since the last subsidy was added (Figs 1 C, D). In fact, there was a significant time effect on abundance for both resource types (Appendix D, Table D2, $p < 0.01$).

On rats, invertebrate abundance rose slowly in the beginning of the experiment, peaked during the “warm” months, between May and July of the first year (months 5 through 7) but had declined by month 9 (August). Abundance peaked again in month 10 (September) before declining for most of the second year. There was a slight increase in abundance in July and August of the second year, but invertebrate abundance did not reach the high levels observed during the first year (Fig 2A). On leaves, abundance remained relatively low and constant throughout the duration of the experiment (Fig 2A). Examining paired monthly differences, total invertebrate abundance on rats was significantly higher than on leaves in ten months,

particularly the summer of 2006 and the spring of 2007 (Fig 2A, Appendix D, Table D3).

Total invertebrate abundance differed between treatments over the duration of the experiment and over the temporal “life” of the individual resources, which were restocked upon exhaustion. Leaf packs decayed very slowly and were restocked on average every 371 days, whereas rats quickly decomposed and were restocked on average every 136 days. When accounting for the age of the rat subsidy, abundance initially decreased, then peaked twice later during in decomposition (Fig 2B).

Because the rate of decomposition of leaves was much slower than that of rats, leaf packs lasted longer, and the time since last resource addition was greater.

Consequently, the pattern of abundance on leaves varied little whether time was measured since the beginning of the experiment (Fig 2A) or since the resource was renewed (Fig 2B). Incorporating the “age” of the resources, total abundance on rats was significantly higher than on leaves during four months of decomposition (the first, second, fourth and sixth (Fig 2B, Appendix D, Table D3).

Overall, the two treatments did not differ in invertebrate species richness (Appendix D Table 1D, $p > 0.05$). When accounting for the temporal effect, there was a significant interaction of “season” and “month since start of the experiment” on richness on rats (Appendix D, Table 2D, $p < 0.001$). On leaves, there was only a significant temporal effect when the data were grouped by Orders ($p = 0.019$) or when both the most common and most rare species were removed from the analysis ($p = 0.048$, Appendix D, Table 2D).

On rats, the number of morphospecies generally rose during the first year and declined in the second year (Fig 2C). The number of morphospecies on leaves also increased towards the end of the first year (Fig 2C). During the second year, the rat and leaf treatments yielded samples of comparable species richness. Richness was only significantly higher on rats in the first year, during the summer season (June – October) (Fig 2C, Appendix D, Table 3D).

Factoring in the age of the resource, both treatments exhibited fluctuations in the number of morphospecies over time (Fig 2D). Richness was significantly higher on rats in only the first and seventh months of decomposition (Fig 2D, Appendix D, Table 3D).

Hierarchical cluster analysis

In July 2005, prior to resource removal, caves that were to receive rats versus leaf packs did not exhibit any clear associations in cluster analyses, whether based on species presence (Fig 3A) or based on abundance (Fig 3B). In January 2006, after the caves had been empty of resources for five months and immediately prior to the first stocking event, the caves did not show clear associations in either species occupancy (Fig 3C) or abundance (Fig 3D). On the last day of the experiment (November 2007), the rat and leaf sites (now 18 treatment sites) appear similar to each other in both species presence (Fig 3E) and abundance (Fig 3F). With occurrences summed across the entire experiment, the communities at rat sites are more similar to each other than to the communities from leaf sites (Fig 3G), with all nine of the rat sites clustering together. Two leaf sites represent the most basal sites on the dendrogram representing the presence of species throughout the experiment, and there is a clear distinction

where all of the leaf sites separate from all of the rat sites. Based on species abundances, rat sites are also more similar to each other than to leaf sites (Fig 3H), with seven of the rat sites clustering together. This distinction occurs at the most basal node of the dendrogram, suggesting strong dissimilarities between these two clusters. Interestingly, there are two rat sites (both within one cave, Posthole Pit), that cluster together within the leaf sites.

Redundancy analysis

The RDA biplot demonstrates how caves separate based on the resource addition treatments that they received (Fig 4). The RDA based on the first two axes explains 6.9 % of the variance in the morphospecies data and 47.7% of the variance in the fitted morphospecies data. Incorporating the covariables (season, time, and trap number) explained 14.6% of the variation. The first canonical eigenvalue, 0.035, was statistically significant ($F = 17.769$, $p = 0.002$). The relationship between the species and environmental variables was also highly significant (trace eigenvalue = 0.124, $F = 6.348$, $p = 0.002$).

The biplot (Fig 4) shows the striking separation of caves on the first ordination axis, which sets the caves apart based on the treatments that they received. Interestingly, several pits also separate based on the second axis. There were seven morphospecies for which at least 10% of the variation was explained by the ordination. Five of these morphospecies, two types of flies (calliphorids and phorids), two collembola (an isotomid and an unidentified entomobryid), and the earthworm (a lumbricid) have a significant positive relationship with the rat treatments. The

remaining two, both chordeumatid millipedes, have a significant positive relationship with the majority of the leaf treatments.

Time lag analysis

Directional change was observed at all of the treatment sites, as evidenced by the positive relationship between community dissimilarity and time (Fig 5). Mantel tests with 10,000 permutations show that these time lag regressions are statistically significant for seventeen of the eighteen treatment sites ($p < 0.05$). On average, the temporal rate of change in community composition (i.e., divergence from communities observed earlier in the experiment) was faster in leaf treatment sites than in rat treatment sites (leaf average slope \pm se: 0.041 ± 0.005 vs. rat average slope \pm se: 0.035 ± 0.003), excluding the non-significant rat site (Fieldstation Pit). Directional change in community composition was also stronger in the leaf treatment sites, when compared to rat treatment sites (average leaf r^2 : 0.076 ± 0.014 vs. average rat r^2 : 0.061 ± 0.008). The strongest directional changes were observed in three leaf sites, with r^2 values of greater than 0.100.

Discussion

In many natural systems, spatial resource subsidies can have significant impacts on the composition and structure of the recipient communities. In a recent meta-analysis, Marczak et al. (2007) reviewed 32 studies of resource subsidies, none of which focused on terrestrial habitats subsidized by resources of terrestrial origin. Caves represent one such donor-controlled habitat wherein such a terrestrial-terrestrial link can be investigated. Despite the cave environment being consistently

highlighted as an ideal “donor-controlled habitat” (Polis et al. 1997, Moore et al. 2004), ours is the first study in which an ecosystem-level manipulation experiment has tested the effects of detrital resource subsidies on the terrestrial invertebrate community in caves (but see Jessor 1998). Overall, our results show how the type of detrital subsidy can influence both community structure and dynamics. Over the two year experiment, we found that the invertebrate community utilizing each resource type was changing over time. In addition, community composition and abundance differed between the two experimental treatments, though overall morphospecies richness did not differ on the rats vs. the leaf packs. Our results show how resource subsidies can drive community composition but suggest that richness may be constrained by other factors in these cave ecosystems.

Richness changed over time on the rats, whereas richness on leaves remained relatively constant over time (Fig 2C). Throughout the entire duration of the experiment, however, the two treatments did not differ in richness. This result was surprising because we hypothesized that the higher quality resource (the rat carcass) would be able to support a great number of individuals, as well as species (as proposed by the species energy theory (Wright 1983) and its recent extension (Srivastava and Lawton 1998)). Over the ecological time scale investigated, both communities may be at saturation (Cornell and Lawton 1992). Though investigators have examined interactions between detritus and detritivores (Yang 2006), it is still unclear which factors control species richness in detrital communities (Moore et al 2004). We suggest that this stabilization in species richness may be attributed in part

to niche differentiation over time (Cornell and Lawton 1992), and in part to the dispersal limitation in this system (MacArthur and Wilson 1967).

As predicted, the treatments did differ in abundance, with rat treatments supporting more individuals, in support of species energy theory. Higher quality resource subsidies have been shown to increase primary productivity in both aquatic and terrestrial systems (Anderson and Polis 1999 and references therein). Higher quality resources can also support an increased number of individuals (Rose and Polis 1998, Sanchez-Piñero and Polis 2000), increased biomass (Kawaguchi et al. 2003) and an increase in the consumer rate of growth (Yee and Juliano 2006).

We also found differences in community composition between the two treatments. Both the cluster analysis (Fig 3) and the RDA (Fig 4) indicate that caves receiving the rat treatments differed in community composition from those receiving leaf treatments. In both analyses, one specific rat cave (Posthole Pit) behaved more similarly to the leaf caves than to the other rat caves. Though it can be seen from the RDA that Posthole Pit had more millipedes and fewer flies than would be expected based on the treatment it was assigned (Fig 4), no physical or biological characteristics of this cave are evident that would explain this result (Appendix A, Table 1A). At this point, the precise explanation for why these results were found remains unknown.

Using time lag analysis (Collins et al. 2000), we found that both types of resources harbored communities that were increasingly divergent over time (Fig 5). This type of directional change is commonly seen in communities after disturbance (Platt and Connell 2003 and references therein), and may reflect shifts in the

community after the initial shock to the system (Thibault et al. 2004). The observation that the rate of change was faster in the leaf sites may thus be attributed to the longer persistence time of this resource, whereas the community on the rat carcasses was exposed to multiple “disturbances” with each restocking event, possibly reshuffling the community to an earlier stage in decompositional succession (Fuller 1934, Schoenly 1992).

The effect of resource availability on cave communities is especially important when focusing on the obligate cave invertebrates (i.e. “troglobionts”). Here, we included the entire invertebrate community in our analyses, including species found on the surface as well as cave-dwellers. Yet the obligate cave invertebrates are the most important players in this system; for, unlike their surface counterparts who can disperse freely, cave species are intricately linked to and dependent upon allochthonous subsidies into caves. Organic subsidies into caves are of utmost importance to obligate species and the depletion of such resources can lead to decline of cave populations (Humphreys 1991). Though nutrient enrichment has been shown to lead to competitive exclusion of cave species by surface species (Sket 1977), the experimental addition of resources can also rejuvenate populations (Humphreys 1991). For example, in this experiment, we found that obligate cave carabids (*Pseudanophthalmus grandis*) responded favorably to supplementation by leaf packs. Anecdotally, we observed a rarely-seen mating event of this species underneath a leaf pack subsidy, which strongly suggests environmental conditions conducive to population growth (Baber Pit 2, 07 April 2007).

The availability of resources is likely to influence the distribution of species in cave environments (Gibert and Deharveng 2002). Through resource removal, we collected a substantial amount of leaf detritus, combined with an impressive array of vertebrate remains (ranging from cow to rodent bones, Schneider unpublished data). In caves, like other systems, the impact of resource subsidies depends, in part, on the flux rate (the frequency and relative contribution of different resource types [Polis et al. 1997, Cole et al. 2006] and the rate of input of resources (the pulse and duration of specific resources [Cloe and Garman 1996]). Both of these rates can vary temporally and depend on factors of the recipient habitat (e.g. ecosystem size, perimeter-to-area relationships (Polis and Hurd 1995)), and this variation can ultimately influence the coexistence and exclusion of species (Yee et al. 2007) and the strength of trophic cascades (Leroux and Loreau 2008). In these temperate caves, leaf litter constitutes a major contributor of energy. In forests, for example, up to 90% of net primary productivity may enter the detritus based food web (Cebrian 1999). In fact, the bulk of organic matter in forests is 62% dead material (Hairston and Hairston 1993), and thus the pool of this resource type is substantial. The rate of input of animal remains, which is less predictable in time but represents a larger nutrient pulse, is the subject of ongoing investigation.

Concern over cave-limited species has heightened within the past two decades, and particular attention has focused on the impacts of allochthonous nutrients on cave-resident species. Though many studies focus on aquatic subterranean species (recently the topic of a special journal issue of *Freshwater Biology* (April 2009)), terrestrial cave-limited species are equally threatened, whether

by such factors as careless human visitation or the disruption of the flow of organic matter into caves (Culver et al. 2000). The flow of energy into caves can be disrupted by the manipulation of cave entrances via enlargement, closure, or by the installation of improper cave gates (Elliott 2000). In addition, circumstances that alter the flow of energy via animal vectors (e.g. cricket feces and eggs or bat guano) are also common. For example, changes to the vegetation structure surrounding cave entrances can have dramatic effects on populations of cave crickets, who routinely leave the cave to forage (Taylor et al. 2005, Fagan et al. 2007). The mysterious and horrendous disease that is killing hundreds of thousands of bats in the Northeastern United States (white nose bat syndrome) is also likely to affect the invertebrate cave species that rely on the guano of these species. As seen in our experiment, allochthonous resource subsidies are of major importance in cave ecosystems, and they can ultimately drive changes in the invertebrate community in caves. This study, which describes the impacts of terrestrial subsidies into a terrestrial system fills a void in the spatial subsidy literature, and increases our awareness of the effects of allochthonous resources on arthropod consumers.

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Tables

Table 1. Designation of morphospecies to order, and the number of individuals observed within each morphospecies throughout the 23 months of the resource manipulation experiment.

Class	Order	Number of Morphospecies	Number of Individuals Observed
Arachnida	Acari	9	928
	Araneae	3	72
	Opiliones	1	21
	Pseudoscorpiones	3	47
Chilopoda	Geophilomorpha	1	5
	Lithobiomorpha	1	3
	Scolopendromorpha	1	2
Oligochaeta	Haplotaxida	1	208
Copepoda	Harpacticoida	1	28
Malacostraca	Isopoda	3	142
Diplopoda	Chordeumatida	4	2434
	Julida	1	165
	Polydesmida	4	40
	Spirostrepida	1	34
	Unknown	2	99
Gastropoda	Pulmonata	1	72
Hexapoda	Blattaria	1	1
	Coleoptera	14	814
	Collembola	18	6488
	Dermaptera	1	5
	Diplura	3	8
	Diptera	19	7322
	Hemiptera	1	8
	Hymenoptera	1	15
	Lepidoptera	1	3
	Orthoptera	2	702
Siphonaptera	1	11	
Nematoda	Unknown	1	186
Symphyla	Cephalostigmata	1	2
Tubellaria	Seriata	1	1
Totals		102	19866

Table 2. Classification of morphospecies found utilizing rat treatments, but not leaf treatments.

Class	Order	Morphospecies only in Rat	Number of Individuals Observed
Arachnida	Acari	1	2
	Pseudoscorpiones	1	20
Chilopoda	Geophilomorpha	1	5
	Scolopendromorpha	1	2
Diplopoda	Polydesmida	1	1
	Spirostrepida	1	34
Hexapoda	Coleoptera	3	20
	Collembola	3	7
	Diplura	1	3
	Diptera	6	1211
	Hemiptera	1	8
	Lepidoptera	1	3
Totals		21	1316

Table 3. Classification of morphospecies found utilizing leaf treatments, but not rat treatments.

Class	Order	Morphospecies only in Leaves	Number of Individuals Observed
Arachnida	Araneae	1	9
Chilopoda	Lithobiomorpha	1	3
Diplopoda	Polydesmida	1	1
	Unknown	1	1
Hexapoda	Blattaria	1	1
	Coleoptera	1	1
	Collembola	2	2
	Diplura	1	1
	Diptera	1	4
Symphyla	Cephalostigmata	1	2
Tubellaria	Seriata	1	1
Totals		12	26

Figure legends

Figure 1. Photographs of the exclusion boxes placed at the top of Fieldstation Pit (left) and Raceway Pit (right).

Figure 2. Overall trends in the mean (\pm se) abundance (A,B) and number of morphospecies (C,D) averaged by treatment over the entire experiment (left) and averaged across the time since the last resource addition (right). Asterisks denote significant differences ($p < 0.05$) between the two treatments at that time period based upon t-tests with Bonferroni corrections.

Figure 3. Dendrograms depicting the hierarchical clustering of presence/absence (Jaccard Indices, left panels) and community similarity (Bray-Curtis Indices, right panels) for the twelve caves (A,B) prior to resource removal (July 2005) and (C,D) prior to the first stocking event (Jan. 2006), and for the eighteen treatment sites using data for (E,F) the last day of the experiment (Nov. 2007) and (G,H) all species ever recorded during the experiment (post-manipulation). These same dendrograms are repeated in Figure 3(I) through 3(M) using cave names instead of treatment designations, with caves that received rats shaded in gray.

Figure 4. RDA ordination biplot of the distribution of the pits (triangle = centroid) relative to the log transformed species abundances. \blacktriangle : Caves that received rat treatments, \triangle : Caves that received leaf treatments. Eigenvalues: axis 1, 0.035, axis 2, 0.024. The biplot only includes the seven morphospecies for which at least 10% of

the variation was explained by the ordination (including millipedes, dipterans, collembolans, and an earthworm). Additional information about the caves and the morphospecies are supplied in Appendices B and C, respectively.

Figure 5. Results from the Time Lag Regression Analysis (TLA), showing the relationship between community dissimilarity (1 - Bray Curtis Index) and the time lag (in months, square root transformed) between each pair of samples for a given treatment site. The first set of nine panels show the leaf sites, denoted in each plot by L1 for leaf site one, L2 for leaf site two. Pit names are abbreviated in parentheses. Slope and r^2 values are from the linear regression, the p values are from 10,000 Monte Carlo simulations. Results from the rat treatments (R1, R2) are shown in the second block of 9 panels.

Figures

Figure 1.



Figure 2.

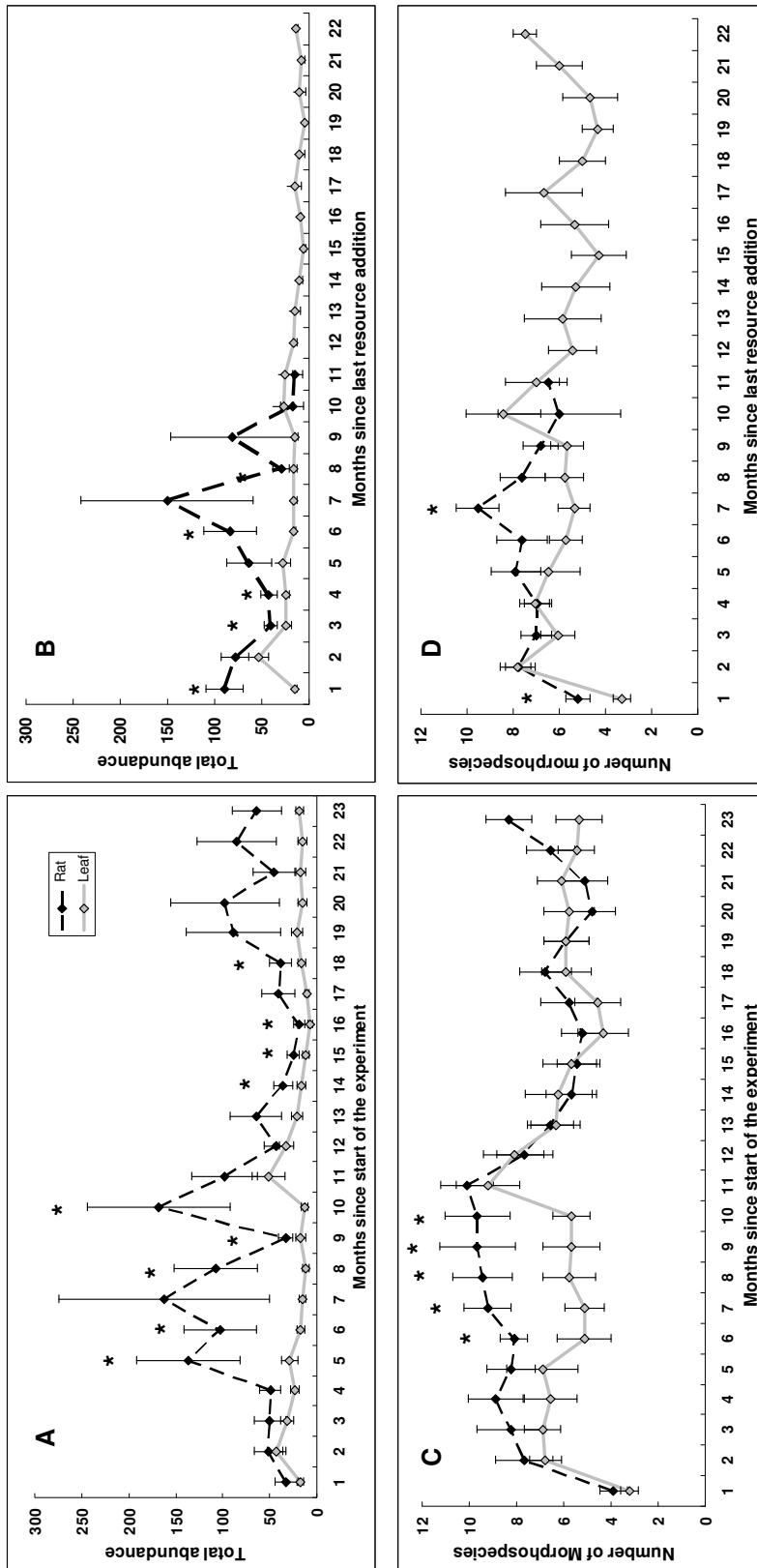


Figure 3.

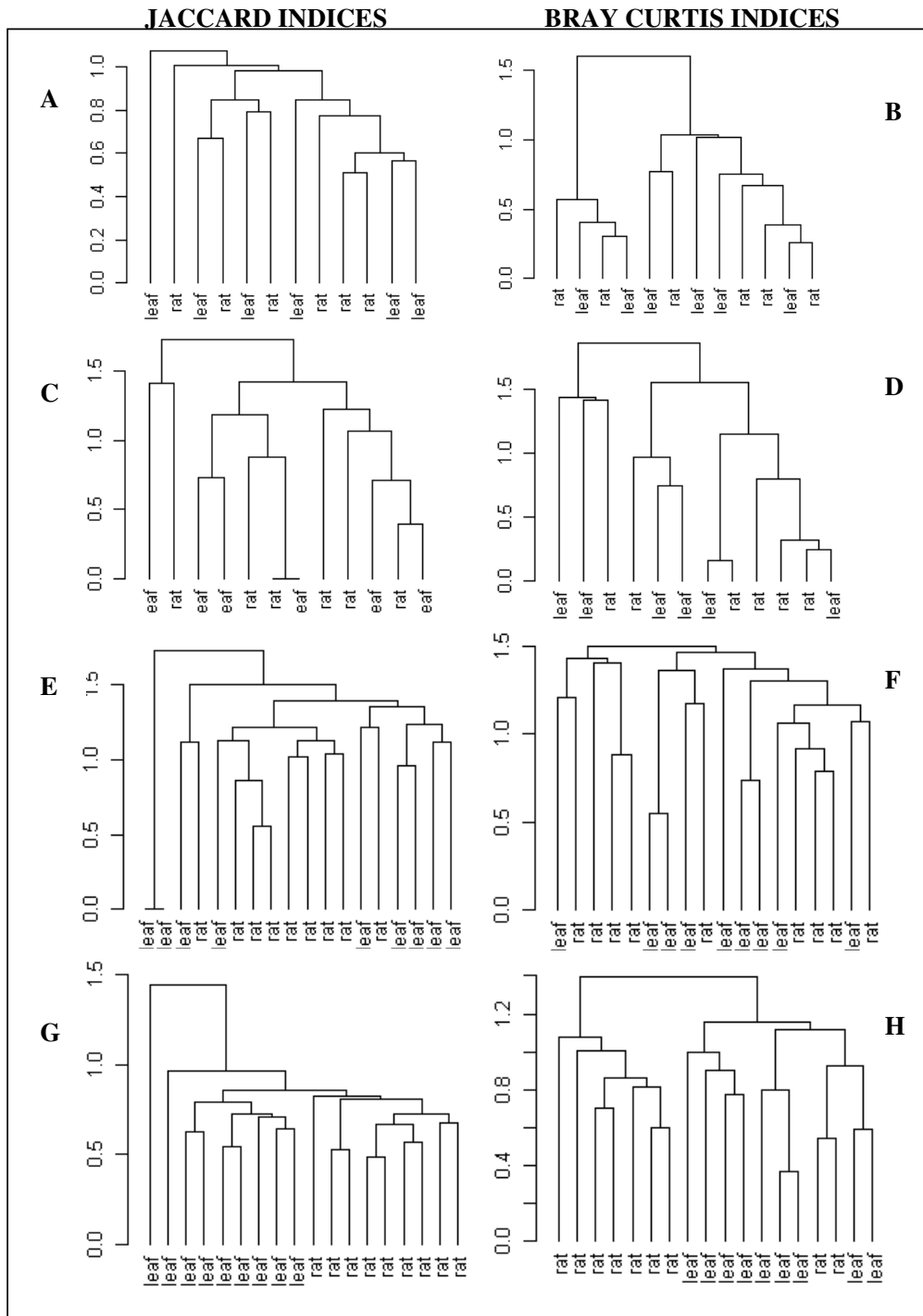


Figure 3 (continued).

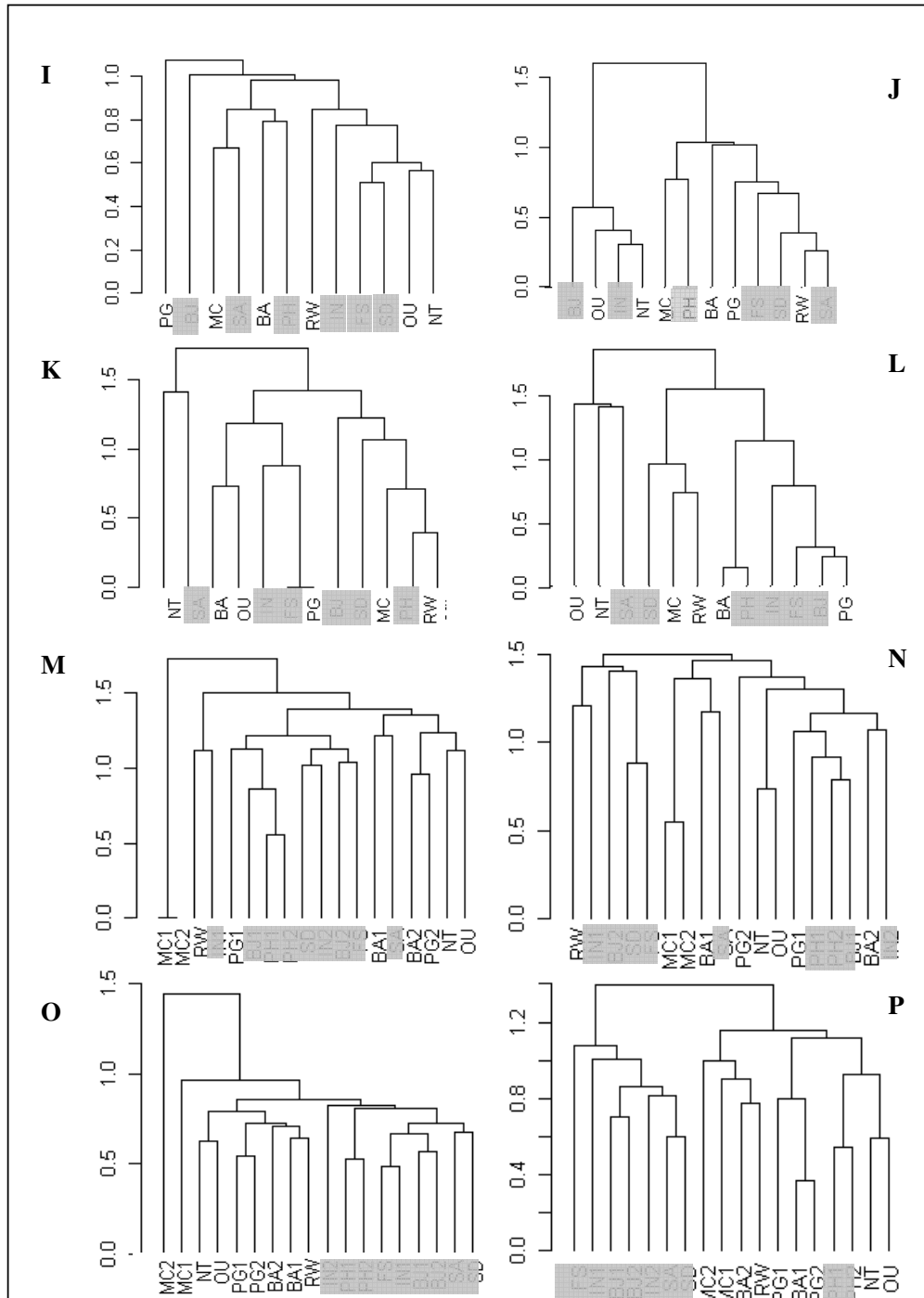


Figure 4.

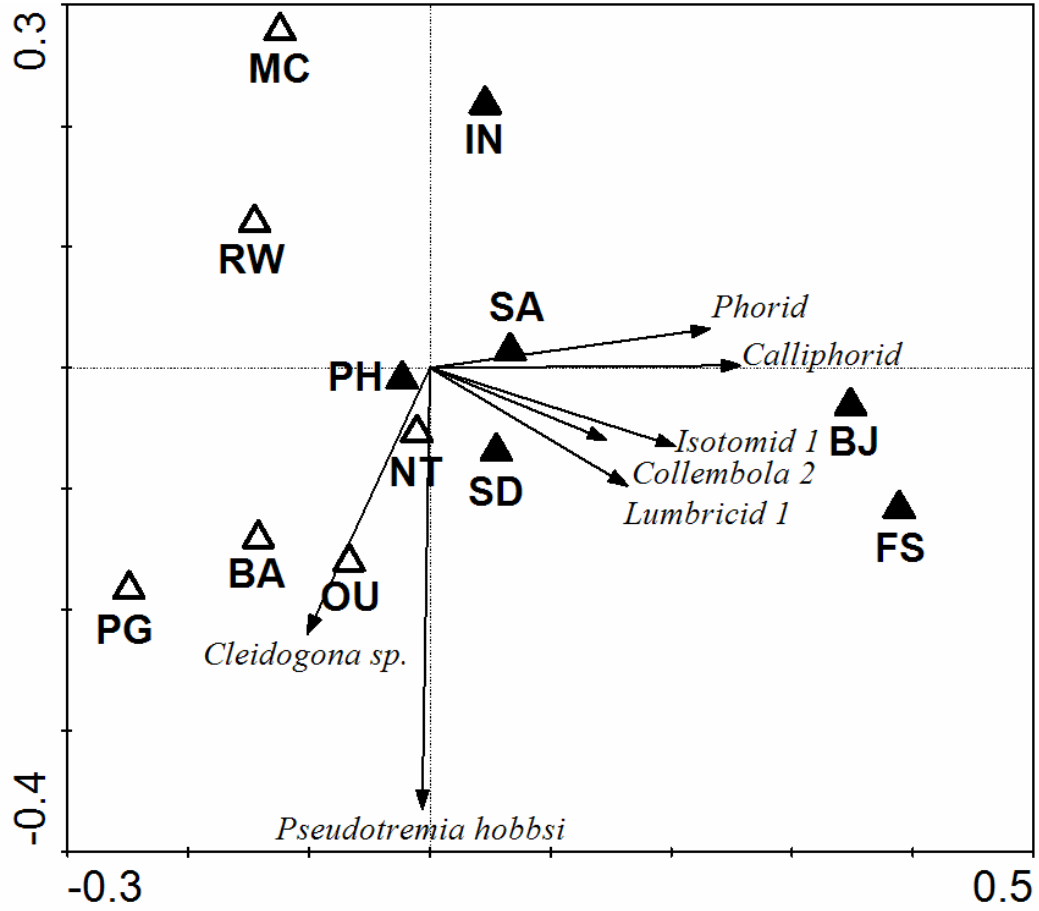


Figure 5.

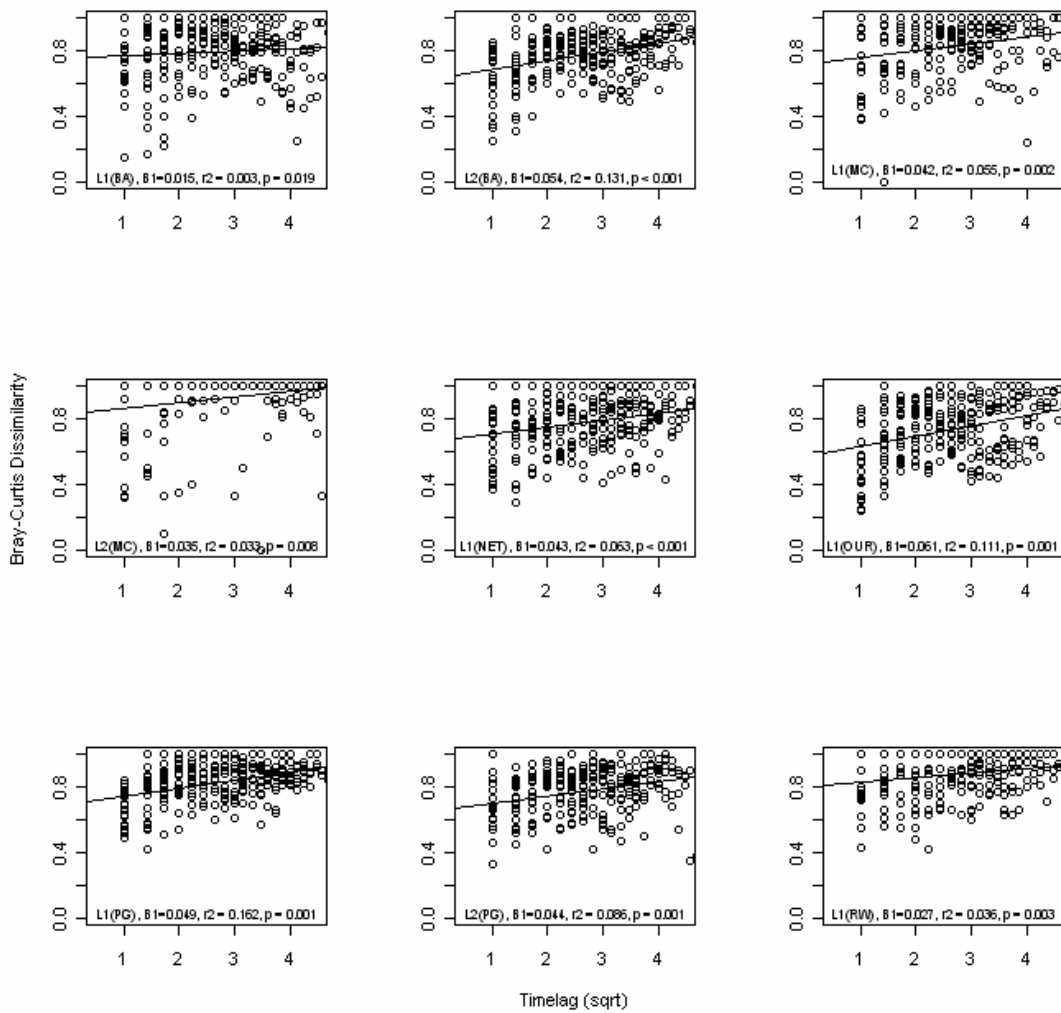
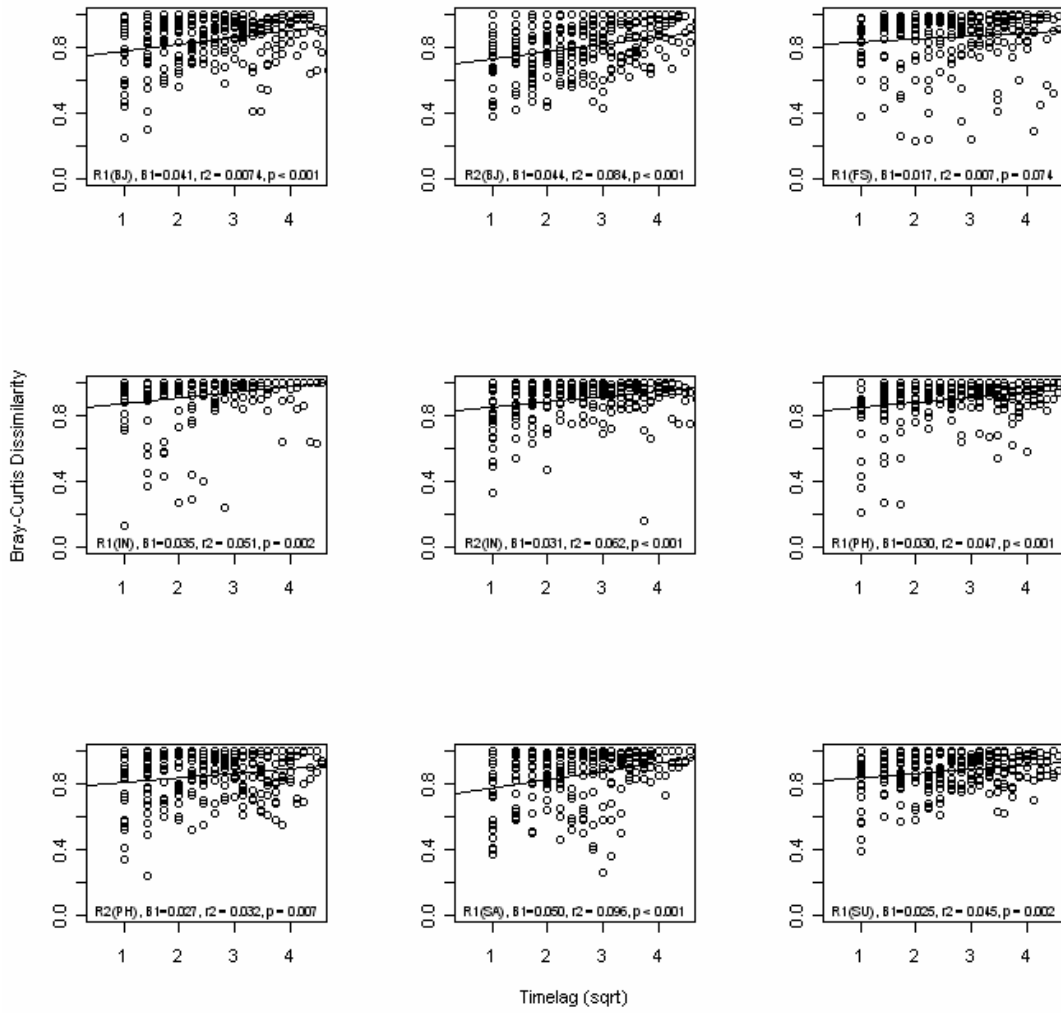


Figure 5 (continued).



Chapter IV: The blind misleading the blind: Modeling occupancy patterns of cave arthropods

Co-authored with: E.H.C.Grant, D.C. Culver, M.C. Christman and W.F. Fagan

Abstract

Obligate cave invertebrates are spatially rare and often hard to detect within a coarse biological inventory. Inventories therefore prove to be expensive and time consuming endeavors, necessitating repeated visits and many person-hours of searching to collect a majority of the species present within a given cave. We used multiple years of data regarding the distribution of terrestrial obligate cave species across 65 caves to examine occupancy patterns of these rare species and assess the sampling intensity necessary to accurately assess regional richness patterns. Previous research suggests that cave species richness is badly underestimated based on one-time biological surveys. Here, we applied classical estimators of species richness to our data set, and, taking advantage of sampling effort repeated through time, we compared these values to more recently developed statistical estimators that incorporate detection. Values of both classical and recently developed estimators indicate that despite multiple years of surveys, numerous cave species go undetected from this region. The values of both types of estimators are very high due to the rarity of cave animals. The estimate that incorporates detection indicates that undetected species result from low rates of both occurrence and detection. In addition, the number of uniques (species known from only one cave) increases with additional

sampling, influencing the classical estimators. Occupancy modeling techniques that incorporate temporally replicated sampling were used to estimate species richness for each cave and determine how the species richness across a series of caves is related to the physical attributes of the caves (e.g. length, proximity to other caves, entrance geometry). Several models fit the data well, and we found substantial support for models that incorporate cave covariates (length, connectivity, and entrance area). These models also demonstrate that cave richness is underestimated at the level of the cave, with an average of one species undetected from each cave. Results from this study suggest that additional sampling is likely to yield both new records of species and new occurrences of species. These results further suggest that incorporating physical characteristics of caves can improve our ability to examine spatial patterns of these rare species and make predictions about patterns of occupancy, both of which would aid species preservation.

Introduction

Caves harbor rare and unique species, but the processes underlying the spatial distributions of terrestrial cave species remain unknown. Obligate cave species spend their entire life cycles in caves and are usually characterized by troglomorphic characteristics, such as the lack of pigment, reduced or absent eyes, reduced dispersal patterns, and the presence of extra-sensory appendages (Christiansen 1962, Culver et al. 2000, Howarth 1993). Research on cave biodiversity suggests that approximately 1/3 of all obligate cave species are single-site endemics (Culver et al. 2000). Across diverse ecological systems, high endemism is often associated with increased extinction risk (Brown 1995, Frankham 1998). Given their high endemism and small

populations, it is not surprising that more than 95% of obligate cave species in the United States are considered vulnerable or imperiled (Culver et al. 2000).

In an ideal world, a single cave visit would be sufficient to provide an entire biological inventory for that cave. However, during any survey, a species can be in one of three 'states': present and detected, truly absent, or present but undetected. Due to the nature of the cave environment, cave animals can be difficult to detect, especially if they retreat into cracks and crevices that are not human-accessible. In addition, some species are numerically rare which decreases the chances of detecting a species even though it may be present (Eberhard et al. 2009). Recent studies have examined the role of sampling effort in caves, elucidating this phenomenon (Krejca and Weckerly 2007, Pipan and Culver 2007, Schneider and Culver 2004).

The primary goal of this paper is to determine environmental factors related to the distribution of obligate cave invertebrates. Here, we use three years of bioinventories of all accessible caves in a small karst area in West Virginia to estimate species richness and explore occupancy patterns of terrestrial cave species. Previous research suggests that cave species richness in this area is highly underestimated when using data from single sampling visits (Schneider and Culver 2004). We hypothesize that a more accurate and reliable estimate of species richness will be obtained by using multiple years of data. In addition, recent studies have shown the importance of including detectability into estimates of species richness, especially because classical estimates may fail when communities contain many rare species or when species are difficult to detect (Dorazio et al. 2006). Here, we apply three classical estimators of species richness, and, taking advantage of our repeated

sampling efforts, we compare those estimators to more novel estimators that incorporate detection probabilities (Dorazio and Royle 2005, Dorazio et al. 2006, Royle et al. 2007). Subsequently, we use occupancy models (MacKenzie et al. 2006) to investigate the role of cave characteristics in determining patterns of species richness while reducing bias in the richness estimators by explicitly accounting for the probability of detecting a species. Specifically, we investigate covariates that are classically associated with patterns of species richness based on biogeographical theory, namely, the size and connectivity of the caves, and the entrance area of the cave (a surrogate for energy input, which, in a sense, can be compared to the perimeter: area measurement of an island [Polis and Hurd 1995]). These analyses of covariation are worth considering because they can provide information regarding where terrestrial obligate cave species are likely to occur and can lead to predictive models that can inform conservation and management of these unique ecosystems.

Methods

Field methods and data collection

In the 2002 study of Schneider and Culver (2004), a biological inventory of 68 caves was performed in a ~11km² area located between the towns of Lewisburg and Frankford, in Greenbrier County, WV (Figure 1). In May and June of 2004 and 2007, the same area was revisited and the majority of the caves were inventoried again. The 2004 survey was part of a statewide effort to document cave invertebrates (Fong et al. 2007). Of the 68 original caves (Schneider and Culver 2004), three were not revisited because of stability and access concerns. In 2007, thirteen additional caves were not resampled. In two cases, these caves could not be sampled because they were

inaccessible. The additional eleven caves were eliminated in 2007 because of their use in an ecosystem-level resource manipulation experiment (Schneider et al. in prep, see Chapter III). Thus, in total, 52 caves were repeatedly sampled in the three years (2002, 2004, 2007). The identities of the caves and the number of times they were sampled within the three annual surveys are provided in Table 1. The detectability analyses described below can take advantage of all caves in the dataset, as long as they were sampled at least twice.

To assess how detectability and cave attributes influence occupancy, we focused on the community of terrestrial cave-limited species. We restricted our analyses to the terrestrial species because our hypotheses concerning the distribution of terrestrial species (i.e. resource availability, connectivity) are somewhat different from those factors that influence aquatic species richness (e.g. pore size, dissolved oxygen concentration, Dole-Olivier et al. 2009). Sampling protocols for terrestrial caves species follow a standard procedure in which visual censuses are combined with baited pitfall traps (using limburger cheese) that were set for three days (Schneider and Culver 2004). The number of traps placed in each cave was a function of the size of the cave and was fixed for each cave across years. Invertebrate specimens were sorted and sent to expert taxonomists for identification. In the analyses that follow, we only included cave obligate species (*i.e.* species known to spend their entire life cycles in caves).

For the analyses described below, one important assumption is that the system is closed, meaning there are no changes in occupancy based on dispersal between sampling locations during the time between surveys. Therefore, if a species is present

in a site, it is presumed to have always been present there (i.e. there is no new colonization). If a species is absent, these techniques assume that that species was either not present in that location or was present but undetected in that particular survey. This is a reasonable assumption for several reasons. Caves are naturally isolated habitats (Culver 1970), and cave species have delicate forms and can not physically survive surface conditions (to facilitate surface migration) (Barr 1967). The assumption of a closed system has been tested and satisfied for other obligate cave species (Krejca and Weckerly 2007).

Estimating species richness for the area

Species richness was estimated with Chao 2 and Jackknife 2, the two classical richness estimators that were applied in Schneider and Culver (2004); for the current study, data from all three years were used for these estimates. For an additional comparison, the bootstrap estimator was also used. For these classical estimators of richness, we examined each year separately and, subsequently, all three years in combination. For the combined data, a species was considered present if it was ever recorded in a cave and absent if it was never recorded. Both of the classical estimates used were based on the quantification of rare species: uniques (species found only in one site [Q_1]) and duplicates (species found in only two sites [Q_2]). The equation for the Chao 2 Estimator (Chao 1984) is:

$$S_{Chao2} = S_{obs} + \frac{Q_1^2}{2Q_2}, \quad (1)$$

and the equation for Burnham and Overton's (1978) Jackknife 2 Estimator is:

$$S_{Jack2} = S_{obs} + \left[\frac{Q_1(2m-3)}{m} - \frac{Q_2(m-2)^2}{m(m-1)} \right], \quad (2)$$

where m is the number of samples (caves). Lastly, the equation for the bootstrap estimator (Smith and van Belle 1984) is:

$$S_{boot} = S_{obs} + \sum_{k=1}^{S_{obs}} (1 - p_k)^m, \quad (3)$$

where p_k is the fraction of caves that contain species k .

After quantifying these estimates based on the above calculations, we created species accumulation curves based on observed Mao-Tau estimates of species richness (Colwell et al. 2004) and compared the projection of those curves to the calculated estimates of richness. Computations and resulting graphs were generated for each year separately, and all years combined, using EstimateS (version 8.0.0, Colwell 2006).

The probability of detecting a species given that it is present is generally low for cave species (estimated range: 0.1875 to 0.2424, Krejca and Weckerly 2007). Therefore it may be more appropriate to use a richness estimator that incorporates detectability. One such estimator is a hierarchical Bayesian (HB) multispecies site-occupancy model that allows for estimation of richness while incorporating detection (Dorazio and Royle 2005). This model estimates parameters associated with species-specific rates of occupancy and detection probabilities based on the marginal density of the observed data. Using the distribution of rates of occupancy and detection probabilities, the model estimates the expected occurrence of species that were not found during any surveys at a particular site, but that are known to occur at other sites. In addition, using the posterior probabilities of occupancy and detection

obtained from the original data, the model uses a data augmentation approach to estimate the number of species that were not detected at all in any of the sites, but are likely to be present in the community. The augmented data represent members of an arbitrary (but sufficiently large) supercommunity, a fraction of which are likely to be present in the study area. Following estimation, one can create species accumulation curves based on the mean of the posterior predictive distribution of the data. This method takes advantage of all of the data acquired from the repeated sampling, including when species were not detected, which allows for a more refined prediction of species richness. This is in stark contrast to relatively coarse predictions of the classical estimators, which are entirely based on binary presence or absence data. The code for the implementation of the model uses WinBugs (version 1.4.3, Lunn et al. 2000), R (version 2.7.0, R Development Core Team 2008), and the R2WinBugs package (Sturtz et al. 2005) and is provided in the electronic supplement of Dorazio et al. (2006).

Modeling occupancy patterns for each cave

We next used single state occupancy models (MacKenzie et al. 2006) to examine the relationship between site characteristics and site-specific patterns of species richness. In our models, we included covariates about the caves that we predicted to influence the “suitability” of caves for occupancy by cave-obligate species. Specifically, we hypothesized that cave length, proximity to other caves, and cave entrance size would influence occupancy. Before running the models, we performed linear (or log linear) regressions to examine the relationship between each covariate and the observed species richness data. We then compared these slopes to

the slopes determined by the occupancy models. The reasoning behind including each of the covariates is outlined below.

Cave length, a proxy for available habitat, is likely to influence both occupancy and detectability. Consistent with biogeography theory, the number of species found in a site should increase with an increase in available habitat. Indeed, a significant positive relationship between species richness and cave length has already been observed in these caves (Schneider and Culver 2004) and therefore should be incorporated as a covariate for occupancy in this model. We also predicted that length may influence detection, with larger caves being more difficult to sample and providing additional places for species to “hide”. Cave length data were provided by the West Virginia Speleological Survey and represent the total length (as the caver crawls) of passageways accessible to small-bodied humans.

Another important covariate to be tested is proximity to other caves, with the hypothesis that caves that are closer to other cave systems will have a greater probability of being occupied. To quantify proximity, we used two separate measures of isolation. First, we used the simple measure of Euclidean distance between cave entrances and created a half-matrix of straight-line distance between each pair of the 65 caves using their UTM coordinates. Second, we calculated the connectivity between caves based upon the smallest distance between their mapped passages. For 12 caves, raw survey data was generously provided by cave cartographers and scientists. For 9 caves we surveyed the passages ourselves. For the remaining 43 caves, we measured the magnetic direction of the cave entrance in the field using a Brunton Survey Master 360 LA Sighting Clinometer. Using this value for the

direction of the first cave passage, we estimated the remaining distances and directions from published cave maps and cave descriptions (Dasher and Balfour 1994). This method does not take into account the elevational changes within the cave, and only results in a two dimensional image of the cave passage. There was one cave for which we did not have accurate survey data and instead substituted Euclidean distance for the passage covariate. We entered all cave survey data into the free software COMPASS (www.fountainware.com/compass) from which we exported a polygon shapefile of each cave into ArcMap (version 9.2, ESRI 2009). The distances between the polygons were calculated in ArcMap using the XTools Pro extension (version 5.1.0, Delaune and Chikinev 2005), and a matrix of nearest passage distances between each of the 64 caves was generated.

Because only one value of isolation for each cave will enter into the model as a covariate, we used the incidence function connectivity metric of Hanski (1994) to calculate the potential contribution of every cave to the cave of interest. This potential contribution of propagules is a function both of the distance to the focal cave as well as the size of the contributing cave and is formulated as:

$$connectivity = \sum \exp(-\alpha d_{ij}) A_j \quad (4)$$

where d_{ij} is the distance between caves, A is the length of the contributing cave, and α is the dispersal parameter of the cave species. Because of the large distances between sites, we set α to 0.01. Using a constant α assumes that variation among the species in dispersal ability is minimal relative to the distances between caves; regardless of the exact level of α , cave rank based on connectivity will be equivalent. For each

cave, we calculated connectivity from Eq. (4) using both the Euclidean and nearest passage distances.

Species energy theory predicts that in similar-sized areas, species richness will be determined by energy flux per unit area (Currie 1991). Therefore, the occupancy model includes covariates that are predicted to affect the availability of food resources to cave resident species. Because cave species are completely dependent on allochthonous resources, primarily in the form of leaves and detritus, or the fecal matter, eggs, and decaying bodies of occasional cave visitors, resource constraints (e.g., food energy, nutrients, and available habitat) may help determine where particular cave obligate species occur (Poulson and Lavoie 2000). At the entrance to each cave, we measured the length of the major and minor axes of the entrance ellipse, hypothesizing that entrance area may, in part, determine resource availability inside the cave. Likewise, because cave entrance circumference may facilitate passage of cave crickets and other species that can crawl along vertical surfaces, we also used circumference as measure of cave entrance size.

We approached the occupancy modeling as a 2-step process: first, we attempted to find a covariate structure on the detection probability (p), using a model incorporating all factors on occupancy (ψ). We investigated a constant detection model as well as detection models in which detection probability varies as a function of sampling year, cave length, and the additive effects of year and length. Once we determined the most parsimonious structure on p , we fit 13 models which incorporated key combinations of the six covariates that were hypothesized to influence occupancy (cave, cave length, entrance area, entrance circumference,

connectivity based on Euclidean distance, and connectivity based on nearest cave passage) (Table 4). All models included the cave identity, as we were interested in obtaining an estimate of species richness for each cave. All continuous covariates were normalized prior to running the models. We excluded caves where no species were detected in any of the surveys from the analyses and ran the models on the remaining 59 caves. Models were fit using the program PRESENCE (version 2.2, Hines 2006). We assessed model fit and estimated an overdispersion parameter using the parametric bootstrap approach incorporated in the program PRESENCE (MacKenzie and Bailey 2004, MacKenzie et al. 2006). A global model (containing cave, length, area, and connectivity as factors for occupancy, and the additive effects of length and year as factors for detection) was fit to the data, and the resulting \hat{c} estimate was used to adjust model selection and parameter precision (QAIC, Burnham and Anderson 2002).

Focusing on the models where the ΔQAIC was <2.0 (and thus still had substantial support [Burnham and Anderson 1998]), we recalculated the model weights (w_i), including only the four top models. We then used model-averaging to generate overall estimates of occupancy for each covariate of interest. First, we calculated the model-averaged estimated slope of overall occupancy, cave identity, and detection based on the top four models.

For each of the covariates of interest, we calculated the probability of occupancy while holding every other covariate constant and altering the values of the covariate of interest. We multiplied this estimate of occupancy by the number of

species in the data set, and plotted the estimated richness against each covariate (length, connectivity, entrance geometry).

Results

A total of 22 terrestrial cave-limited species, representing three classes, seven orders, and ten families were collected in the three annual surveys (Table 2). The most widespread species was the entomobryid collembolan, *Pseudosinella gisini*, which was found in 51 of the 65 caves sampled. Other widespread species were the cleidogonid millipede *Pseudotremia fulgida*, the carabid beetle *Pseudanopthalmus grandis* and the sminthurid collembolan *Arrhopalites clarus*, which were each found in more than 30% of the caves. Three species (one millipede and two beetles) were each found in only two of the 65 caves. Eight species, including two spiders, a millipede, two collembolans, and three diplurans were collected from only one cave each. Four of these species (one collembolan and three dipluran species) are currently undescribed. Though undescribed species are frequently excluded from community analyses until they are named, these species are known to be both troglobiotic (obligate cave-dwellers) and unique from the species known in the study area, and therefore we included them in the analyses below.

Estimating species richness for the area

The number of species collected from a single site (uniques) ranged from two (2007) to five (2002) to seven (2004), and finally to eight using the union of these annual data sets (Table 3). The number of duplicates (species collected from two sites) was low during all years; and in 2007, no duplicate species were collected. The

values of all richness estimators are provided in Table 3. The annual bootstrap estimates are the most conservative, with estimates of richness only two or three species above the number of species that were collected each year. Estimates based on Jackknife 2 and Chao 2 are much higher than the observed number of species, suggesting that as many as 50% of the species were not found. The exception is the 2007 data, where the estimates from both Chao 2 and the Bootstrap are equal to the number of species collected that year. Using the Chao 2 estimate of species richness with the union of the three years of data, the estimated number of species in this area is 33, very similar to the Jackknife 2 estimate of 35 (Table 3).

Species accumulation curves should reach an asymptote when no new species are acquired. The species accumulation curves, with the calculated Chao 2 estimates for each data set marked for reference, are provided in Figure 2. The accumulation curve for the 2007 data set rises rapidly and quickly reaches a plateau at the Chao 2 estimate. The species accumulation curves for the 2002 and 2004 data rise much more slowly in comparison, and are not yet approaching an asymptote. The Chao 2 estimates for samples from 2002 and 2004 are much higher than their respective curves. Lastly, the curve for the union of the three data sets rises more rapidly than the 2002 and 2004 data, and also does not reach an asymptote, nor come close to the predicted Chao2 estimate. The relatively large estimates from the Jackknife and Chao 2 techniques may be attributed to the large increase in the number of uniques (Figure 2, inset).

Based on the Dorazio and Royle (2005) data augmentation approach, more than half of the estimated species were not collected (25 of the 47 estimated species).

According to the hierarchical Bayesian model, the estimated mean and median values of species richness region-wide are 46.5 and 43, respectively. Model parameters of the estimate show that heterogeneity in occurrence among species ($\hat{\sigma}_u = 2.63$) is higher than the heterogeneity in detection ($\hat{\sigma}_v = 1.91$), suggesting that detection failures of cave species are attributed to low rates of occurrence, but that detection is a major issue (Figure 3). The spatial rarity (= endemism) of the cave species is the reason that the predicted species accumulation curve fails to reach an asymptote even if number of caves sampled were quadrupled (Figure 4).

Modeling occupancy patterns for each cave

Although both year and cave length were important factors in detection, incorporating length alone as a factor influencing detection was not favored in the model selection analysis. In contrast, the ψ (Cave) p (year) model had the most support (with ~30% of the weight). We used p (year) to investigate covariates on ψ . We found no differences in occupancy models that included entrance area vs. entrance circumference, nor Euclidean vs. nearest passage measures of distance, and thus only one member of each pair of metrics appeared in the analysis (entrance area, Euclidean connectivity). The global model, ψ (Cave, Length, Connectivity, Entrance) p (year, length) was not a top ranking model. Our global model had a variance inflation factor (\hat{c}) = 2.615, indicating some extrabinomial variability unexplained by the global model (MacKenzie and Bailey 2004). Under the method of MacKenzie and Bailey (2004), one can calculate the Pearson's chi-square statistic (χ^2) for the observed occupancy data under the global model and find the probability that the calculated statistic is greater than the bootstrapped χ^2 test statistic ($\chi^2 = 0.834$, $p =$

0.002, $\hat{c} = 2.615$). Because of this high \hat{c} value (which is common in ecological data [MacKenzie et al. 2006]), we used the \hat{c} to modify the AIC criterion (now QAIC, Burnham and Anderson 2002).

Four models had a ΔQAIC of ≤ 2 and were considered models with substantial support (Table 5). Each of these four models included a different estimate for each cave, and one additional covariate. The three top models had equivalent support via QAIC. In fact, the point estimates for each cave were equal across the four models (these are given in Table 1). Because these multispecies models assume that the covariates affect all of the species the same (MacKenzie et al. 2006), this point estimate reflects the probability that a species will occupy that site, and this probability is constant across all 22 species for the site. Thus, multiplying the point estimate by 22 (the number of terrestrial species in the data set), we calculated the estimated site-specific species richness. For all caves, estimated richness was higher than or equal to observed richness (Figure 5).

Using the raw data of species richness, we found significant relationships between richness and log cave length ($F_{1, 57} = 32.82$, $p < 0.001$, $R^2 = 0.36$), and entrance area ($F_{1, 57} = 6.91$, $p = 0.011$, $R^2 = 0.11$), but not with log connectivity ($F_{1, 57} = 0.03$, $p > 0.05$) (Figure 6, left panels). The model-averaged estimates for the slopes of overall occupancy, occupancy per cave, and detection probability per year are provided in Table 6. Using these model averaged estimates, we calculated the estimated richness across the range of each of the three covariates of interest (Figure 6). For length, we found a positive relationship between estimated richness and log cave length, with the largest effects apparent as the length increases over 2.5 km.

Similarly, for entrance area, we found that estimated number of species increased with increasing area. Lastly, the results of the model show a negative slope for connectivity (Table 6), indicating a negative relationship between predicted richness and connectivity, such that the few caves that are the most connected have a slightly decreased probability of being occupied (Figure 6). However, these well-connected caves are also quite small in length, which probably explains the downturn in occupancy.

Discussion

In systems dominated by rare species, bioinventories are challenging and time-consuming, requiring that many sites be repeatedly sampled for an accurate characterization of species richness and occupancy patterns. When such substantial efforts are not possible, information on where species are likely to occur is crucial. Here, we applied novel statistical models to data from repeated biological surveys of caves to examine classical questions of biogeographic theory while incorporating information about the detection of these rare species.

Despite three years of sampling, the species accumulation curves indicate that only about half of cave obligate species have been collected in this region. This phenomenon of the underestimation of species richness is nearly ubiquitous for cave-dwelling species (Culver et al. 2004, Culver et al. 2006, Zigmajster et al. 2008, Eberhard et al. 2009), with the exception being epikarstic copepods (Pipan and Culver 2007). Such underestimates are likely due to the rarity of cave species. Classical estimates, like the Chao 2 and Jackknife estimators are based on the assumption that with increased sampling, the number of unique species will decline to zero (Soberón

and Llorente 1993). This is not what we observed. In fact, we found that the number of uniques was still increasing with increasing effort after three years of sampling (Figure 2 inset). Similar increases in unique species with additional sampling effort have been observed for several subterranean groups of species and are attributed to the high endemism of cave species (Pipan and Culver 2007, Zigmajster et al. 2008).

The bootstrap estimate, on the other hand, is much more conservative than these estimators. In their recent analysis of subterranean beetles, Zigmajster et al. (2008) recommend the use of the bootstrap estimate because it is not as sensitive to spatially rare species (Poulin 1998). If we focus on the bootstrap estimate, only two additional species are likely to be present in this system. However, if we had only considered the bootstrap estimate in 2002, we would have been satisfied when 14 species were collected, and thus we would have missed eight cave-limited species subsequently documented from the same suite of caves (Table 3).

The estimates of obligate cave species richness based on Chao 2 and Jackknife 2 are high, but not nearly as high as the estimate based on the Bayesian model, which are more than double the number of species that were collected over the three years. The distribution of the parameters from this model suggests that heterogeneity in occupancy is the predominant explanation for why so many species remain undetected in this system. This combination of low rates of occurrence and low detection probability is not unexpected in a system dominated by species that are rare both spatially and numerically (Eberhard et al. 2009). Similarly high estimates of species richness due to low occupancy rates are also attributed to undetected bird species based on the extensive Breeding Bird Survey data, in which the number of

sites would need to be doubled to collect the estimated number of species in the community (Dorazio et al. 2006). For our data on cave species, even if we quadrupled the number of caves sampled, we would still not capture 47 species (Figure 4). This estimate is also high compared to the known biodiversity of Greenbrier County, West Virginia (Culver, unpublished data). In the entire county (which contains a limestone area of approximately 300 km²), there are 31 known terrestrial cave species or 35 if the four undescribed species are included (Table 2). These totals, although still substantially lower than the Bayesian estimate, are virtually identical to the classical (Chao 2, Jackknife 2) estimates for our 11 km² area (Table 3). Thus, it seems plausible that some of these as-yet unknown species may actually be present within our study area. Additional support for this possibility derives from the fact that our previous sampling efforts have greatly extended the geographic ranges of several cave obligate species.

It is not clear whether additional sites or additional visits would be more effective at increasing occurrence records for cave-resident species. Culver et al. (2004) describe instances where scientists discover undocumented species within caves after many previous visits. Krejca and Weckerly (2007) agree, and suggest that with recommendations of ten visits are needed to collect ascertain occurrence patterns of several obligate cave species in a series of Texas caves.

We too, have observed this type of scenario, even within these caves. Recall that eleven caves were removed from our annual survey (Table 1) for their use in a resource manipulation project (Schneider et al. in prep, see Chapter III). In this project, all natural food was removed from these caves and standardized quantities of

alternative food sources were provided. The community utilizing each resource within each cave was monitored monthly for 23 months. These efforts resulted in significant increases in our knowledge of occupancy of these sites. For example, this intensive sampling yielded three new site occurrences of the pseudoscorpion *Kleptochthonius henroti*, five new sites occurrences for the beetle *Pseudanophthalmus grandis* (and two for *P. fuscus*), and three new sites for the dipluran *Litocampa fieldingae* and one new site for the new species of the genus *Orientocampa*. Lastly, the collembola *Pseudosinella gisini* and the millipede *Pseudotremia fulgida* were found in all eleven sites, whereas before they were only known from six and three of the sites, respectively. (None of these new occurrence records related to the experimental effort were included in our analyses here.)

The point estimates derived from the multispecies occupancy model (Table 1) support the conclusion that cave species richness in this system is underestimated, even at the local scale (Figure 5). In fact, when detection was incorporated, local species richness for every cave was underestimated by an average of one species per cave. Summed across all caves, the detectability analysis suggests 77 occurrence records are as-yet missing from this suite of caves. Using the Chao 2 estimate of richness, at least 14% of these missing records should yield new species system-wide.

Model averaged estimates of richness based on covariates supported our hypotheses that species richness increases with cave length (a gauge of habitat area) and entrance area (an indirect measure of allochthonous resource input). The hypothesis that species richness will be higher in larger caves was supported using both the raw data and the data based on the occupancy models. A larger cave is likely

to support more species because of an increase in available habitat, increased probability of finding mates because of larger population sizes, and protection from predators (Begon et al. 2006). A larger cave also means decreased competition with cave-transient species that can also live on the surface. Transient cave occupying species, such as flies, salamanders, and crickets, are known to compete with and prey upon cave obligate species (Howarth 1993, Culver 1982). Though the relationship between species richness is less pronounced when using estimated richness (Figure 6), we expect that if larger caves were present in the study area, this relationship would be more evident.

The hypothesis that species richness will be higher in caves with a larger entrance area was supported using both the raw data and the data based on occupancy models. Here, we hypothesized that caves with a larger entrance would provide more food resources, in a habitat often classified as “food-poor”. The productivity hypothesis, which is often used as an explanation for increased diversity in the tropics, contends that a large resource base can support increased species richness and increased specialization (Hutchinson 1959, Connell and Orias 1964, Brown 1981, Wright 1983, Currie 1991). Though the species energy hypothesis has been proposed for cave invertebrates before (Christman et al. 2005, Culver et al. 2006), these studies focus on large scale differences in geographic areas, where high species richness and endemism is in part attributed to high surface productivity. In their global study of cave biogeography, Culver et al. (2006) proposed a “biodiversity ridge” in which cave species richness is a reflection of high productivity that has remained relatively constant through geological time. Here, we suggested that the influence of resource

availability can also be observed even at the local scale, such that caves with larger entrances support more species because of increased detrital resource base. Indeed, we found that the model averaged estimates exhibited a positive relationship between entrance size and richness, though the overall change in the estimated number of species is small (ranging from three to five species). This is because the model assumes that all species are going to respond equally to the covariates. An additional hypothesis that should be tested could incorporate species-level traits, such as trophic level, which may make the effect of entrance size even more evident.

We did not observe a compelling relationship between richness and connectivity among caves, even when measuring connectivity based on the distance between cave passages. The lack of a strong predictive value of connectivity is thus attributed to the scale of our analysis. Over evolutionary time, it is predicted that proximity to other caves would be an important characteristic, as it may be expected that clusters of caves would experience the same degree of initial colonization, with site characteristics determining extinctions (Culver et al. 2006). However, the restricted dispersal capabilities of terrestrial cave species suggest that proximity to other caves should not affect patterns of species richness on an ecological scale. Our results are driven largely by a few well-connected caves, which are extremely small and have relatively low species richness. This pattern, which may reflect the geologic age of the large sites or the selective colonization of the large sites, contrasts with the results of Christman et al. (2005), who, in their study of all karst areas of the eastern United States, found that cave endemism was highest in karst areas with increased connectivity.

In the past several decades, several studies have examined diversity patterns in terrestrial cave communities (Poulson and Culver 1969, Sket 1999, Culver and Sket 2000, Hobbs 2005). General factors hypothesized to control species distribution are those that affect invasion, isolation, and movements of cave organisms (Christman and Culver 2001), such as evolutionary and ecological time, cave size and density, and hydrological and geological connectivity (Poulson and Culver 1969), as well as physical rigor (e.g. flooding), substrate diversity and organic content (Poulson and Culver 1969), and surface productivity over geologic time (Culver et al. 2006). Here we examined these biogeographical questions on a more local scale, investigating all caves within a small karst area. We found that, as predicted by biogeographical theory, species richness was influenced primarily by cave length and to a lesser extent by cave entrance size (a gauge of resource availability) and by cave connectivity. Historically, a major impediment to accurately describing cave biodiversity patterns has been that richness is often underestimated because of the rarity of cave species (Culver et al. 2004). In this paper, we show that novel applications of statistical methods can be incorporated to cave bioinventory data such that information from biological surveys, even if incomplete, can provide insights into the spatial occupancy patterns of rare species.

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Tables

Table 1. List of the caves biologically inventoried in at least one of the three repeated surveys. Included for each cave is the richness based on raw survey data and the point estimated occupancy probability (derived from the occupancy models).

Cave name	No. annual surveys	Explanation if not sampled three years	Length (m)	Entrance area	Connectivity based on Euclidean distance	Terrestrial richness based on annual surveys	Point estimate occupancy probability (?) + SE
Allisons	3		134	1.38	99.61	6	0.37 ± 0.13
Al's Insurgence	3		5	2.12	2.60	1	0.06 ± 0.06
Apple	3		11	1.51	484.96	1	0.06 ± 0.06
Boothe Cave	3		15	1.81	9.36	1	0.06 ± 0.06
Buckeye Crawl	3		2	0.54	3117.88	1	0.06 ± 0.06
Buckeye Creek	3		3719	44.30	80.41	9	0.56 ± 0.15
Buckeye Overflow	3		8	6.50	2007.53	3	0.19 ± 0.1
Buckeye Storage	3		5	0.66	3229.73	0	
Callison's Pond Cave	3		76	0.88	0.23	1	0.06 ± 0.06
CB's blowhole	3		2	0.63	7.20	1	0.06 ± 0.06
Clutetown	3		61	3.99	95.88	5	0.31 ± 0.12
Crabapple	3		2	0.42	435.31	1	0.06 ± 0.06
Deer Insurgence	3		5	0.67	29.70	1	0.06 ± 0.06
Fuells Fruit	3		346	4.12	11.79	9	0.56 ± 0.15
Goat Cave	3		9	3.21	11.06	6	0.37 ± 0.13
Hannah Caverns	3		772	7.85	22.15	4	0.25 ± 0.11
Hannah Overhang	3		3	11.16	179.80	0	
Hannah Water (Upper Spout)	3		39	1.89	243.49	3	0.19 ± 0.1
Hell of a Pit	3		20	1.98	12.27	2	0.19 ± 0.1
Hell of a Pit 2	3		20	2.27	12.15	3	0.12 ± 0.08
Hillside Pit	3		30	45.74	5.73	7	0.43 ± 0.14
Hit N Head	3		6	0.29	99.06	1	0.06 ± 0.06
JJ Spring Cave	3		41	0.53	130.38	0	
Looks Good From Afar	3		2	11.38	4.45	1	0.06 ± 0.06
Mary McFerrin Cave	3		8	34.68	344.00	2	0.12 ± 0.08
Matts Black	3		490	19.09	64.46	6	0.37 ± 0.13
MC Cave	3		15	0.28	70.29	3	0.19 ± 0.1
McFerrin Breakdown	3		155	34.33	195.69	6	0.37 ± 0.13
McFerrin Water (Spur Cave)	3		453	47.00	27.01	2	0.12 ± 0.08
Nellies	3		431	2.24	88.59	4	0.25 ± 0.11
Oak Sang Cave	3		3	1.10	4.14	1	0.06 ± 0.06
Osborne Pit	3		5	0.28	14.18	2	0.12 ± 0.08
Pilgrims Rest Church Cave 1	3		42	0.19	13.97	4	0.25 ± 0.11
Pilgrims Rest Church Cave 2	3		51	9.49	9.59	3	0.19 ± 0.1
Point Pit	3		3	3.65	2.66	2	0.12 ± 0.08
Rapps	3		1829	8.14	71.56	7	0.43 ± 0.14
Seep Cave 2	3		30	14.09	4.75	2	0.12 ± 0.08
Short Stuff Cave	3		56	1.62	15.64	1	0.06 ± 0.06
Spencer Cave	3		304	17.15	121.72	2	0.12 ± 0.08
Spencer Trap Cave	3		61	0.95	182.28	3	0.19 ± 0.1
Spencer Waterfall Cave	3		103	13.67	305.21	1	0.06 ± 0.06
Spout Cave	3		300	33.68	42.13	6	0.37 ± 0.13
Teetering Rock Pit	3		24	1.26	0.43	6	0.37 ± 0.13
Tin Cave	3		6	9.87	4.76	5	0.31 ± 0.12
Turner Cave 1	3		9	1.89	135.68	1	0.06 ± 0.06
Turner Pit 2	3		117	21.41	26.38	5	0.31 ± 0.12
Unnamed Insurgence	3		5	0.44	4.05	0	
Upper Buckeye	3		436	2.07	250.62	8	0.5 ± 0.14
Upper Turner	3		3	0.17	13.84	1	0.06 ± 0.06
US 219	3		387	5.81	0.02	4	0.25 ± 0.11
Water Trough Cave	3		11	1.24	26.01	1	0.06 ± 0.06
Zimmerman Pit	3		17	0.90	6.12	4	0.25 ± 0.11
Baber Pit 2	2	Removed for RM (2007)*	28	16.62	0.62	0	
Bill Jones FRO	2	Removed for RM (2007)*	20	1.13	119.17	2	0.17 ± 0.11
Fieldstation Pit	2	Removed for RM (2007)*	6	0.35	24.97	2	0.17 ± 0.11
Inspired Pit	2	Removed for RM (2007)*	5	27.14	134.82	3	0.25 ± 0.14
MC Pit	2	Removed for RM (2007)*	9	1.47	10.54	3	0.25 ± 0.14
Our Pit	2	Removed for RM (2007)*	26	1.26	39.09	0	
Pignut Pit	2	Removed for RM (2007)*	18	0.79	29.79	2	0.17 ± 0.11
Posthole Pit	2	Removed for RM (2007)*	5	0.61	165.57	1	0.08 ± 0.08
Raceway Pit	2	Removed for RM (2007)*	14	2.91	2.77	5	0.42 ± 0.17
Salamander Suicide Pit	2	Removed for RM (2007)*	10	0.42	11.74	2	0.17 ± 0.11
Sunnyday Pit	2	Removed for RM (2007)*	8	48.49	2.99	3	0.25 ± 0.14
Trilium Cave	2	Turkey vulture in cave (2007)	3	1.23	11.97	3	0.19 ± 0.1
Spade Cave	2	Inaccessible after 2004	20	5.60	0.02	1	0.06 ± 0.06
McFerrin Resurgence	1	Water too high after 2002	-	-	-	0	
One Little Room Cave	1	Inaccessible after 2002	-	-	-	0	
Wake Robbin Cave	1	Unstable after 2002	-	-	-	2	

*RM=Resource Manipulation Project (see text)

Table 2. Terrestrial cave-obligate species collected in the multiyear survey of the caves of Greenbrier County, West Virginia. Data are from caves visited at least twice over the survey period (2002, 2004, 2007).

Order	Family	Genus species	Number of caves occupied
Acari	Rhagidiidae	<i>Rhagidia varia</i>	13
Araneae	Linyphiidae	<i>Phanetta subterranea</i>	10
Araneae	Linyphiidae	<i>Bathypantes weyeri</i>	1
Araneae	Linyphiidae	<i>Porrhomma cavernicola</i>	1
Araneae	Linyphiidae	<i>Anthrobia coylei</i>	5
Pseudoscorpiones	Chtoniidae	<i>Kleptochthonius henroti</i>	8
Chordeumatida	Cleidogonidae	<i>Pseudotremia fulgida</i>	20
Chordeumatida	Cleidogonidae	<i>Pseudotremia schneiderae</i>	1
Chordeumatida	Trichopetalidae	<i>Zygonopus packardi</i>	2
Coleoptera	Carabidae	<i>Pseudanophthalmus fuscus</i>	4
Coleoptera	Carabidae	<i>Pseudanophthalmus grandis</i>	23
Coleoptera	Carabidae	<i>Pseudanophthalmus higinbathami</i>	2
Coleoptera	Carabidae	<i>Pseudanophthalmus hypertrichosis</i>	2
Collembola	Entomobryidae	<i>Sinella hoffmani</i>	9
Collembola	Entomobryidae	<i>Pseudosinella gisini</i>	51
Collembola	Onychiuridae	<i>Onychiurus n. sp.</i>	1
Collembola	Sminthuridae	<i>Arrhopalites clarus</i>	22
Collembola	Sminthuridae	<i>Arrhopalites carolynae</i>	1
Diplura	Campodeidae	<i>Eumesocampa spp.</i>	1
Diplura	Campodeidae	<i>Orientocampa n. sp.</i>	1
Diplura	Campodeidae	<i>Litocampa n. sp.</i>	1
Diplura	Campodeidae	<i>Litocampa fieldingae</i>	7

Table 3. Values of estimators for species richness in this ~11km² area of West Virginia.

Data Set	Number of Caves	Observed Number of Species	Number of Uniques ^a	Number of Duplicates ^b	Bootstrap	Jackknife 2	Chao 2	HB Estimate*
2002	65	12	5	1	14.0	20.8	24.5	
2004	65	18	7	2	20.9	29.8	30.3	
2007	52	11	2	0	11.9	14.9	11.0	
All data [†]	65	22	8	3	25.3	34.8	32.7	46.5

^a Uniques: species collected from only one cave
^b Duplicates: species collected from two caves
* HB Estimate is the Hierarchical Bayesian estimate of Dorazio and Royle (2005) (see text).
[†] The data set "All data" represents the union of the three annual data sets.

Table 4. Model selection procedure for estimating occupancy (ψ) and detection (p) for the multispecies model, testing factors hypothesized to influence occupancy (Cave, length, Euclidean and nearest passage connectivity, and entrance area and circumference (“EntranceCirc”). We also tested factors on detection, including the effects of year, length, or the hypothesis that detection was constant “p(.)”.

Models	Par	QAIC	Δ QAIC	QAIC weight
ψ (Cave) p (Year)	63	733.58	0.00	0.3726
ψ (Cave, Length) p(Year)	64	735.58	2.00	0.1371
ψ (Cave, EuclideanConnect) p (Year)	64	735.58	2.00	0.1371
ψ (Cave, AreaEntrance) p(Year)	64	735.58	2.00	0.1371
ψ (Cave, EuclideanConnect, AreaEntrance) p (Year)	65	737.58	4.00	0.0504
ψ (Cave, Length, EuclideanConnect) p (Year)	65	737.58	4.00	0.0504
ψ (Cave, Length, EntranceArea) p (Year)	65	737.58	4.00	0.0504
ψ (Cave, Length, EuclideanConnect, EntranceArea) p (Year)	66	739.58	6.00	0.0186
ψ (Cave, Length, PassageConnect, EntranceArea) p (Year)	66	739.58	6.00	0.0186
ψ (Cave, Length, EuclideanConnect, EntranceArea) p (Year)	66	739.58	6.00	0.0186
ψ (Cave, Length, EuclideanConnect, EntranceArea) p (Year, length)	67	741.07	7.50	0.0088
ψ (Cave, Length, EuclideanConnect, EntranceArea) p (.)	64	746.96	13.38	0
ψ (Cave, Length, EuclideanConnect, EntranceArea) p (Length)	64	759.24	25.66	0

Table 5. The four best fit models for estimating probabilities of occupancy (ψ) and detection (p) and their associated recalculated QAIC weights.

Models	Par	QAIC	ΔQAIC	QAIC weight
$\psi(\text{Cave}) p(\text{Year})$	63	1714.81	0.00	0.3711
$\psi(\text{Cave, Length}) p(\text{Year})$	64	1716.81	2.00	0.1365
$\psi(\text{Cave, EuclideanConnect}) p(\text{Year})$	64	1716.81	2.00	0.1365
$\psi(\text{Cave, EntranceArea}) p(\text{Year})$	64	1716.81	2.00	0.1365

Table 6. Slope estimates for occupancy (psi), cave (psiCave), and detection (pyear) from the multispecies occupancy models.

Source	Slope	Estimate
Model-averaged estimates	psi	-1.219
	psiCave1	0.838
	psiCave2	-0.869
	psiCave3	-0.848
	psiCave4	0.005
	psiCave5	-0.868
	psiCave6	-0.738
	psiCave7	0.955
	psiCave8	0.166
	psiCave9	-0.871
	psiCave10	-0.864
	psiCave11	0.620
	psiCave12	-0.846
	psiCave13	-0.863
	psiCave14	0.005
	psiCave15	1.398
	psiCave16	0.840
	psiCave17	0.297
	psiCave18	0.107
	psiCave19	0.099
	psiCave20	-0.276
	psiCave21	0.905
	psiCave22	-0.859
	psiCave23	0.323
	psiCave24	-0.898
	psiCave25	-0.364
	psiCave26	0.747
	psiCave27	0.107
	psiCave28	0.400
	psiCave29	0.735
	psiCave30	-0.458
	psiCave31	0.349
	psiCave32	-0.866
	psiCave33	-0.268
	psiCave34	0.002
	psiCave35	0.390
	psiCave36	0.072
	psiCave37	-0.279
	psiCave38	-0.606
	psiCave39	0.990
	psiCave40	0.858
psiCave41	0.004	
psiCave42	-0.315	
psiCave43	-0.872	
psiCave44	-0.882	
psiCave45	-0.346	
psiCave46	0.105	
psiCave47	-0.902	
psiCave48	0.717	
psiCave49	0.250	
psiCave50	0.845	
psiCave51	0.604	
psiCave52	0.103	
psiCave53	-0.863	
psiCave54	0.557	
psiCave55	1.211	
psiCave56	-0.862	
psiCave57	0.339	
psiCave58	-0.866	
psiCave59	0.389	
pyear1	-0.994	
pyear2	0.725	
pyear3	0.627	
Estimates from full model	psiLength	0.296
	psiArea	0.195
	psiEuclidean	-0.110
Constants	psiLength	0.373
	psiArea	0.310
	psiEuclidean	-0.140

Figure legends

Figure 1. Map of the study area from Google Earth. Circles denote cave entrances. For large caves, the entire cave passage is mapped as white lines extending from the entrance. The figure is oriented such that North is upward.

Figure 2. Species accumulation curves. Incidence functions of number of terrestrial cave obligate species plotted against the number of caves sampled. The estimates of richness based on the Hierarchical Bayesian model (HB estimate) and Chao 2 estimator (marked with asterisks), are provided for reference at the number of species corresponding to the estimate (in parentheses). Inset: The number of uniques (i.e. species collected from only one cave) continues to rise based on the union of the three annual data sets.

Figure 3. Distributions of the probabilities of occupancy (black line) and detection (gray line) based on estimates of model parameters ($\hat{\alpha} = -1.77$, $\hat{\sigma}_u = 2.63$, $\hat{\beta} = -3.22$, $\hat{\sigma}_v = 1.91$).

Figure 4. Predicted species accumulation curve based on the Bayesian approach of Dorazio and Royle (2005). Each point is the estimate of the mean of the posterior predictive distribution of the data. Error bars represent 90% prediction intervals. The vertical white line indicates the number of caves that were sampled in this study. The horizontal white line indicates the estimated number of species based on this approach.

Figure 5. Observed number of terrestrial obligate cave species (across all years of data), plotted against the estimated number of species based on the multispecies occupancy model. The line represents a 1:1 line where the observed richness is equal to the estimated richness.

Figure 6. Observed numbers of species (left) and estimated numbers of species (right) plotted against length (log transformed), entrance area, and connectivity (log transformed, based on Euclidean distance [see text]). Estimated species richness based on model-averaged estimates of the slopes of occupancy, site, and detection, holding the slopes of the other covariates constant. Regression lines on the left panels represent the linear regression of the covariate and observed richness.

Figures

Figure 1.

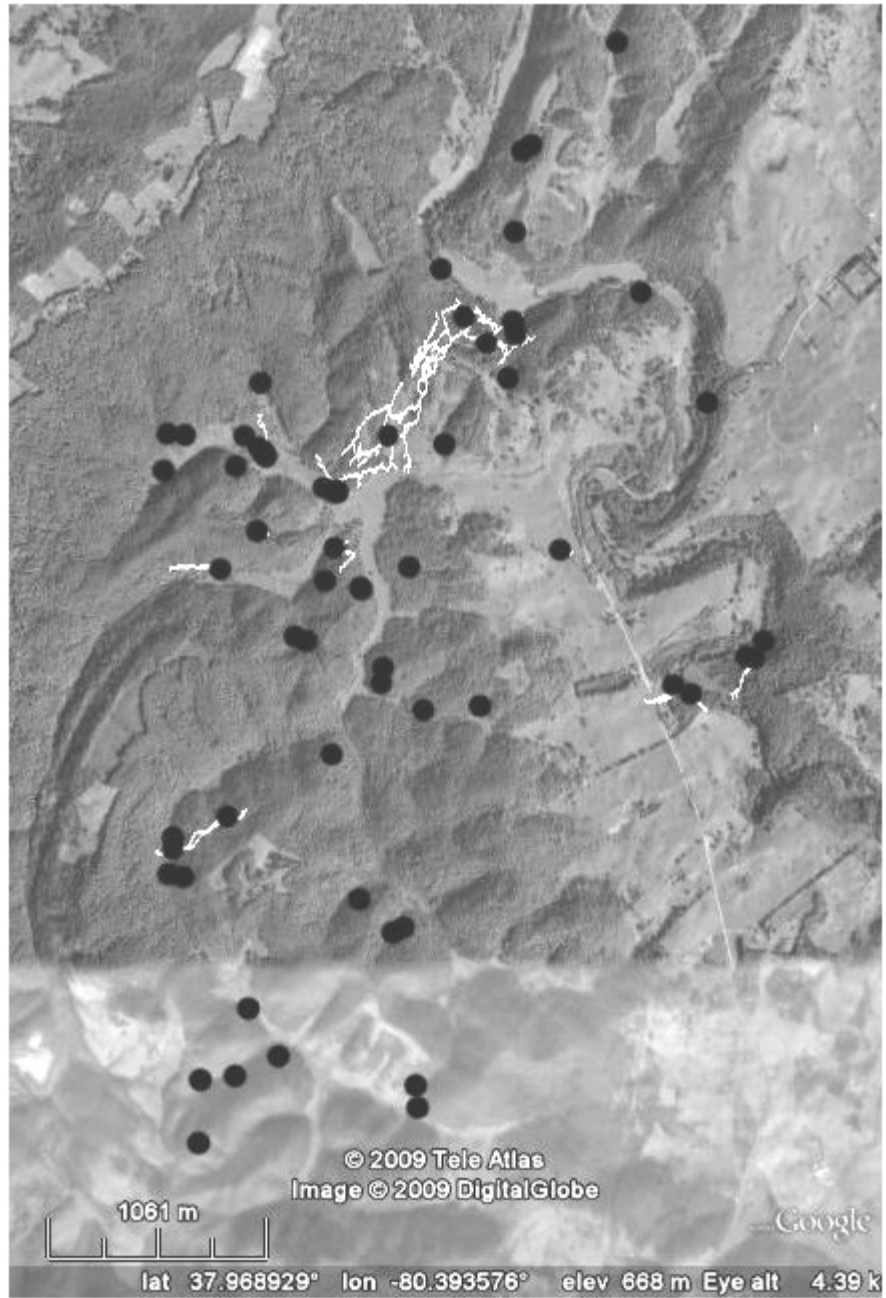


Figure 2.

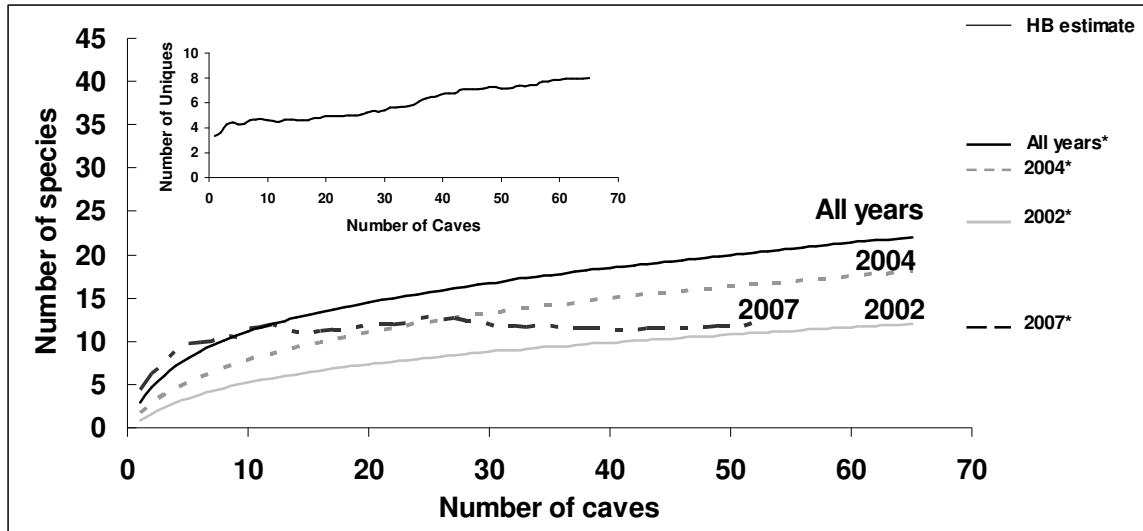


Figure 3.

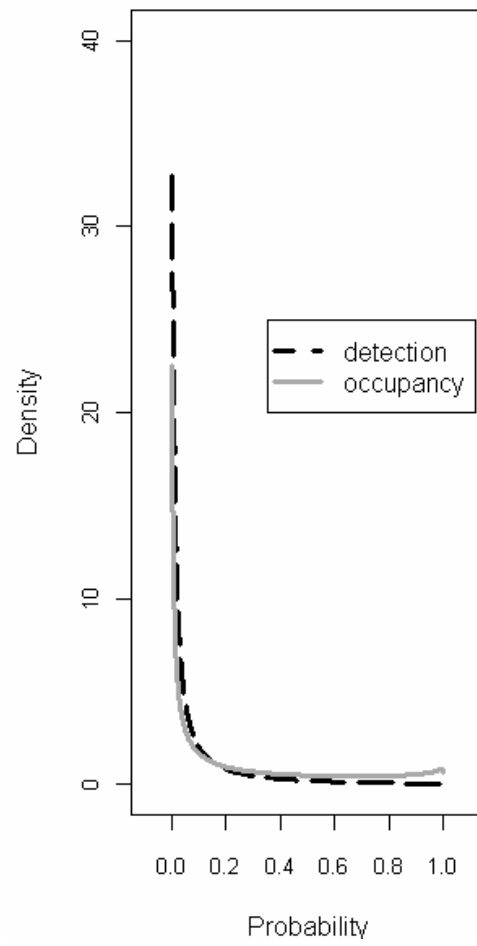


Figure 4.

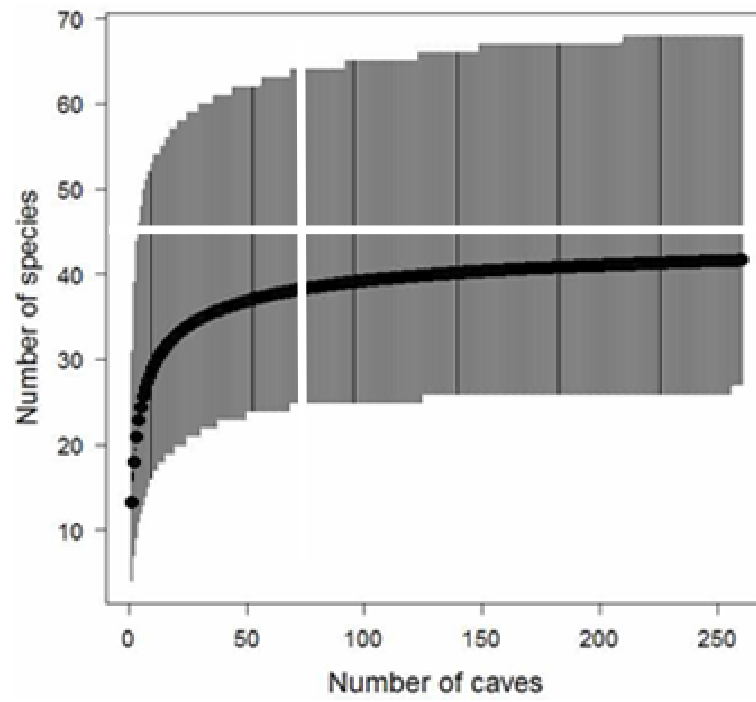


Figure 5.

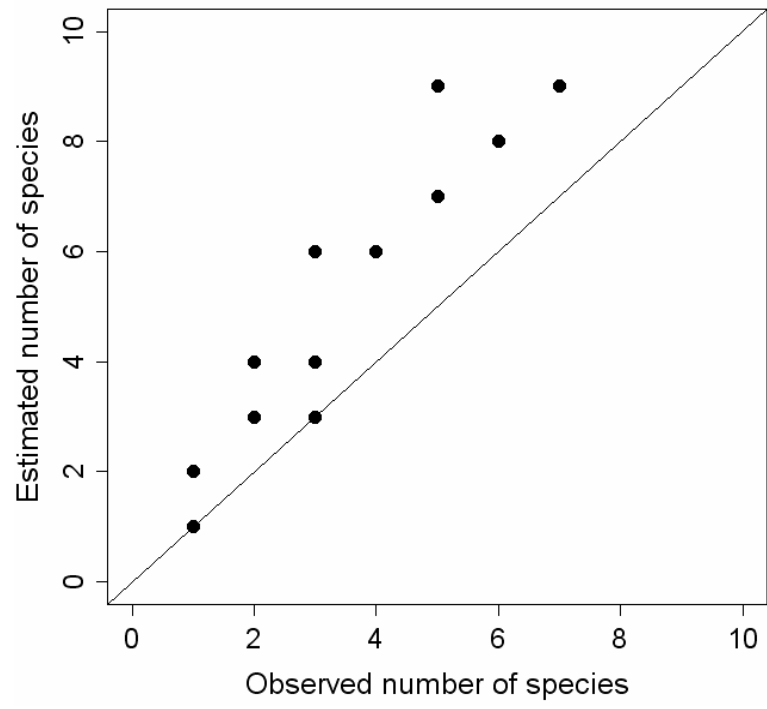
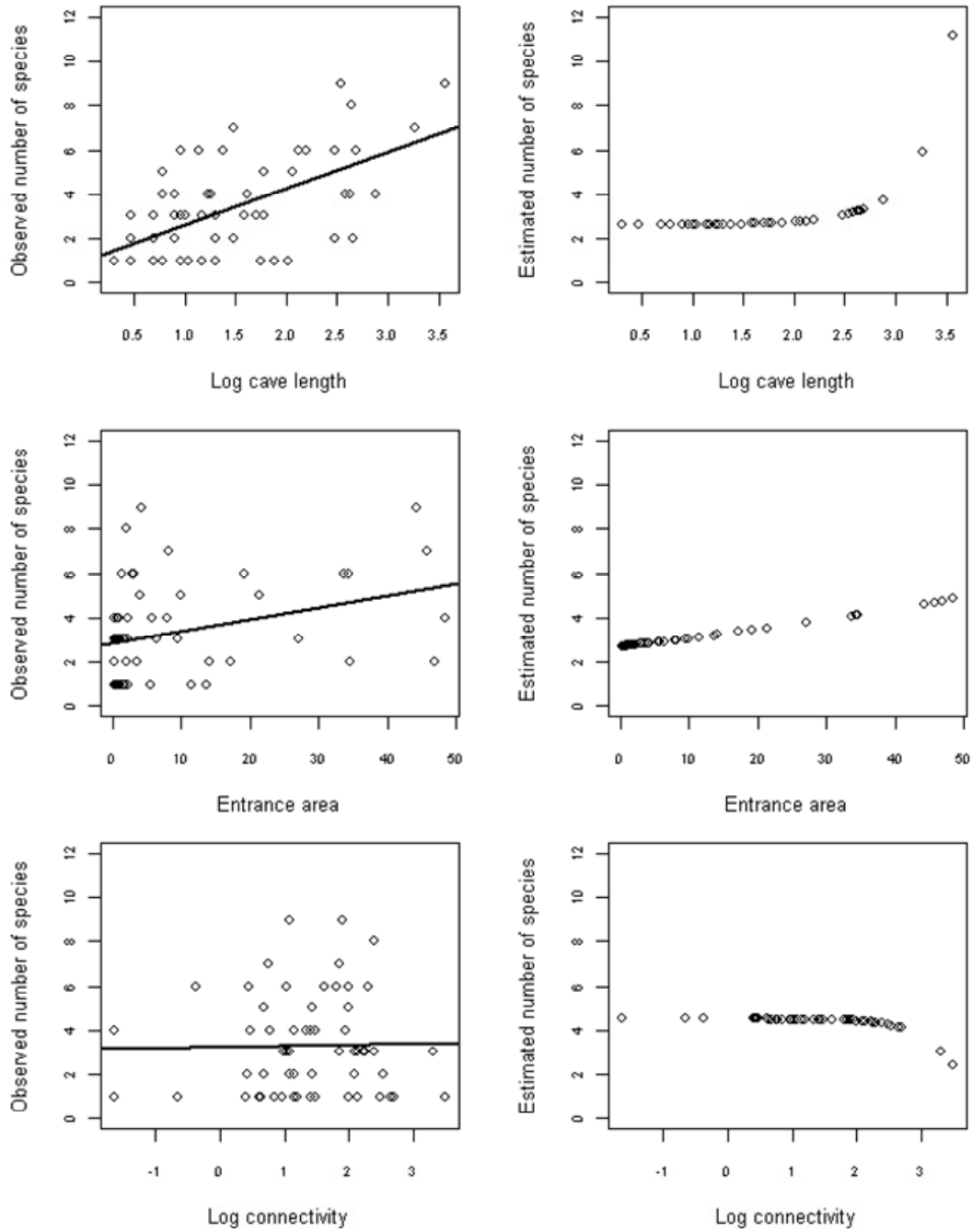


Figure 6.



Appendices

Appendix A. Average amino acid concentrations in two species of millipedes, amphipods, and ants (data from Williams et al. 1987). The numbers of replicates (n_r) and the total average FAA concentrations are also presented.

Amino Acid	%N by mass in side chain	transient millipede ($\mu\text{mol/g}$) $n_r = 9$	obligate millipede ($\mu\text{mol/g}$) $n_r = 5$	surface amphipod ($\mu\text{mol/g}$) $n_r = 20$	cave amphipod ($\mu\text{mol/g}$) $n_r = 17$	normal ant ($\mu\text{mol/g}$)	depigmented ant ($\mu\text{mol/g}$)
AABA	0	0.10	0.18	0.85			
α ALA	0	24.65	22.69	7.64	43.88		
ALA	0					8.35	3.72
ASP	0	3.78	3.16	2.49	4.15	0.15	6.68
β ALA	0	0.05	0.23	0.49			
GABA	0	1.49	1.25	0.52			
GLU	0	14.54	11.60	7.71	14.61	6.02	8.60
GLY	0	11.01	9.13	4.07	12.79	5.50	8.28
HomoSER	0	4.74	4.08	0.66			
ILE	0	4.44	3.65	1.19	10.54	1.73	2.48
LEU	0	10.13	8.20	1.94	23.04	0.91	6.96
MET	0	2.83	2.15	1.52	8.98	0.97	1.36
PHE	0	5.23	3.93	1.12	14.01	4.85	4.52
PRO	0	8.68	7.83	5.06	8.87	12.75	25.72
SER	0	10.21	7.67	5.68	14.51	8.46	9.60
THR	0	8.25	6.53	2.89	11.02		
TYR	0	2.64	1.41	1.73	15.36	2.19	6.16
VAL	0	8.36	6.44	2.43	18.92	4.73	10.00
ORN	10.60	0.49	0.58	0.20	3.06		
TRP	10.76	0.87	0.72	0.32	3.01		
LYS	19.42	12.56	8.21	3.83	38.13	10.53	3.68
GLN	19.43	13.61	7.34	17.14	74.05		
ASN	24.13	4.34	2.90	12.45	8.84		
HIS	34.55	4.63	3.58	6.44	9.74	6.83	3.92
ARG	41.96	21.31	16.48	17.35	28.40	3.18	1.76
Total		178.95	139.94	105.72	365.91	77.15	103.44

Appendix B. List of the 12 caves investigated for the resource manipulation experiment (Chapter III) experiment, including their code used in the RDA (Fig 4), the treatment that they received (either one or two leaf packs or rat carcasses), descriptive characteristics (length, depth, presence of standing water). The number of obligate cave species recorded is based on the literature (Schneider and Culver 2004, Fong et al. 2007). The number of individuals and number of morphospecies are broken down by trap (as half of the caves received two subsidy treatments); the number of unique morphospecies includes only those that were found in the cave at least once, regardless of whether or not they were found on only one trap.

Cave	Code	Trt	Number of subsidies	Length (m)	Depth (m)	Standing water	Number of obligate species prev. recorded	Number of individuals observed in trap one	Number of individuals observed in trap two	Number of morphosp. observed in trap one	Number of morphosp. observed in trap two	Number of unique morphosp.
Nettle	NET	Leaf	1	4.6	4.6		1	644	NA	40	NA	40
Our Pit	OUR	Leaf	1	25.9	12.2	Y	2	878	NA	40	NA	40
Raceway Pit	RW	Leaf	1	13.7	13.7		6	272	NA	38	NA	38
Baber Pit 2	BA	Leaf	2	27.7	18.6	Y	3	582	400	32	33	41
MC Pit	MC	Leaf	2	9.1	9.1		3	459	153	32	15	37
Pignut Pit	PG	Leaf	2	18.3	3	Y	4	546	588	47	43	55
Fieldstation Pit Salamander	FS	Rat	1	6.1	6.1	Y	4	4328	NA	36	NA	36
Suicide Pit	SA	Rat	1	10.4	10.7		3	1294	NA	44	NA	44
Sunnyday Pit	SU	Rat	1	7.6	9.1		5	1382	NA	48	NA	48
Bill Jones Pit	BJ	Rat	2	19.8	13.7		3	970	2044	40	39	49
Inspired Pit	INS	Rat	2	4.6	4.6		3	993	2133	43	54	62
Posthole Pit	PH	Rat	2	4.6	4.6		3	1139	1061	40	41	48

Appendix C. List of the 102 morphospecies collected during the 23 months of the manipulation experiment (Chapter III). Also included are the number of caves (out of 12), the number of sites (out of a potential 18), and the number of months in which the morphospecies was observed, as well as the number of individuals observed during the course of the experiment. Asterisks denote cave obligate species (“troglonbionts” **) or likely cave obligate species (*) that are incompletely identified, but troglomorphic.

Class	Order	Family	Identification	No. sites	No. caves	No. months observed	No. individuals observed
Arachnida	Acari	Gamasidae	Gamasid mite	10	8	11	450
		Oribatidae	Oribatid mite	17	12	20	253
		Rhagidiidae	Rhagidiid mite*	18	12	22	159
		Tetranychidae	Tetranychid mite	1	1	1	2
		Trombiculidae	Trombiculid mite	2	2	2	2
		Unknown	Mite 1	4	4	5	6
		Unknown	Mite 2	11	9	11	31
		Unknown	Mite 3	5	4	6	11
		Unknown	Mite 4	2	2	1	14
		Araneae	Agelenidae	<i>Circurina sp.</i>	9	7	8
	Araneae	Tetragnathidae	<i>Meta ovalis</i>	3	3	6	9
	Araneae	Unknown	Araneae 1	10	8	16	45
	Opiliones	Unknown	Opiliones 1	4	2	9	21
	Pseudoscorpiones	Chthoniidae	<i>Kleptochtonius henroti**</i>	6	4	13	23
	Pseudoscorpiones	Chthoniidae	<i>Apochthonius sp.</i>	2	2	4	4
Pseudoscorpiones	Neobisiidae	<i>Microcreagris sp.</i>	3	2	4	20	
Chilopoda	Geophilomorpha	Geophilidae	<i>Arenophilus bipunctatus</i>	2	2	3	5
	Lithobiomorpha	Lithobiidae	<i>Nampabius sp.</i>	2	1	3	3
	Scolopendromorpha	Cryptopidae	<i>Scolopocryptops sexspinosus</i>	1	1	2	2

Class	Order	Family	Identification	No. sites	No. caves	No. months observed	No. individuals observed	
Oligochaeta	Haplotaxida	Lumbricidae	Lumbricid 1	14	10	23	208	
Copepoda	Harpacticoida	Harpacticidae		3	2	2	28	
Malacostraca	Isopoda	Oniscoidea	Isopod 1	10	6	22	137	
			Isopod 2	2	2	3	3	
			Unknown	2	2	1	2	
Diplopoda	Chordeumatida	Cleidogonidae	<i>Pseudotremia fulgida</i> **	14	10	18	113	
			<i>Pseudotremia</i> juvenile	10	8	12	260	
			<i>Pseudotremia hobbsi</i>	18	12	23	1486	
			<i>Cleidogona</i> sp.	18	12	17	575	
	Julida	Julidae	<i>Ophiulus pilosus</i>	11	8	22	165	
			Polydesmida	Polydesmidae	<i>Pseudopolydesmus</i> sp.	8	7	12
	<i>Scytonotus</i> sp.	1			1	1	1	
	<i>Xystodesmidae</i>	1			1	1	1	
	<i>Nannaria</i> sp.	1			1	1	1	
				<i>Apheloria virginiensis</i>	3	3	5	8
		Spirostreptida	Cambalidae	<i>Cambala</i> sp.	5	3	7	34
		Unknown	Unknown	Diplopod 1	12	10	13	98
Diplopod 2	1			1	1	1		
Gastropoda	Pulmonata	Unknown	Pulmonata 1	15	11	20	72	
Hexapoda (Insecta)	Blattaria	Unknown	Blattaria 1	1	1	1	1	
			Coleoptera	Carabidae	<i>Pseudanophthalmus fuscus</i> **	2	2	2
	<i>Pseudanophthalmus grandis</i> **	9			7	16	57	
	Carabid 1	12			9	20	135	
	Carabid 2	9			7	6	74	
	<i>Pseudanophthalmus</i> larvae**	7			5	9	26	
	Coccinellidae	Coccinellid			4	3	6	35
	Dermestidae	Dermestid			3	3	2	5
	Silphidae	Silphid			5	5	3	42
	Staphylinidae	Staphylinid 1	16	12	22	360		

Class	Order	Family	Identification	No. sites	No. caves	No. months observed	No. individuals observed
			Staphylinid 2	1	1	1	1
		Trogidae	Trogid	1	1	2	2
		Unknown	Coleoptera larvae 1	14	10	18	56
			Coleoptera larvae 2	4	3	1	14
			Coleoptera larvae 3	2	1	1	4
	Collembola	Entomobryidae	<i>Pseudosinella gisini</i> **	18	12	20	1428
			<i>Sinella sp.</i>	18	12	23	935
			Collembola 2	9	7	13	285
			Collembola 4	7	7	9	16
			Collembola 5*	18	12	22	2525
		Hypogastruridae	Hypogastrurid 1	1	1	1	1
		Isotomidae	<i>Folsomia candida</i>	13	9	16	270
			Isotomid 1	12	11	20	473
			Isotomid 2	12	11	12	365
		Sminthuridae	Arrhopalites 1*	12	9	13	27
			Arrhopalites 2	5	5	6	8
			Arrhopalites 3	1	1	1	1
		Tomoceridae	Tomocerus sp	16	12	22	129
		Unknown	Collembola 1	1	1	1	1
			Collembola 3	1	1	1	1
			Collembola 6	3	3	3	8
			Collembola 7	5	5	2	10
			Collembola 8	1	1	1	5
	Dermaptera	Unknown	Dermaptera 1	4	3	3	5
	Diplura	Campodeidae	<i>Litocampa fieldingae</i> **	3	3	3	4
			<i>Orientocampa n.sp.</i> **	1	1	3	3
			Campodeid 1	1	1	1	1
	Diptera	Calliphoridae	Calliphorid	13	10	19	5274
		Culicidae	<i>Culex sp.</i>	1	1	1	1

Class	Order	Family	Identification	No. sites	No. caves	No. months observed	No. individuals observed
		Heleomyzidae	<i>Amoebaleria sp.</i>	6	4	11	26
		Mycetophilidae	Mycetophilid	13	9	10	44
		Phoridae	Phorid	18	12	21	478
		Psychodidae	Psychodid	2	2	2	2
		Sciaridae	Sciarid	1	1	1	1
		Sphaeroceridae	Sphaerocerid	13	11	14	104
		Tipulidae	Tipulid	2	2	2	13
		Unknown	Diptera 1	2	2	1	4
			Diptera 2	1	1	1	1
			Diptera 3	3	3	2	4
			Dipteran larvae 4	16	12	12	73
			Dipteran larvae 1	9	6	5	703
			Dipteran larvae 2	5	4	6	158
			Dipteran larvae 3	9	6	15	347
			Dipteran larvae 5	4	3	3	6
			Dipteran larvae 6	12	10	11	79
			Dipteran larvae 7	3	3	3	4
	Hemiptera	Cicadellidae	Cicadellid	4	3	5	8
	Hymenoptera	Formicidae	<i>Formica sp.</i>	5	4	5	15
	Lepidoptera	Noctuidae	<i>Scoliopteryx libatrix</i>	1	1	3	3
	Orthoptera	Gryllacrididae	<i>Ceuthophilus sp.</i>	12	7	11	53
		Rhaphidophoridae	<i>Euhadoenecus fragilis</i>	16	11	23	649
	Siphonaptera	Pulicidae	Siphonaptera 1	6	5	8	11
Nematoda	Unknown	Unknown	Nematoda 1	15	11	23	186
Symphyla	Cephalostigmata	Scutigereidae	<i>Hanseniella vandykei</i>	1	1	2	2
Turbellaria	Seriata	Planariidae	Planaria 1	1	1	1	1

**obligate cave species, *likely to be obligate cave species

Appendix D. Results from all statistical tests in Chapter III.

Table D1. Results from the generalized mixed model testing only the effect of treatment on richness and abundance, while including the random effects due to subsampling (see text). To test the effects of common and rare species, the analyses were performed without the most common morphospecies (n = 4), without the singletons and doubletons (n = 19), and without the most common and rare combined (n = 23). To test the effects of taxonomic groupings, we also performed the test with the morphospecies assigned to Order (n = 28 Orders, excluding the two diplopods and the nematodes that could not be assigned to Order). Lastly, to test the effects of unidentifiable juveniles, we excluded these 13 morphospecies from the analysis.

Only testing the effect of treatment					
	Data Set	estimate	SE	z value	p-value
Abundance	All data	1.026	0.310	3.314	< 0.001
	W/O most common	0.774	0.237	3.261	0.001
	W/O singletons & doubletons	1.027	0.310	3.320	< 0.001
	W/O most common & most rare	0.776	0.237	3.271	0.001
	With higher groupings	1.057	0.306	3.452	< 0.001
	W/O juveniles	0.972	0.327	2.976	0.003
Richness	All data	0.217	0.170	1.280	NS
	W/O most common	0.263	0.202	1.305	NS
	W/O singletons & doubletons	0.217	0.169	1.289	NS
	W/O most common & most rare	0.263	0.200	1.317	NS
	With higher groupings	0.190	0.137	1.388	NS
	W/O juveniles	0.216	0.160	1.346	NS

Appendix D. Results from all statistical tests in Chapter III.

Table D2. Results from the generalized mixed model testing the temporal aspects of the experiment. The data were parsed as in the statistical tests presented in Table D1 (see legend).

Examining the temporal effects						
	Data Set	Interaction of season and month since start	Estimate of interaction if significant	Effect of season	Effect of month since start	
RICHNESS	Rat	All data	p < 0.001	-0.042		
		W/O most common	p < 0.001	-0.046		
		W/O singletons & doubletons	p < 0.001	-0.042		
		W/O most common & most rare	p < 0.001	-0.046		
		With higher groupings	p < 0.001	-0.041		
		W/O juveniles	p < 0.001	-0.038		
	Leaves	All data	NS		NS	NS
		W/O most common	NS		NS	NS
		W/O singletons & doubletons	NS		NS	NS
		W/O most common & most rare	p = 0.048	-0.024		
With higher groupings		p = 0.019	-0.025			
W/O juveniles	NS		NS	NS		
ABUNDANCE	Rat	All data	p < 0.001	-0.083		
		W/O most common	p < 0.001	-0.052		
		W/O singletons & doubletons	p < 0.001	-0.084		
		W/O most common & most rare	p < 0.001	-0.052		
		With higher groupings	p < 0.001	-0.083		
		W/O juveniles	p < 0.001	-0.096		
	Leaves	All data	p < 0.001	-0.033		
		W/O most common	p < 0.001	-0.052		
		W/O singletons & doubletons	p < 0.001	-0.034		
		W/O most common & most rare	p < 0.001	-0.053		
		With higher groupings	p < 0.001	-0.030		
W/O juveniles	p = 0.010	-0.021				

Appendix D. Results from all statistical tests in Chapter III.

Table D2. Paired t-tests (with Bonferroni adjustments) to test differences in abundance and richness between the treatments in the months since the start of the experiment and the months since the last resource addition. Though all pairwise monthly samples were examined, only those which were significantly different are presented here. These numbers correspond to the data presented in Figure 2.

Months since start of the experiment					
	Month	Month number in graph	t	df	p-value
Abundance	April 2006	4	-2.262	10.915	0.023
	May 2006	5	-1.94	8.412	0.043
	June 2006	6	-2.199	8.203	0.029
	August 2006	8	-2.167	8.088	0.031
	September 2006	9	-1.712	13.608	0.055
	October 2006	10	-2.039	8.025	0.038
	February 2007	14	-1.775	11.116	0.052
	March 2007	15	-1.855	12.224	0.044
	April 2007	16	-1.881	10.531	0.044
	June 2007	18	-1.822	10.123	0.049
Richness	June 2006	6	-2.347	10.300	0.020
	July 2006	7	-3.153	15.376	0.003
	August 2006	8	-2.19	15.801	0.022
	September 2006	9	-1.986	14.883	0.033
	October 2006	10	-2.516	12.856	0.013
Months since last resource addition					
	Month	Month number in graph	t	df	p-value
Abundance	Jan 2006	1	-3.628	47.486	< 0.001
	March 2006	3	-2.041	47.716	0.023
	April 2006	4	-2.067	40.120	0.023
	June 2006	6	-2.421	12.278	0.016
Richness	Jan 2006	1	-2.932	77.408	0.002
	July 2006	7	-3.623	19.125	< 0.001

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