EVALUATION OF THE TROPHIC STATUS AND FUNCTIONAL FEEDING GROUP STATUS OF THE COMAL SPRINGS RIFFLE BEETLE

EAHCP PROJECT NO. 148-15-HCP

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EXECUTIVE SUMMARY

In this Final Report, we present the results of a series of experiments conducted as a part of the Edwards Aquifer Habitat Conservation Plan (EAHCP) (EAHCP Project No. 148-15-HCP) Applied Research Program. In this study, we assessed the diet and functional feeding group of the endangered Comal Springs riffle beetle (*Heterelmis comalensis*) through the use of stable isotopes and a genetic analysis of its gut contents. To date, the potential food resources for *H. comalensis* in the wild have not been clearly identified, but the literature posits that riffle beetles are generally biofilm scrapers that can utilize detrital materials. The standard capture and census method for *H. comalensis* in Comal Springs is through the use of cotton-poly cloth lures and it is thought that beetles are are attracted to the lures to gain access to the biofilms that grow there. In addition, captive populations in the lab are maintained by supplying adult and larval beetles with well-conditioned leaf litter. However, the precise food sources and trophic ecology of *H. comalensis* remains to be elucidated.

To evaluate the feeding ecology of *H. comalensis*, we primarily used a stable isotope approach to determine the feeding ecology of the riffle beetle and other invertebrate species found in the Comal Springs system. We additionally characterized the microbial communities associated with biofilms in Comal Springs and compared them to the communities found within the guts of riffle beetles using NextGeneration sequencing techniques (Illumina).

Whole organism (bulk) isotope data for $\delta^{13}C$ and $\delta^{15}N$ indicated that *H. comalensis* adults and larvae utilized terrestrially-derived course particulate organic matter (CPOM), such as leaf litter and well-rotted wood. Mixing models indicated that *H. comalensis* obtained 70 – 92% of its diet from CPOM sources. In contrast, another riffle beetle species which frequently occupies the same habitats in Comal Springs (*Microcylloepus pusillus*) derived much of its diet from grazing algal-based periphyton biofilms.

We additionally characterized the $\delta^{13}C$ signatures of essential dietary-derived amino acids in *H. comalensis* and found that this species grazes bacterial-based biofilms and obtains ~67% of its diet from this food source. Algal-based foods contributed a much smaller fraction to its diet (~11%).

Finally, the microbial sequencing data for *H. comalensis* gut contents and the various sources of biofilms found in the Comal Springs system indicated that the proportions of major bacterial groups found in the guts of *H. comalensis* were to a large extent reflective of the bacterial groups found in the biofilms they were grazing. This finding suggests that *H. comalensis* is largely a non-selective grazer and that its gut microbial flora is quite similar to a variety of other insects.

Our results indicate terrestrial-derived materials are important for the diet and thus conservation of *H. comalensis*. Lower spring discharge and declining flows can lead to disconnection of the aquatic environment from the bank and to smaller water surface areas of aquatic habitats, potentially leading to decreased inputs of terrestrial material. In addition, decreased flow rates and water velocities could also lead to lower rates of downstream transport of terrestrial materials from more canopy-covered upstream areas (e.g., the more canopy-covered Spring Runs) to more open areas with lower rates of terrestrial inputs (e.g., Spring Island area).

BACKGROUND AND SIGNIFICANCE

In 2013, the United States Fish and Wildlife Service (USFWS) issued an Incidental Take Permit (ITP) to the Edwards Aquifer Authority, Texas State University (TXSTATE), the City of San Marcos, the City of New Braunfels, and the San Antonio Water System (SAWS) for the use of the Edwards Aquifer and its spring-fed ecosystems. The ITP is maintained through the Edwards Aquifer Habitat Conservation Plan (EAHCP). As a part of the first phase of the EAHCP (Phase 1), applied research projects examining the ecology of spring-associated ecosystems and the organisms covered by the ITP will be conducted alongside ecological modeling efforts. The organisms covered by the ITP are the fountain darter (*Etheostoma fonticola*), Texas wild rice (*Zizania texana*), the Comal Springs riffle beetle (*Heterelmis comalensis*), the San Marcos salamander (*Eurycea nana*), the Texas blind salamander (*Eurycea rathbuni*), the Peck's Cave amphipod (*Stygobromus pecki*), the Comal Springs dryopid beetle (*Strygoparnus comalensis*), Edwards Aquifer diving beetle (*Haideoporus texanus*), Comal Springs salamander (*Eurycea* sp.), the Texas troglobitic water slater (*Lirceolus smithii*), and the San Marcos gambusia (*Gambusia georgei*; assumed extinct). Much of the focus of this applied research effort is associated with determining the effects of low-flow conditions in the Comal and San Marcos Springs ecosystems on these organisms.

The Edwards Aquifer Recovery and Implementation Plan (EAHCP) currently sets the long-term mean and minimum daily discharge objective for Comal Springs at 225 cfs (cubic feet/second) and 30 cfs, respectively. However, modeling results from Phase 1 of the EAHCP predict that the mean and minimum daily discharge will be 197 cfs and 27 cfs, respectively (EARIP 2012). Consequently, there are significant concerns on the above-mentioned species, including the Comal Springs riffle beetle. Historical data and modeling results indicate some of the potential loss of habitat and habitat degradation associated with the reduction in spring flows. It has been observed that Spring Runs 1 and 2 generally cease to flow when total Comal Springs flow is ~130 cfs and Spring Run 3 generally ceases to flow when Comal Springs total flow is about 50 cfs (LBG Guyton 2004). Modeling results suggest that discharge will be less than 120 cfs for a total of 127 months and less than 45 cfs for a total of 7 months during a repeat of the drought of record (in the 1950s) with Phase 1 of the HCP implemented (EARIP 2012). Modeling efforts also indicate that a repeat of the drought of record (with Phase 1 of the HCP fully implemented) will lead to the total flows in the Comal Springs system to be < 30 cfs for a two-month period (EARIP 2012). If flows drop below 30 cfs, it is expected the main spring runs in the system (Spring Runs 1 through 6) will be dry for a considerable time period and the remaining aquatic habitat within the Comal Springs system will be limited to portions of Landa Lake and the Spring Island area. Cumulatively, this information indicates that it is possible for several if not most of the spring runs in the Comal system to cease flowing for

Figure 1 – Spring site along Spring Run 3 in the Comal system where *H. comalensis* commonly occurs.

extended periods of time (from months to years) and for a significant reduction of aquatic habitat to occur if there is a recurrence of the drought of record.

It is currently thought that the occurrence of Comal Springs riffle beetles within the Comal system is largely limited to habitats immediately adjacent to spring outflows which are also associated with the presence of leaf litter, wood, and other terrestrial plant organic matter (OM) (Fig. 1). Many aquatic Coleoptera, and some Elmid species in particular, exhibit an association with course woody debris (CWD) in stream habitats (Phillips 1995). Therefore, a reduction in spring flow that leads to loss of habitat (via desiccation) or prevents them from gaining access to the terrestrial OM may impact the fitness and survival of beetles. However, it is unknown whether the OM that beetles are associated with serves as a food source or as habitat or both (Fig. 2).

Figure 2 – Example of the spring associated terrestrial OM where the highest densities of *H. comalensis* are commonly found.

Trophic Ecology of Elmidae and H. comalensis

To date, the potential food resources for *H. comalensis* have not been clearly identified. Most literature sources state that riffle beetles are generally biofilm scrapers that can utilize detrital materials (Brown 1987). Currently, the standard capture and census method for *H. comalensis* in Comal is through the use of cotton-poly cloth lures. Presumably, Comal Springs riffle beetles are attracted to the lures to gain access to the biofilms that grow there, but it is not known if this is the reason why beetles are attracted to the lures. However, other closely-related riffle beetle species are frequently associated with terrestrially-derived OM. For example, a more widely-distributed but closely-related elmid species, *Heterelmis vulnerata*, is often associated with coarse woody debris with biofilm coverage and loose bark and/or interstitial spaces in the wood (Phillips 1995). The biofilm and interstitial spaces are thought to be used as concealment from the predators and biofilms may serve as algal and fungal food sources for the beetles (Phillips 1995). Seagle (1982) found that the gut contents of larvae and adults of three different riffle beetle species (*Stenelmis crenata*, *Stenelmis mera*, and *Optisoservus trivittatus*) was dominated by detritus-like materials, including included wood xylem, unidentified organic matter, and mineral particles, while algal materials were consumed to a much lesser extent. Thus, it has been suggested that elmids should be reclassified as detritivores-herbivores rather than as strictly herbivores, with the exception of known xylophagus genera (i.e., *Lara* spp.; Seagle 1982). Cannibalistic foraging has been observed in some elmids (i.e., *M. pusillus*), but this behavior was attributed to nutritional deprivation, and is probably not a common foraging strategy (Brown and Shoemake 1969).

Currently, the precise food sources and trophic ecology of *H. comalensis* remains to be elucidated. Recently collected preliminary diet data based on stable isotopes indicate that both adult and larval *H. comalensis* gain much of their diet through the use of woody material and leaves (BIO-WEST 2015). In contrast, *Microcylloepus pusillus*, another riffle beetle species commonly found in the Comal Springs system with *H comalensis* appears to largely rely upon algal-derived resources. Because of this apparent reliance on terrestrially-derived OM, it is unknown whether a reduction in spring flow may lead to the

disconnection of *H. comalensis* from potential or preferred food sources, such as terrestrial organic matter and detritus which may be most concentrated along the bank. Despite these preliminary data, it is unknown what aspect of the terrestrial OM and woody materials that *H. comalensis* is using as a food source, but it is likely scraping biofilms attached to the materials. Currently, the standard capture and census method for *H. comalensis* in Comal is through the use of cotton-poly cloth lures. Presumably, Comal Springs riffle beetles are attracted to the lures to gain access to the biofilms that grow there, but it is not known if this is the reason why beetles are attracted to the poly-cotton lures.

In the study detailed here, we examined the tropic level status and functional feeding group categorization of the Comal Springs riffle beetle in the Comal Springs system. In order to evaluate the feeding ecology of the Comal Springs riffle beetle, we primarily used a stable isotope approach to determine the feeding ecology of the riffle beetle and several other invertebrate species found in the upper Comal system. A stable isotope-based approach is preferable to other methods of examining trophic ecology in this case because examination of gut contents can be extremely time consuming, visual identification of materials in the guts of biofilm grazers is extremely difficult, and gut contents only provide information on what the organism has been consuming in very recent time. In contrast, stable isotopes often provide a clear long-term picture of what an organism has been consuming over longer periods of time and do not require laborious efforts in extracting and visually identifying gut contents. These stable isotope data were combined with preliminary stable isotope data that was associated with a previous EAHCP project (BIO-WEST 2015).

In addition to using stable isotope analysis, we characterized the microbial communities associated with biofilms in Comal Springs and compared them to the communities found within the guts of riffle beetles. Although stable isotopes are a powerful method for identifying potential food sources and trophic ecology of organisms, relying solely upon isotopes can sometimes lead to erroneous conclusions (Fry 2006). Thus, it is often recommended to add at least some additional information on feeding or foraging relationships to supplement isotope data (Fry 2006). In this study, we collected information on the microbial composition of the biofilms found on rocks, organic matter, and in the guts of adult beetles through two different techniques. The first technique will identify and quantify microbial communities through removing biofilms from rocks, terrestrial OM, lure cloths, and the gut contents of adult riffle beetles using fluorescent in-situ hybridization (FISH) (Amman et al. 1992; Amman et al. 1997). FISH counts allow for direct quantification of the density of major microbial groups present in various biofilms and in the guts of riffle beetles. Additionally, we characterized the taxonomic composition and diversity of biofilms through sequencing rRNA genes in the same environmental samples. Collectively, both techniques were used determine the microbial composition of biofilms and compare that to the material in the guts.

Stable isotopes and their use in diet and trophic ecology studies

In ecology, stable isotope analyses have increased in practice over the last several decades (Griffiths 1998) and have become one of the primary means to analyze food webs structure (Layman et al. 2012). Nitrogen and carbon are two most commonly used elements in food web context. The ratios of ^{15}N to ^{14}N (expressed relative to $\delta\ ^{15}N$) gets enriched in a stepwise pattern with trophic transfers and is a powerful tool for estimation of trophic position of organisms. The ratios of carbon isotopes ($\delta\ ^{13}C$) change little with trophic transfers, but shows substantial variations in primary producers with different photosynthetic pathways (C_3 vs. C_4 pathway in plants (Fry 2006, Post 2002, Layman et al. 2012).

Although, bulk-tissue isotope analysis is a useful tool for understanding the factors controlling food

web complexity in aquatic and terrestrial ecosystems, there are number of confounding factors that can complicate its interpretation (McMohan et al. 2010). From an ecological perspective, variations in flow in small or large river can alter $\delta^{13}C$ and $\delta^{15}N$ over the distances of few meters or over a period of time (weeks-years) even in most pristine freshwater ecosystems. The bulk tissue analysis from metabolic perspective has two main drawbacks. First, the biomagnification of $\delta^{15}N$ along the trophic chain is poorly understood for many ecological situations and isotopic signature can vary with nutritional stress, overall body conditions, differences in prey consumed, and seasonal and temperature changes (Bowes et al. 2014, Bowes and Thorp 2015; Wyatt et al. 2010). Second, among the tissues in the same organism, diet to tissue discrimination factors differ in $\delta^{13}C$ and $\delta^{15}N$. The $\delta^{13}C$ value of the higher trophic level consumers is determined by $\delta^{13}C_{base}$. It is difficult to interpret consumer $\delta^{13}C$ values using bulk SIA in light of potential changes in food web structure vs. variations in $\delta^{13}C_{base}$ (Post 2002) without suitable estimates of $\delta^{13}C_{base}$, which can vary at both temporal and spatial scales (Graham et al. 2009).

Due to recent advances in gas chromatography-combustion-isotope ratio monitoring mass spectrometry, the specificity of dietary studies has increased by analyzing $\delta^{13}C$ values of specific biochemical compounds including amino acids (AAs) (Sessions 2006). Stable isotope analysis of individual AAs provides more accurate and precise estimates of trophic position and food chain length and requires fewer sample replicates (Bowes and Thorp 2015).

An effective tool to determine the organic matter sources from consumers and trophic relationships is through the use of "stable isotope fingerprints" (Larsen et al. 2009). These isotopic fingerprints can be evaluated in a variety ecosystems including freshwater environments with strong allochthonous influences (Larsen et al. 2013). Amino acids have been shown to hold significant variations in their $\delta^{13}C$ in regards to metabolism (Abelson and Hoering 1961). Origins can be distinguished between plants, bacteria, and fungi through in situ assays of amino acids identifying each unique ^{13}C fingerprint. Amino acids contribute half of the total carbon in organisms (Hedges et al. 2001) granting the capacity to trace these sources ($\delta^{13}C_{AA}$) that are conserved through food chains. We can greatly advance our understanding of nutrient cycling and trophic relationships by tracking the origins and fluxes of essential AAs (EAAs) when it is passed from food source to consumer without alteration to their carbon skeletons (McMohan et al. 2010).

Categorization and identification of microbial biofilms

Although we will evaluate the trophic status and functional feeding group of the Comal Springs Riffle Beetle through stable isotopes, we will also collect additional data on derived from culture-independent methods to identify and classify the biofilms associated with specific potential food types. Culture independent methods for identifying biofilms have recently gathered interest amongst microbial ecologists due to its ability to characterize organismal influence on biotic and abiotic exchange (Haig et. al, 2015; Grob et. al, 2015). Thus, pairing the stable isotope data with the identification of gene sequences responsible for the translation of enzymatic products offers methods for modeling systematic interactions unavailable to either stand-alone method (Chen and Murrell 2010).

Traditional methods of microbial ecology are dependent on developing general and defined medias for culturing microscopic organisms (microbes) for the purpose of enumeration and identification (Buck, 1979). Culture-dependent methodology for identifying microbial composition regularly leads to sample bias inherent in the development of culture media engineered to support organisms with specific metabolic activity. Developing microbial cultures limits research to organisms that are viable

in vitro while the majority of microbial life exists in a viable but non-culturable (VBNC) state that will not be represented in the cultured population (Su et al., 2013; Xu et al., 1982). Recent developments in genetic analysis provide techniques for representing the microbial community previously rare in ecological model reporting. Utilizing contemporary genetic analysis in conjunction with bioinformatic modeling of metagenomic libraries allows practitioners to identify the prominent transcript production of ecological systems.

Nucleic acid-base cultivation-independent surveys (CIS) have been in regular use since implementation in a variety of formats initially developed for microbial evolution analyses (Pace et al., 1985; Olsen et al., 1986). CIS have been predominantly used to interpret phylogenetic relationships of communities using primer-based applications targeting ribosomal RNA (rRNA) gene sequences (Woese, 1987). Methods were based on isolating genetic material directly from the environment for cloning in specially designed phage vectors (Schmidt et al., 1991). Sequences obtained are catalogued in varying formats to construct a library of transcribed gene sequence data similar to those first developed by the creators of "Zoolibraries" (Healy et al., 1995). Methodology of rRNA sequencing disciplines require skilled development of cloning vectors and mandate the use of a template sequence that has been highly conserved (commonly 5S rRNA or 16S rRNA) which may render a bias depiction of the community structure (Simon and Daniel 2010; Kakirde et al., 2010).

Next-Generation sequencing techniques are now a mainstay of modern metagenomic sequencing. The discipline offers vast savings in time and resources due to the streamline processes utilizing minimal equipment which allows technicians to sequence genes directly from a DNA source sample without the use of a cloning treatment requiring bacterial vectors (Mardis, 2008). Enzymes specific to varying commercially available Next-Generation sequencing platforms cleave DNA samples into small genomic fragments (sequences <1kb) which are then linked to adaptor sequences which serve as the source for polymerase-amplification activity (Mardis, 2013). Common to all Next-Generation sequencing methodology is a platform containing amplification sites with adaptors complimentary to those of the prepared genomic fragments (Mardis, 2013). Sequence amplification in this format allows for the production of vast amounts of short genomic sequences each tagged with an adaptor correlating to the amplified sample that are utilized in the formation of sequence libraries.

The construction of sequenced based metagenomic libraries provides a venue for analyzing the products of DNA sequence amplification. Short genomic fragments produced by Next-Generation sequencing techniques are aligned by sample associated adaptors applied during the amplification process. Once aligned fragments may be used to construct overlapping sequences that may represent a contiguous region of DNA or fragments may be independently assorted for inter-comparative sample analysis.

METHODS

Bulk isotope methods

The review of the literature and preliminary data (see above) suggest that riffle beetles are biofilm scrapers, but there are a diversity of biofilm scraping strategies that can be utilized by stream organisms. Thus, in order to examine if, *H. comalensis* is utilizing OM or biofilms associated with OM, we conducted a study in which we assessed the diet of the Comal Springs riffle beetle and other invertebrate consumers in the Comal Springs food web using stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$). We collected *H. comalensis* and other organisms from sites where they are present

(Spring Run 3 and Spring Island) for analysis of stable isotopes. Stable isotopes are a commonly-used method in diet studies, and the ratio of stable isotopes of carbon (^{13}C:^{12}C or $\delta^{13}C$) can be used to determine which food items a consumer it utilizing (Fry 2006; Boecklen et al. 2011; Layman et al. 2012).

In early August 2016, we collected macroinvertebrates (including adult and larval *H. comalensis*) by hand picking along Spring Run 3 and along Spring Island. Invertebrates were generally sorted in the field while alive and active and kept in vials containing water from the site for 1-2 hours to allow them to void gut contents. Organisms were then brought back to the lab, sorted, and then dried at 60°C for 48 h in plastic weigh boats.

In addition to organisms, we also collected several types of basal resources that would potentially support consumers in the Comal food web. We collected in-stream detritus of three types that are present in the system: well-conditioned leaves (typically sycamore and pecan, *Carya illinoinensis*, leaves), well-rotted wood, and fine (<64 μm) sediments. We chose to collect wood because *H. comalensis* (and the Comal Springs dryopid beetle, *Stygoparnus comalensis*) is frequently found on pieces of porous rotted woody material (W.H. Nowlin, *pers. obs.*). Because wood was relatively rotted, we did not attempt to identify wood to species. Leaves and well-rotted wood and sediment samples were collected in triplicate from each location (SR3 and Spring Island). Materials were stored in plastic bags and brought back to the lab. Wood and leaf material was dried at 60°C for 48 h in plastic weigh boats. We additionally collected at least 6 relatively flat rocks from each location for algal biofilms. Rocks were placed in plastic bags within a cooler and brought back to the lab to remove biofilms. Biofilms were removed by scrubbing the upper rock surface with a clean nylon bristle brush and washing the material with DI water into a clean HDPE beaker. A portion of this slurry was then filtered onto ashed and pre-weighed 25-mm diameter Whatman GF/F filters; for each rock, we collected two filters. One filter (to be used for $\delta^{15}N$ analyses) was immediately placed into an oven and at dried at 60°C for 48 h. The other filter was placed into a fuming HCl chamber for at least 8 hours to remove inorganic precipitated carbonates from the algal biofilms, which affect bulk $\delta^{13}C$ values (Pound et al. 2011); this filter would be used for $\delta^{13}C$ values only. After fuming, filters were dried at 60°C for 48 h. We also collected triplicate samples of an aquatic moss (*Amblystegium riparium)* from spring run 3. It was stored in plastic bags, bought back to the lab, and dried at 60°C for 48 h in plastic weigh boats.

After samples were sorted, treated and dried, we prepared samples for analysis at the University of California – Davis Stable Isotope Facility. All organisms, leaves, and wood samples were homogenized and ground to a fine powder using a mill grinder or a mortar and pestle that was thoroughly cleaned between samples with DI water and acetone. In a few cases, individual organism was of great enough mass for individual analysis, but in most cases we were forced to create composite samples of 2-10 individuals so that there was enough mass to run ^{13}C and ^{15}N analyses. For all organisms, leaves, and wood samples, we analyzed at least 3 and up to 6 independent samples for stable isotopes of C and N after packing homogenized material into tin capsules.

Stable isotopes of C and N were analyzed using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Samples were combusted at 1000°C in a reactor packed with chromium oxide and silvered copper oxide. Following combustion, oxides were removed in a reduction reactor (reduced copper at 650°C). The helium carrier then flowed through a water trap (magnesium perchlorate) and the resulting N_2 and CO_2 were separated on a Carbosieve GC column (65°C, 65 mL/min) before entering the IRMS.

Glass fiber filters were analyzed for ^{13}C and ^{15}N isotopes using an Elementar Vario EL Cube or Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Samples were combusted at 1000°C in a reactor packed with copper oxide and lead chromate. Following combustion, oxides were removed in a reduction reactor (reduced copper at 650°C). The helium carrier then flowed through a water trap (magnesium perchlorate). N_2 and CO_2 were separated using a molecular sieve adsorption trap before entering the IRMS. For each isotope run, samples were interspersed with several replicates of at least two different laboratory standards. The laboratory standards, which are selected to be compositionally similar to the samples being analyzed, were previously calibrated against NIST Standard Reference Materials (IAEA-N1, IAEA-N2, IAEA-N3, USGS-40, and USGS-41). Each sample's preliminary isotope ratio is measured relative to reference gases analyzed with each sample. These preliminary values are finalized by correcting the values for the entire batch based on the known values of the included laboratory standards. The long-term standard deviation during analysis is ± 0.2‰ for ^{13}C and ± 0.3‰ for ^{15}N. Final delta values are expressed relative to international standards Vienna Pee-Dee Belemnite for C and Air for N.

Compound specific stable isotope analysis of amino acids (CSIA-AA) methods

Most animals incorporate essential AAs directly from their diet (Reeds, 2000). As a result, the $\delta^{13}C$ value of consumer essential AAs, such as phenylalanine and leucine, must represent the isotopic fingerprint of primary producers at the base of the food web ($\delta^{13}C_{BaSe}$) (McMohan et al. 2010). Therefore, in this study CSIA AAs were used as a potential tool to complement data obtained from bulk C isotope analysis since amino acid more directly reflects information on the primary producer. Therefore, we selected a smaller group of samples to additionally analyze for $\delta^{13}C$ of specific amino acids. For CSIA, approximately 2 mg of invertebrate tissue, terrestrial organic matter (well-conditioned leaves, well-rotted wood) and algal biofilm collected from the rocks (slurry dried in a weigh boat) was acid-hydrolyzed in 1-2 mL of 6 N HCl at 110°C for 20 h to isolate the total free AAs. Samples were evaporated to dryness under a gentle stream of N_2. The total free AAs were derivatized by esterification with acidified isopropanol followed by acylation with trifluoroacetic anhydride (Silfer et al. 1991) and were brought up in dichloromethane (DCM) for stable isotope analysis. For AA $\delta^{13}C$ analyses, the derivatized AAs were injected into a column in split mode at 250°C and separated on a DB-5 column (Agilent Technologies, Santa Clara, CA) in a Thermo Trace Ultra gas chromatograph (GC) at the University of California, Santa Cruz, CA. The separated AA peaks were analyzed on a Finnegan MAT DeltaPlus XL isotope ratio monitoring mass spectrometer (irm-MS) interfaced to the GC through a GC-C III combustion furnace (960°C) and reduction furnace (630°C).

We analyzed two consumer species for CSIA: *H. comalensis* and *M. pusillus* and three types of sources: well-conditioned leaves (typically sycamore and pecan, *Carya illinoinensis*, leaves), well-rotted wood and algae collected from rocks. These two consumers were selected because they are the focal species of this study and a potential competitor for the focal species, respectively. All CSIA samples were analyzed in triplicate along with AA standards of known isotopic composition (Sigma-Aldrich Co., St. Louis, MO, USA). Standardization of runs was achieved using intermittent pulses of a CO_2 or N_2 reference gas of known isotopic value. Mean reproducibility of a laboratory algal standard across all individual AAs was ±0.73 for $\delta^{13}C$.

For all consumers and sources mentioned above, $\delta^{13}C$ values of twelve individual AAs was analyzed. These AA's included six essential AA (threonine (Thr), leucine (Leu), isoleucine (Ile), valine (Val),

Lysine (Lys) and phenylalanine (Phe)) and six non-essential AA (glutamic acid (Glu), aspartic acid (Asp), alanine (Ala), proline (Pro), glycine (Gly), and serine (Ser)). Although data were provided on a suite of non-essential AAs, we only present analyses for the essential AAs.

Gut content and biofilm identification methods

Adult *H. comalensis* were collected from SR3 and SI by hand-picking from the various substrates upon which they might be grazing biofilms. Adult beetles were hand-collected from well-rotted wood, leaf material, and rocks and were placed in 95% EtOH in the field and immediately transported to the lab at Texas State University in a cooler on ice. In addition, representative substrates of each type were collected in the field, stored in plastic bags in coolers on ice and transported to the lab. In the lab, beetles had gut contents removed with the aid of a dissecting microscope. For FISH counts, beetle gut contents and approximately 0.75g of biofilm materials were placed into sterile microcentrifuge tubes and tubes were centrifuged at 3000 x *g* for 1 h at 4°C and supernatant was removed. The remaining pellet was fixed with 1.5 ml of 4% paraformaldehyde/phosphate-buffered saline fixative for 12–24 h. The pellet was then re-suspended, transferred to autoclaved 2 ml centrifuge tubes, washed twice with PBS after re-pelleting at 4000 x *g* for 5 min, and stored in a 1:1 mixture of PBS and ethanol at -20°C (Zarda et al., 1997). For NextGeneration sequencing, beetle gut contents were placed in nucleases-free water and centrifuged at 13.2 k RPM, and then stored at -80°C for later DNA extraction. Biofilm materials from rocks, well-rotted wood, and well-conditioned leaves were gently scraped with a cleaned razor blade to remove biofilms. Biofilms were centrifuged and stored as above.

For initial attempts at enumeration using FISH, microbial cells were stained with 40,6-diamidino-2-phenylindole (DAPI) and EUB338. For slide application, each sample was sonicated for 10 sec and a subsample was sequentially dispersed in 0.1% sodium pyrophosphate to a 1–25% sample concentration. Of this diluted sample, 10 µl was dispersed into the well of a gelatin–coated 8-well slide and dried at 35°C. To improve cell permeability, the samples were treated with 10 µl 1% lysozyme for 30 min at room temperature (Zarda et al., 1997). For counting, slides were mounted with CitifluorTM AF1 solution (London, England) and examined with a Nikon Eclipse 80i microscope (Melville, NY), fitted with a mercury lamp (Nikon X-CiteTM 120). For a suite of initial samples, 10 fields were haphazardly selected from each slide well hybridized with each probe, and cell counts were converted to the average cells/ml in the original sample. For the initial attempts to count samples with FISH, each field replicate was counted for a total ~40 fields per site-probe combination, and the counts from each were averaged.

The initial round of samples both from guts and biofilms either had extremely high autofluorescence of particles or a high degree of clumping of cells, making accurate counts impossible. In order to deal with these issues, we tried a variety of solutions, including a longer sonication period, the addition of manual agitation to the pellet, and/or a higher degree of sample dilution. However, after extensive attempts and repeated failure to fix these issues, we elected to abandon attempts at FISH and instead rely upon the more current method of bacterial enumeration using NextGeneration (Illumina) sequencing.

For Illumina sequencing, DNA was extracted from gut contents and biofilm samples using the SurePrepTM Soil DNA Isolations Kit (Fisher Scientific, Houston, TX) with small modifications as described before (Samant et al. 2012). Extractions of all samples were done in triplicate, and DNA concentrations measured with a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, USA).

For Illumina sequencing, 16S rRNA gene fragments were amplified using primer 515f and barcoded primers 806r, both of which included linker sequences following the instructions from the Earth Microbiome Project (www.earthmicrobiome.org/emp-standard-protocols/16s/) (Caporaso et al. 2012). For analyses of Eukarya, 18S rRNA gene fragments were amplified using primer Euk_1391f and barcoded primer EukBr, both of which included linker sequences following the instructions from the Earth Microbiome Project (www.earthmicrobiome.org/emp-standard-protocols/18s/) (Caporaso et al. 2012). PCR was carried out in a 100 µl volume with 1 x Taq Buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2 µM primers, 2.5 µg µl-1 BSA, 1U Taq polymerase (GenScript, Inc., Piscataway, NJ) and 1 µl DNA extract. PCR conditions followed the Earth Microbiome project with an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, with a final 72°C extension for 10 min. PCR products were cleaned using the UltraClean® 15 DNA Purification Kit (Mo Bio Laboratories, Inc., Carlsbad, CA), and then checked and quantified on a 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) using the Agilent DNA 7500 Kit. Samples were analyzed on an Illumina MiSeq v3 with paired end 2 x 350 bp reads using the respective sequencing and index sequence primers (www.earthmicrobiome.org/emp-standard-protocols/16s/).

Sequences were analyzed using the QIIME software (Quantitative Insights Into Microbial Ecology, version 1.9.1) on a LINUX platform available on the HPC-STAR-Cluster (High Performance Computing environment), Texas State University, San Marcos. The 16S rRNA V4 data was mapped to Reference Sequences following the steps mentioned in the standard EMP protocol for mapping Databases (http://www.earthmicrobiome.org/emp-standard-protocols/16s-taxonomic-assignments/). Fastq files containing the raw reads were received from the sequencing facility with barcodes, primers and adapter/linker sequences removed from them. Quality filtering and de-multiplexing of sequences were done prior to analysis. The sequences were truncated at a point where the quality/phred scores of three consecutive bases fell below 20. The resulting truncated sequences were about 75% of the length of the original sequences. These filtered sequences were screened for ambiguous bases (N) and those containing Ns were discarded. The resulting files containing the de-multiplexed and filtered sequences were used to create open reference OTUs (operational taxonomic units) at 97% sequence identity against the most recent Greengenes database (16s) - gg_otus-13_8-relaese/rep_set/97_otus.fasta (http://qiime.org/home_static/dataFiles.html ,http://greengenes.secondgenome.com/downloads), using UCLUST and alignments of representative OTU's that were done using PYNAST. The OTU assigning step was run in parallel mode by passing the parameters "–a" and "–O" which indicates the number of parallel jobs to be done. The resulting BIOM table without singleton OTU's and sequences that failed to align by PYNAST, was used in further downstream diversity analyses processes.

Core diversity analyses were performed to study alpha (i.e. within-sample) diversity and to generate summarized taxa plots at different hierarchy. Initially, the resulting taxonomy table generated by QIIME, was analyzed at the phylum level to establish the diversity of bacteria in various samples. For the alpha diversity analysis, the samples were rarified at a depth of 150,500 seqs/sample and Chao1 (species richness estimator index) data were generated.

Data Analysis

Bulk isotope analysis - Stable isotope data for the various basal food resources (leaves, rotted wood, and algae) and for consumer species (including *H. comalensis*) were initially compared between sites (Spring Run 3 and Spring Island) to determine if there were any longitudinal changes in stable

isotope values for $\delta^{13}C$ and $\delta^{15}N$ as water moved from Spring Run 3 to the Spring Island area. Food resources of the same type and the same species were compared between sites using separate one-way ANOVAs for $\delta^{13}C$ and $\delta^{15}N$ values. We also initially compared the various potential basal food web resources (the aquatic moss *Amblystegium*, leaves, rotted wood, and algae) within a given site using two-way ANOVAs for $\delta^{13}C$ and $\delta^{15}N$ values with the independent variables being site (SR3 and SI) and OM source (CPOM, periphyton, and *Amblystegium*). We assessed assumptions of normality and homogeneity of variances prior to analysis and significance was inferred at $p \leq 0.05$. Analyses were conducted in the program R.

In order to determine the likely basal food resources for consumers in the food web, we utilized a Bayesian inference approach to food source determination using the stable isotope composition of the various food sources and consumers. Bayesian approaches to isotopic mixing models are becoming more widely used (Layman et al. 2012) because, unlike traditional linear mixing models, they allow for incorporate sources of variability within the model, permit the analysis of multiple dietary sources for consumers, and generate potential dietary solutions as true probability distributions (Parnell et al. 2010). Although, there is some debate about the relative importance and kinds of assumptions that must be made with the use of isotopic mixing models, many most stable isotope ecologists use a Bayesian approach because of their utility when compared to traditional models.

In order to determine the relative importance of different basal food resources to different consumers in the Comal Springs food web, we followed the "best practices" guidelines as described in Phillips et al. (2014). We ran all isotope mixing models in the program Stable Isotope Analysis in R (SIAR) Version 4 (Inger et al. 2014). We first identified the potential basal food resources as *Amblystegium*, leaves, rotted wood, and epilithic algae (periphyton) and used these as our resources in models (see Results below). Trophic enrichment factors (TEFs) for the models were taken from a meta-analysis of the literature (Caut et al. 2009) and used the mean (± 1SD) trophic enrichment factors of 1.33 ‰ ± 0.454 and 2.75 ‰ ± 1.637 for ^{13}C and ^{15}N, respectively. These values are the $\delta^{13}C$ for all "freshwater organisms" and the "overall" $\delta^{15}N$ for all organisms reported by Caut et al. (2009). We ran isotope mixing models to determine the proportional contribution of each basal food resource to each consumer species in the food web.

CSIA-AA analysis - We initially explored group memberships in our dataset by performing principal component analysis on $\delta^{13}C$ values of essential amino acids that had been normalized to their respective sample means (denoted as $\delta^{13}CAAn$) (Larsen et al. 2013). Prior to applying statistical analysis, the data were tested for univariate normality by visually checking whether there were departures from normality on Q-Q plots. The AA methionine (Met) was excluded from the analyses due to missing measurements caused by concentrations below detection limits. Differences in each amino acid between different producer groups were tested with ANOVA with Tukey HSD post-hoc tests. To examine combinations of independent variables (i.e. $\delta^{13}CAA$ values) that best explained differences between the categorical variables (i.e. the groups defined by the PCA and one-way ANOVA tests) and to construct models for predicting membership of unknown samples, we performed linear discriminant function analysis (LDA) on $\delta^{13}CAA$ values. We ran LDA models with normalized $\delta^{13}CAA$ values for our three potential food sources (leaves and wood, periphyton) and a large data set of $\delta^{13}CAA$ values for bacterial, algal, fungal, and terrestrial plant values groomed from Larsen et al. (2009) and Larsen et al. (2013). Relative contributions of EAAs from diets to consumers was estimated in the software FRUITS (Version 2.0, http://sourceforge.net/projects/fruits) with normalized isotope values (Fernandes et al. 2014; Fernandes 2015). FRUITS can also considers the

biochemical composition of sources and which sources are most likely to contribute the most. FRUITS is executed with BUGS, which is a software package for performing "Bayesian inference Using Gibbs Sampling" that includes an expert system for determining an appropriate Markov chain Monte Carlo scheme based on the Gibbs sampling (Fernandes et al. 2014; Fernandes et al. 2015).

For Illumina sequencing data, statistical analyses of all the three metrics data was carried out using ANOVA followed by Holm- Post hoc analysis. Values were represented as mean ± standard error. Significance was inferred at $\alpha \leq 0.05$.

RESULTS

Bulk stable isotopes

We were able to collect individuals of 15 different taxa in the Comal system for analysis, including several species of concern in the EAHCP (i.e., *Stygobromus pecki*, *Stygoparnus comalensis*, and *H. comalensis*) (Fig. 3). The suite of taxa included a diversity of different feeding types, so that we could elucidate most of the major feeding groups within the invertebrates found in the Comal system. We initially assessed the food web at the two sampling locations separately (SR 3 and SI).

The food web of SR 3 and SI included a diversity of consumers that utilized differing proportions of the three *a priori* identified basal food resources: *Amblystegium*, periphyton, and conditioned leaf litter and partially rotted wood grouped together as course particulate organic matter (CPOM). In terms of the basal food resources that likely support the Comal food web, both the $\delta^{13}C$ and $\delta^{15}N$ values differed among the basal food resources ($\delta^{13}C$: two-way ANOVA, $F_{2, 32} = 237.58$, $p < 0.001$; $\delta^{15}N$: two-way ANOVA, $F_{2, 32} = 14.56$, $p < 0.001$) (Figs. 3 and 4). However, the isotopic values of each basal organic matter (OM) source did not significantly differ between sites ($\delta^{13}C$: $p = 0.939$; $\delta^{15}N$: $p = 0.403$) and there was not a significant OM source *x* site interaction ($\delta^{13}C$: $p = 0.857$; $\delta^{15}N$: $p = 0.064$). Periphyton $\delta^{13}C$ in periphyton was relatively deplete (-32.62‰) when compared to CPOM (-29.50‰). However, *Amblystegium* exhibited the most deplete value when compared to the other two sources (-42.23‰). Similarly, $\delta^{15}N$ values of Amblystegium was the most deplete (2.40), while periphyton (3.48) and CPOM (5.01) were intermediate and relatively enriched, respectively.

The consumers in SR 3 exhibited a range in potential food resources, with one group of consumers, which was composed of *Psephenus* sp., *M. pusillus* (adults and larvae), Baetid mayfly nymphs, and Helicopsychid caddis fly larvae, were likely feeding on periphyton (Fig. 3A). In contrast, other consumers were likely feeding on wood-based biofilms and was composed of *H. comalensis*, *S. comalensis*, and *Hyalella azteca*. Elevated above these consumers (as indicated by relatively enriched $\delta^{15}N$ values were snail species (*Elimia*, *Tarebia*, and *Melanoides*) and the mayfly *Argia*. Snails in stream systems often exhibit somewhat elevated $\delta^{15}N$ values (e.g., Pound et al. 2011) because they may be less selective grazers than other algivorous invertebrate groups, such as *Psephenus* (Anderson and Cabana 2007). *S. pecki* was the consumer exhibiting the highest trophic position in the SR 3 community, indicating that it is likely to be predatory on other invertebrates in the system, particularly *H. azteca*.

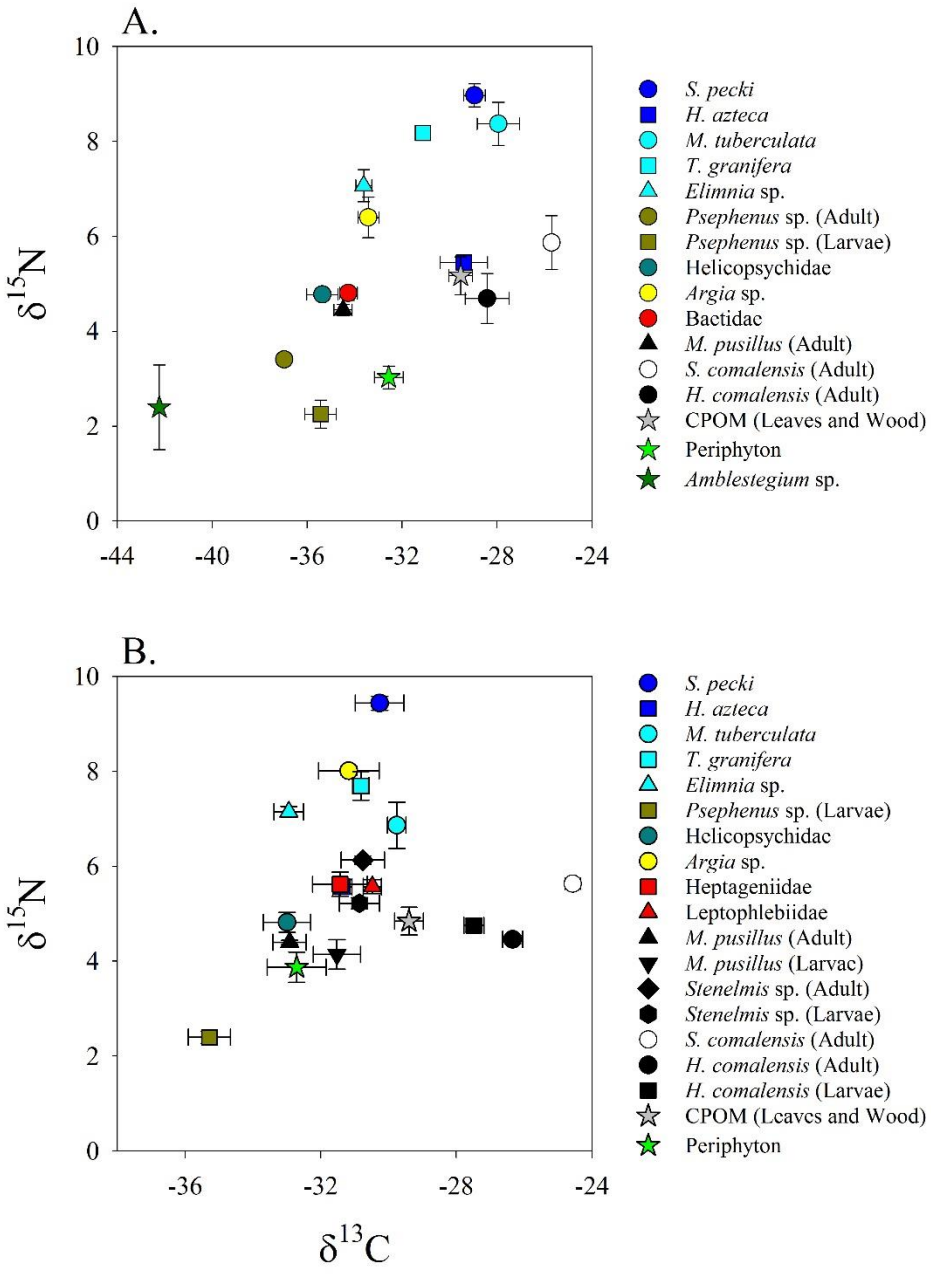


Figure 3 - Stable isotope ($\delta^{13}C$ and $\delta^{15}N$) biplots for Spring Run 3 (A) and Spring Island (B) in the Comal Springs system. Each point represents the mean of each species and bars represent ± 1 SE. Different species and different basal food web resources are portrayed with different symbols and colors.

The food web in the SI section of the Comal system was similar to the patterns found in SR 3 (Fig. 3B). Again, most of the same basal OM resources were present: periphyton, CPOM, but not *Amblystegium*. The consumers in the SI food web exhibited an analogous configuration to SR3. Again, *Psephenus*, *M. pusillus*, and Helicopsychidae were associated with periphyton and larval and adult *H. comalensis* and adult *S. comalensis* were also closely associated with CPOM, as indicated by its

enriched $\delta^{13}C$ values. Heptageniid mayfly nymphs, the large-bodied elmid *Stenelmis*, Leptophlebiid mayfly nymphs, and snails were more associated with periphyton (as indicated by $\delta^{13}C$ values), but exhibited a higher estimated trophic position as estimated by $\delta^{15}N$ values. Again, less-selective grazers like Heptageniids and snails often exhibit higher $\delta^{15}N$ values than more selective grazers such as *Psephenus* (Anderson and Cabana 2007). However, in contrast to the SR3 site, *H. comalensis*, *H. azteca*, and *S. comalensis* had slightly more enriched $\delta^{13}C$ values than at SR3.

When both sites were combined to create a food web for Comal Springs, the same species groupings were still apparent with several species clustered around CPOM and most taxa lying near periphyton or intermediate to periphyton and CPOM $\delta^{13}C$ values (Fig. 4).

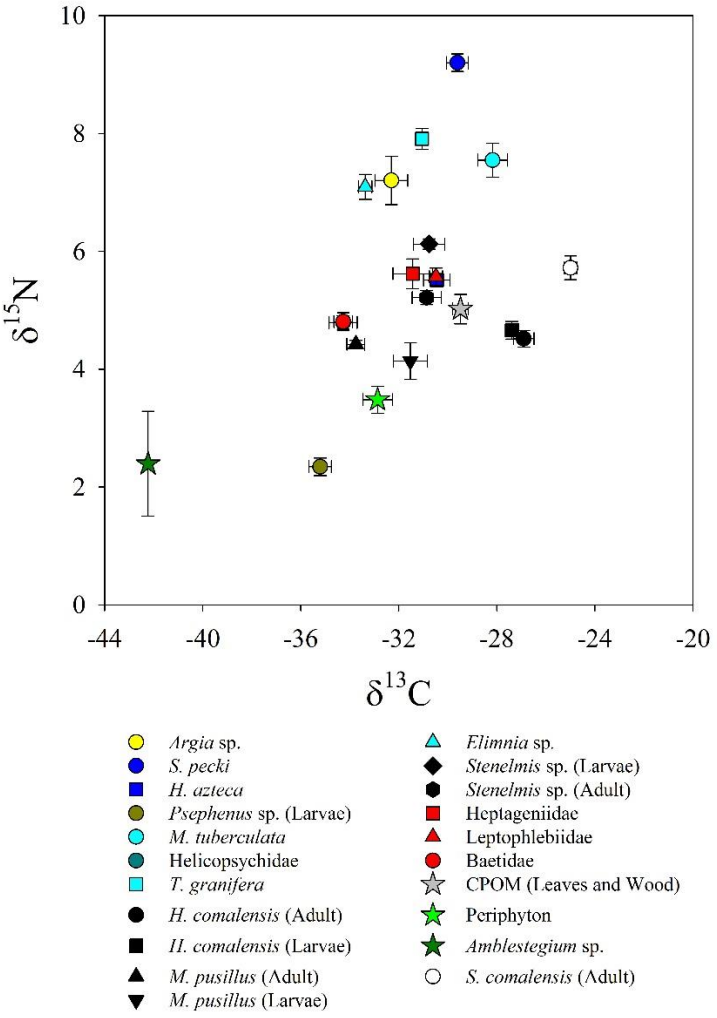


Figure 4 - Stable isotope ($\delta^{13}C$ and $\delta^{15}N$) biplot for the complete Comal Springs system (data from SR3 and SI combined). Each point represents the mean of each species and bars represent ± 1 SE. Different species and different basal food web resources are portrayed with different symbols and colors.

When the combined data set (including data from SR3 and SI) was run in SIAR, the model output for each of these species generally reflected the differences that were apparent in the isotope bi-plot (Table 1). In this report, we provide the mean, mode and 95% credibility intervals for the model outputs for each consumer in the Comal food web; the actual posterior probability distributions and matrix plots are available upon request. Model runs and the resulting means and modes of the posterior probability distributions for a majority of consumers in the food web, including *H. azteca*, *Psephenus*, Helicopsychids, *M. pusillus*, Baetids, *Argia*, Heptageniids, Leptophlebiids, and *Stenelmis* indicated that these consumers had diets in which a majority of their dietary C (>50%) was derived from periphyton and/or *Amblystegium* origin (Table 1). In contrast, several of the consumers including *S. pecki*, the three snail species, *S. comalensis*, and *H. comalensis* had diets which would be considered to be derived from CPOM sources. Indeed, both *H. comalensis* larvae and adults were estimated to have derived >70% of their dietary C from CPOM.

		CPOM			Periphyton			*Amblestegium* sp.		
Site	Taxon	Mean	Mode	95% CI	Mean	Mode	95% CI	Mean	Mode	95% CI
Spring Run 3	*S. pecki*	82%	88%	61 - 98%	14%	0%	0 - 38%	4%	0%	0 - 10%
	H. azteca	32%	33%	3 - 58%	51%	47%	19 - 84%	17%	4%	0 - 42%
	M. tuberculata	70%	78%	42 - 98%	21%	10%	0 - 50%	6%	1%	0 - 20%
	T. granifera	58%	60%	37 - 78%	26%	23%	0 - 51%	16%	17%	6 - 26%
	Elimnia sp.	42%	42%	22 - 62%	25%	28%	0 - 48%	34%	34%	22 - 48%
	Psephenus sp. (Adult)	14%	28%	0 - 31%	25%	62%	0 - 45%	61%	23%	45 - 74%
	Psephenus sp. (Larvae)	19%	5%	0 - 42%	34%	40%	2 - 59%	47%	46%	30 - 64%
	Helicopsychidae	12%	3%	0 - 30%	41%	44%	14 - 64%	47%	47%	33 - 61%
	Argia sp.	34%	34%	1 - 58%	32%	34%	0 - 60%	34%	34%	17 - 48%
	Baetidae	18%	13%	0 - 39%	44%	47%	13 - 70%	38%	38%	28 - 49%
	M. pusillus (Adult)	15%	5%	0 - 38%	47%	55%	15 - 73%	38%	39%	25 - 51%
	S. comalensis	36%	37%	0 - 68%	37%	38%	0 - 69%	26%	32%	0 - 54%
	H. comalensis (Adult)	39%	35%	0 - 79%	40%	39%	1 - 76%	21%	4%	0 - 53%
Spring Island	*S. pecki*	48%	49%	11 - 89%	52%	51%	11 - 88%	--	--	--
	H. azteca	19%	18%	0 - 36%	81%	82%	64 - 100%	--	--	--
	M. tuberculata	51%	52%	19 - 81%	49%	48%	19 - 81%	--	--	--
	T. granifera	37%	37%	14-58%	63%	63%	42 - 86%	--	--	--
	Elimnia sp.	33%	43%	0 - 67%	67%	57%	33 - 100%	--	--	--
	Psephenus sp. (Larvae)	26%	51%	0 - 65%	73%	95%	35 - 100%	--	--	--
	Helicopsychidae	14%	3%	0 - 43%	86%	97%	57 - 100%	--	--	--
	Argia sp.	41%	47%	2 - 78%	59%	53%	22 - 98%	--	--	--
	Heptageniidae	21%	13%	0 - 49%	79%	87%	51 - 100%	--	--	--
	Leptophlebiidae	37%	42%	5 - 63%	69%	58%	37 - 95%	--	--	--
	M. pusillus (Adult)	9%	2%	0 - 30%	91%	98%	70 - 100%	--	--	--
	M. pusillus (Larvae)	29%	22%	0 - 66%	71%	78%	34 - 100%	--	--	--
	Stenelmis sp. (Adult)	34%	36%	3 - 60%	65%	64%	39 - 97%	--	--	--
	Stenelmis sp. (Larvae)	33%	35%	0 - 63%	66%	65%	37 - 100%	--	--	--
	S. comalensis	54%	50%	14 - 100%	46%	50%	0 - 86%	--	--	--
	H. comalensis (Adult)	81%	97%	40 - 100%	19%	3%	0 - 59%	--	--	--
	H. comalensis (Larvae)	87%	97%	57 - 60%	13%	3%	0 - 39%	--	--	--
Comal (All)	*S. pecki*	80%	88%	59 - 96%	14%	3%	0 - 35%	6%	6%	0 - 13%
	H. azteca	32%	30%	8 - 54%	60%	58%	31 - 87%	9%	5%	0 - 22%
	M. tuberculata	73%	78%	48 - 97%	23%	19%	0 - 48%	4%	1%	0 - 12%
	T. granifera	58%	58%	41 - 76%	26%	26%	2 - 49%	15%	15%	7 - 23%
	Elimnia sp.	40%	38%	24 - 58%	26%	31%	1 - 49%	33%	33%	25 - 42%
	Psephenus sp. (Larvae)	17%	4%	0 - 40%	37%	47%	4 - 62%	45%	44%	34 - 58%
	Helicopsychidae	12%	31%	0 - 30%	49%	55%	22 - 73%	39%	37%	25 - 53%
	Argia sp.	42%	40%	16 - 68%	35%	34%	0 - 66%	23%	23%	6 - 38%
	Heptageniidae	34%	36%	4 - 60%	46%	41%	10 - 84%	20%	17%	1 - 40%
	Leptophlebiidae	47%	46%	19 - 76%	42%	44%	4 - 72%	11%	8%	0 - 24%
	Baetidae	23%	42%	1 - 45%	39%	39%	6 - 68%	38%	39%	27 - 50%
	M. pusillus (Adult)	12%	3%	0 - 33%	59%	69%	27 - 81%	30%	28%	20 - 41%
	M. pusillus (Larvae)	38%	40%	4 - 68%	41%	41%	2 - 78%	21%	19%	0 - 43%
	Stenelmis sp. (Adult)	43%	43%	16 - 73%	42%	43%	6 - 74%	15%	11%	0 - 33%
	Stenelmis sp. (Larvae)	40%	40%	6 - 72%	42%	41%	3 - 76%	18%	13%	0 - 43%
	S. comalensis	35%	35%	2 - 66%	41%	40%	7 - 74%	24%	4%	0 - 49%
	H. comalensis (Adult)	68%	92%	5 - 13%	27%	5%	0 - 75%	5%	1%	0 - 17%
	H. comalensis (Larvae)	72%	92%	18 - 100%	22%	4%	0 - 64%	6%	1%	0 - 25%

Table 1 - Summary of SIAR model output from SIAR at SR3, SI and the Comal system, with the percent contribution of basal resources (mean, median, and 95% confidence interval values).

Essential amino acid isotopes

Compound-specific essential $\delta^{13}C_{AA}$ values from the literature data indicated that normalized values of the six amino acids examined by this study [Threonine (Thr), Valine (Val), Leucine (Leu), Isoleucine (Ile), Phenylalanine (Phe), and Lysine (Lys) significantly differed when these AAs are derived from the four major producer groups (fungi, bacteria, algae, and terrestrial plants) (Fig. 5A).

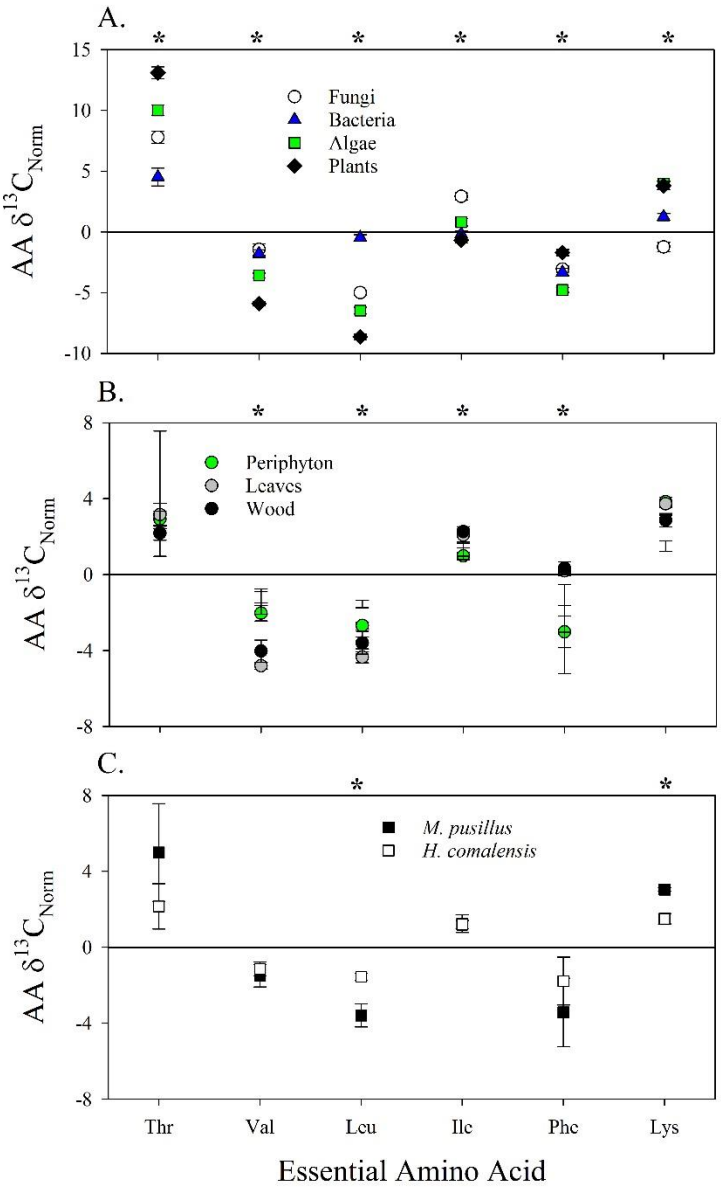
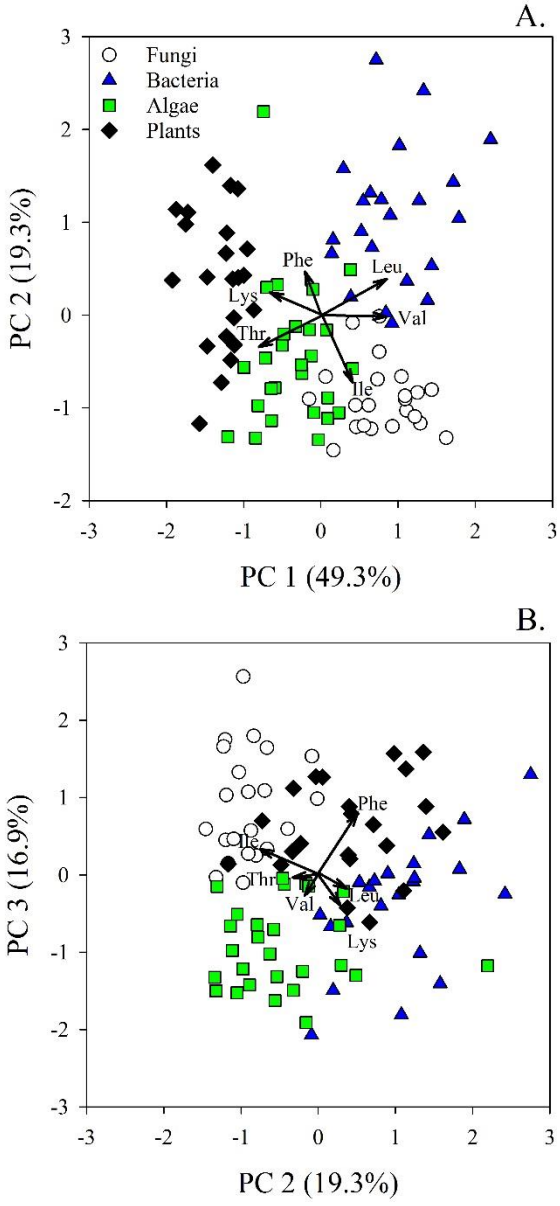


Figure 5 - Normalized essential amino acid $\delta^{13}C$ values for basal sources from the literature (A), basal food resources from Comal (B), and *H. comalensis* and *M. pusillus* (C). Each point represents a mean of $n = 3$ or 4 replicate analyses and bars are ± 1 SE. Asterisks along the top of each panel represent when a significant difference exists among sources or consumers for each amino acid.

Normalized $\delta^{13}C$ values differed among the broad producer groups for Thr ($F_{3, 92} = 47.68$, $p < 0.001$), Val ($F_{3, 92} = 43.27$, $p < 0.001$), Leu ($F_{3, 92} = 195.80$, $p < 0.001$), Ile ($F_{3, 92} = 56.37$, $p < 0.001$), Phe ($F_{3, 92} = 38.79$, $p < 0.001$), and Lys ($F_{3, 92} = 65.72$, $p < 0.001$), indicating that normalized AA $\delta^{13}C$ values for this suite of essential AAs could potentially be used to determine the origin (bacteria, algae, fungi, or plants) of basal food resources for consumers in the Comal system. When the normalized AA $\delta^{13}C$ values basal food resources from Comal (periphyton, leaf biofilms, and wood biofilms) are examined in a similar manner, values significantly differed among basal resources for Val ($F_{2, 8} = 11.42$, $p = 0.009$), Leu ($F_{2, 8} = 9.88$, $p = 0.013$), Ile ($F_{2, 8} = 5.66$, $p = 0.042$) and Phe ($F_{2, 8} = 12.58$, $p = 0.007$), but did not differ among sources for Lys ($F_{2, 8} = 4.10$, $p = 0.075$) and Thr ($F_{2, 8} = 1.26$, $p = 0.348$) (Fig. 5B). Normalized AA $\delta^{13}C$ values for *H. comalensis* and *M. pusillus* from Comal (periphyton, leaf biofilms, and wood biofilms) significantly differed for Leu ($F_{1, 7} = 10.31$, $p = 0.018$) and Lys ($F_{1, 7} = 27.63$, $p = 0.002$), but did not differ for Thr, Val, Ile, and Phe ($F_{1, 7} < 0.997$, $p > 0.357$ for all) (Fig. 5C).



To further assess patterns in $\delta^{13}C_{AA}$ values, we conducted a principle components analysis (PCA) with the data set obtained from the literature (Fig. 6A and B). Overall, we found that literature data clustered according to their major phylogenetic producer category (fungi, bacteria, algae, and plants). In the PCA, variation in Lys, Thr, Leu and Val portrayed on the first principle component, and this axis explained 49.3% of the variation among data points. PC 2 explained an additional 19.3% of the variation, while PC3 explained 16.9% of the variation among samples. PC 1 largely portrayed a gradient of photoautotrophic primary producers (plants and algae) to heterotrophic microorganisms (bacteria and fungi). PC 2 in general separated algae and fungi from the plants and bacteria, and PC 3 largely segregated fungi and plants from bacteria and algae.

Figure 6 - Principle component analysis (PCA) figure for literature-derived and normalized $\delta 13C_{AA}$ values for four major phylogenetically-defined basal producers in food webs: fungi, bacteria, algae, and plants. Each basal producer group is depicted as a different color and the percent variance explained by each PC axis is provided.

We then applied linear discriminant analysis (LDA) to examine the Comal source and consumer data in the context of the literature data. LDA allows users to define categorical groups in analyses (e.g., plants, fungi, bacteria, and algae) and then predict membership of unknown samples (such as Comal basal food resources and consumers) into these categories. Overall, the LDA model was a good fit for the data (Fig. 7; Wilks lambda = 0.014, df = 12, $p < 0.001$). In the LDA, literature-derived data were correctly classified with a 97.8% probability. When Comal basal resources were classified in the LDA, not surprisingly, they were assigned to an area occupied by terrestrial plants and algae. When *H. comalensis* and *M. pusillus* were plotted in LDA space, they were largely classified in the primary producer portion of multivariate space. Closer examination revealed that *M. pusillus* was more strongly associated with a clear photoautotrophic signal, while *H. comalensis* was clearly differently categorized than *M. pusillus* and was moved toward a more microbial (bacteria and fungi) $\delta^{13}C_{AA}$ profile.

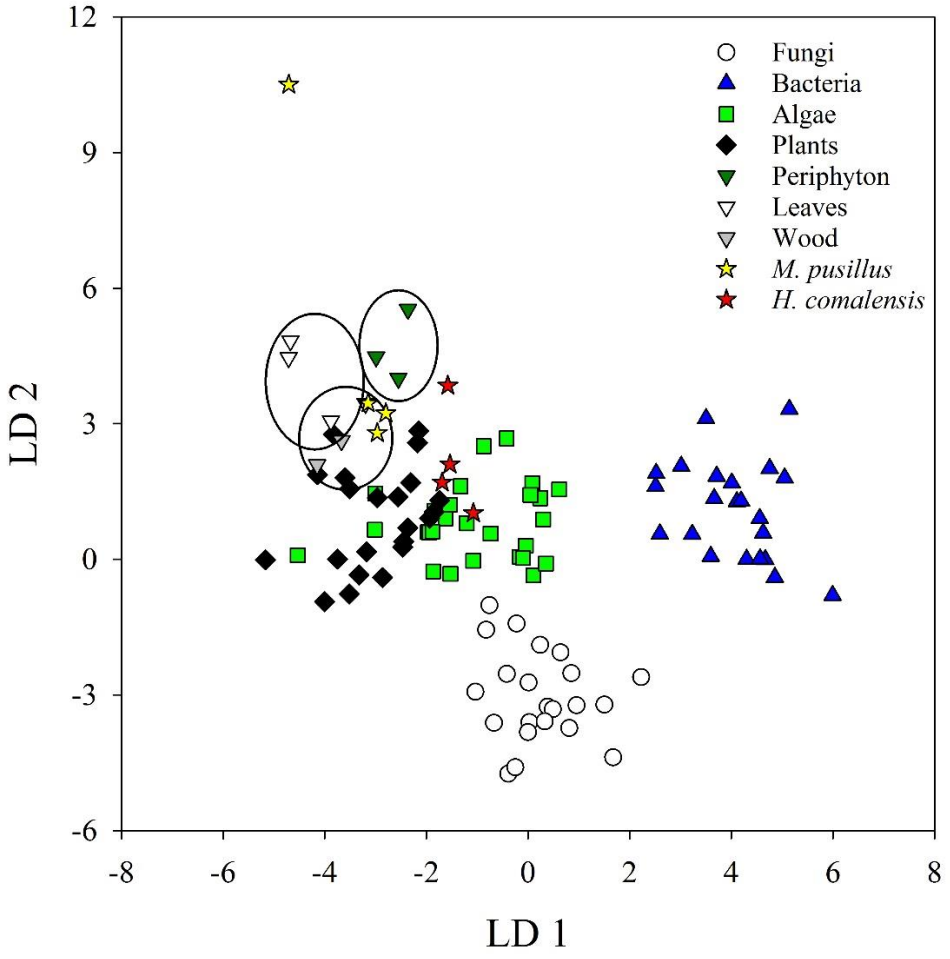


Figure 7 - Linear discriminant analysis figure (LDA) graph portraying normalized $\delta 13C_{AA}$ values for literature basal resources (fungi, bacteria, algae and plants), Comal Springs basal resources (periphyton, leaves, and wood), and *H. comalensis* and *M. pusillus* from the Comal system. Clusters of the points for the Comal basal resources are circled to illustrate their ordination in biplot space.

We then ran an isotopic mixing model (in FRUITS) on these $\delta^{13}C_{AA}$ data sets to determine the proportional contribution of different basal food resources to *M. pusillus* and *H. comalensis*. Models were run with two sources data sets: one run was conducted with basal resources from Comal and one run with basal $\delta^{13}C_{AA}$ values from major phylogenetic groups from the literature. In the Comal basal resources model run, three AAs were utilized (Leu, Phe, and Lys) because these AAs significantly differed among the two consumer species and the basal resources (Fig. 5) and the two basal resources were CPOM (wood and leaves combined) and periphyton. For the literature model run, the same three AAs were utilized and the sources were fungi, bacteria, algae, and plants.

The FRUITS mixing model output for the Comal data indicated that both *M. pusillus* and *H. comalensis* received a majority of their $\delta^{13}C_{AA}$ values from periphyton, while CPOM contributed a smaller fraction (Figs. 8A and B). Model output for the literature data presented a more refined view of the ultimate basal food resources for these two consumer species (Fig. 8C and D). The literatures FRUITS model run found that *H. comalensis* received bacteria and fungi contributed a percentage to the $\delta^{13}C_{AA}$ value of this species (~80%), whereas algae and plants contributed the remainder. In contrast, *M. pusillus* had a much more even contribution of the various basal sources to its $\delta^{13}C_{AA}$ signature, with algae (33%) and bacteria (33%) contributing the largest contributions to its signature.

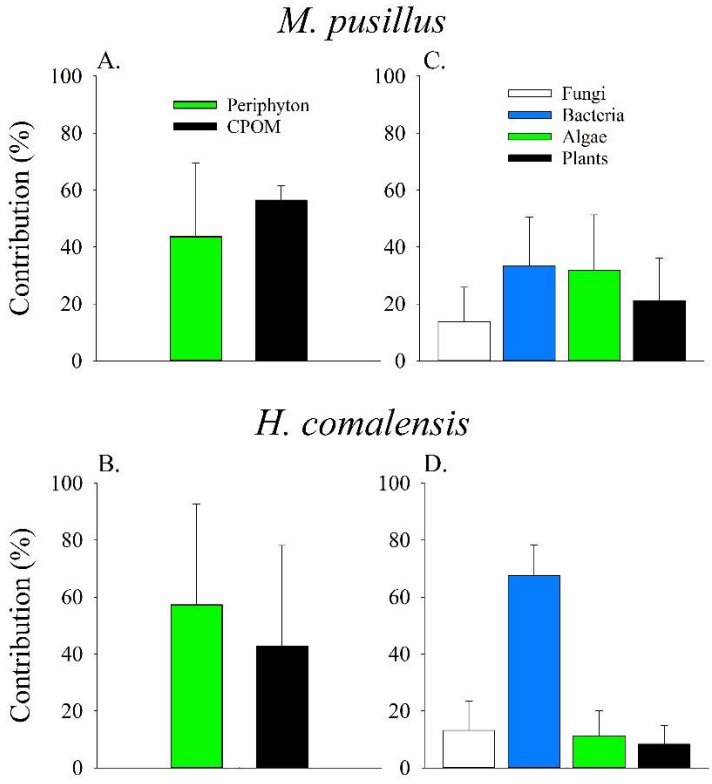


Figure 8 – FRUITS model output for M. pusillus (A) and H. comalensis (B) using in situ OM sources and outputs for runs utilizing literature data for phylogenetically-defined producer groups. Bars represent the mean output from model runs and the bars are ± 1 SD.

Gut content and biofilm sequencing

Illumina sequencing provided between 271,316 and 1,317,065 reads for individual samples, with average reads for the three replicate samples of at least 331,034 reads. Analyses performed at a rarefaction value of 150,500 reads focused on the Domain Bacteria, with 43 phyla identified. Only eight of these phyla (Acidobacteria, Actinobacteria, Bacteriodetes, Chloroflexi, Firmicutes, Planctomycetes, Proteobacteria, and Verrucomicrobia) were generally represented by more than 1% of all reads. The distribution of reads representing these phyla was remarkably similar for biofilm samples, independent of substrate (i.e., wood, rock, leaves), with two phyla dominating: the Proteobacteria represented by 65 ± 5% of all reads, and the Bacteriodetes represented by 15 ± 8% of all reads (Fig. 9). For gut content samples, the distribution of reads representing all phyla was also independent of substrate, but different from distributions in biofilms. Most of the phyla in intestine samples were represented by up to 10-fold lower numbers of reads, compared to biofilm samples, except for the Proteobacteria and the Firmicutes that together accounted for more than 90% of all reads in intestine samples. The Proteobacteria were represented by 50 ± 10% of all reads and thus with numbers comparable to biofilm samples, whereas the number of reads representing the Firmicutes in intestine samples was up to 100-fold higher than those in biofilms. In intestine samples of beetles feeding on biofilms growing on wood or leaves reads representing Firmicutes accounted for 30 and 32% of all reads, respectively, while those from rocks that were only 30-fold higher than those in biofilms accounted for 62% of all reads (Fig. 9).

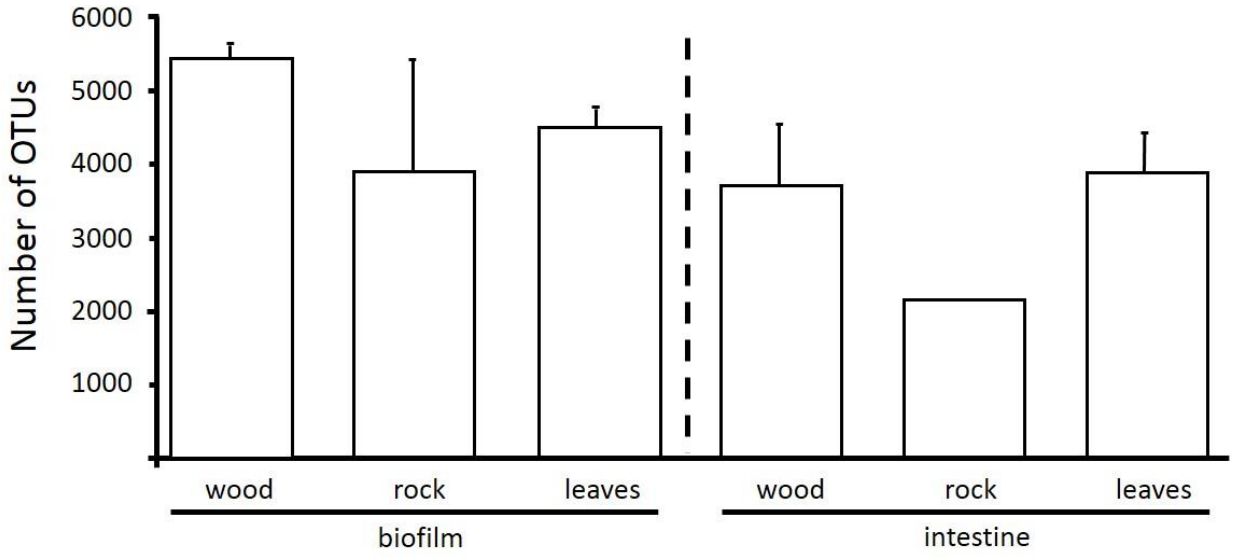


Figure 9 –Alpha diversity (Chao1) of bacteria in biofilm samples from wood, rock and leaves, and gut contents of *H. comalensis* collected from the corresponding microhabitat sites. Columns represent means and the bars are ± 1 SD.

Within the Proteobacteria, only Alpha-, Beta-, Gamma- and Delta-proteobacteria were represented by more than 1% of all reads in most samples. Numbers of reads for Alpha-proteobacteria (one-way ANOVA, $F_{2,6}$ = 3.24, p=0.110) and Gamma-proteobacteria ($F_{2,6}$ = 3.584, p=0.095) did not differ significantly in biofilms from different substrates, while higher numbers of reads were noted for Beta-proteobacteria in biofilms on leaves (Holm-Sidak test, p = 0.008 and 0.009), and for Delta-proteobacteria in biofilms on wood (p = 0.037 and 0.044) (Fig. 10). Similar results were obtained for

reads in intestine samples, where Alpha-proteobacteria ($F_{2,4}$ = 1.785, p = 0.279), Gamma-proteobacteria ($F_{2,4}$ = 1.58, p = 0.312), and Delta-proteobacteria ($F_{2,4}$ = 2.426, p = 0.204) did not differ with substrate, while reads for Beta-proteobacteria were higher when beetles fed on leaves (p = 0.003 and 0.003). In intestine samples, numbers of reads for Alpha-, Beta-, and Delta-proteobacteria were generally lower than in biofilms grown on the same substrate, while those for Gamma-proteobacteria were higher. However, only few of these differences were statistically significant [i.e., Alpha-proteobacteria in intestine from beetles feeding on biofilms on wood (p = 0.002), Beta-proteobacteria on leaves (p = 0.016), and Delta-proteobacteria on wood (p = 0.014)].

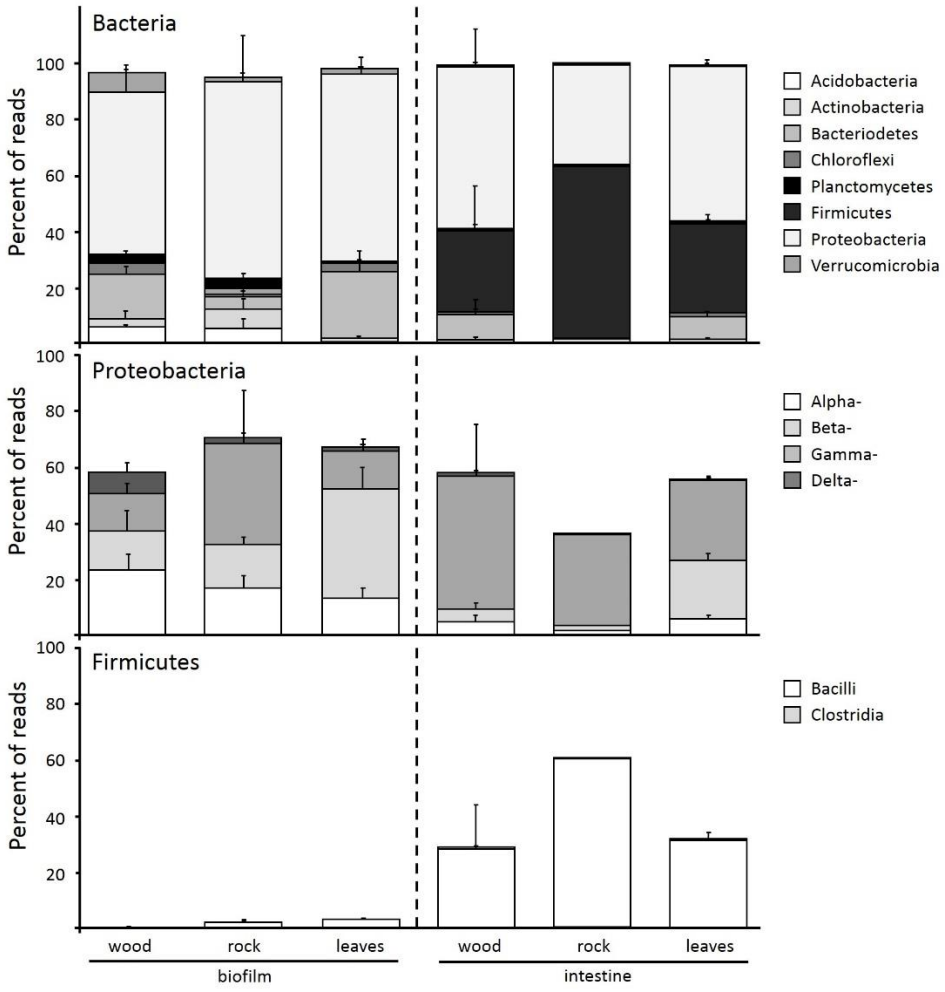


Figure 10 – Percent of all reads identifying bacterial phyla with abundances higher than 1% in biofilm samples from wood, rock and leaves, and intestine contents of riffle beetles collected from the corresponding sites. Two phyla representing the majority of reads within the Bacteria (i.e. Proteobacteria and Firmicutes) were further characterized at the class level.

Within the Firmicutes, nearly all reads represented *Bacilli* and a few *Clostridia* (Fig. 10). Although the small number of reads representing *Bacilli* in biofilms resulted in significant differences in numbers based on substrate, no such differences were obtained for numbers of reads representing *Bacilli* in intestine samples ($F_{2,4}$ = 3.370, p = 0.139). Comparison between reads obtained from biofilms and intestine samples of beetles feeding on biofilms established on the same substrate resulted in

statistically significant differences as a function of substrate with up to 100-fold higher numbers of reads in intestine samples ($p = 0.005$, $p < 0.001$, and $p < 0.001$ for wood, rock, and leaves, respectively).

DISCUSSION

Results of this study indicate that inputs of terrestrial-derived materials are likely important for the diet (and thus conservation) of *H. comalensis* and other species covered under the HCP. *H. comalensis* is generally distributed close to spring openings in the Comal system (Bowles et al. 2003), are commonly found on decaying terrestrial plant material in the field, and individuals can be maintained in the lab by supplying well-conditioned leaf material (W.H. Nowlin and R. Gibson, *unpublished data*). Reliance on leaf- and wood-associated biofilms is not a novel for members of the Elmidae. Indeed, Seagle (1982) found that the gut contents of larvae and adults of three riffle beetle species (*Stenelmis crenata*, *Stenelmis mera*, and *Optisoservus trivittatus*) were dominated by detritus-like materials, including included wood xylem, unidentified organic matter, and mineral particles, while algal material was consumed to a much lesser extent. It was further suggested that elmids as a whole should be reclassified as detritivores-herbivores rather than as strictly herbivores, with the exception of known xylophagus genera (i.e., Lara) (Seagle 1982). The results of the present study do not generally support this proposition given that both *Stenelmis* and *M. pusillus* relied largely upon algal- and *Amblystegium*-based materials as their basal food resources. These results indicate that there is a substantial amount of genus-level variation in dietary preferences in the Elmids and making a family-level trophic designation is unwarranted.

Input and deposition of woody material and plant material to stream and flowing water ecosystems can play an important role in the structure and function of macroinvertebrate communities (Hoffman and Hering 2000; Benke and Wallace 2003). In addition to providing a substrate for microbial biofilms for invertebrates to graze (e.g., Eggert and Wallace 2007), woody material and leaf matter can directly serve as habitat (microhabitat sites) for a diversity of invertebrate taxa and can create larger-scale instream habitats through alteration of flows (snags and CPOM accumulation areas; Benke and Wallace 2003). Colonization and use of terrestrial CPOM and woody material by macroinvertebrates is dependent upon several factors, including the type of CPOM, its state of decay, and the species-specific traits and requirements of the macroinvertebrates in question (Magoulick 1998; Collier and Halliday 2000). In the present study, it is apparent that inputs of terrestrially-derived CPOM is important for maintenance of *H. comalensis* in the wild, but there is a need for greater understanding of the relationships between CPOM decomposition states and utilization of these resources by *H. comalensis*.

In the present study, the bulk stable isotope data clearly showed that *H. comalensis* derived a substantial portion of its resources from terrestrially-derived OM. In bulk isotope mixing models, *H. comalensis* derived 70 – 92% of its diet from CPOM sources, whereas periphyton contributed 5 – 25% of its diet. In contrast, *M. pusillus* derived 21-25% of its diet from CPOM sources and 26 – 50% of its diet from periphyton. In addition, *M. pusillus* derived 19-30% of its diet from OM derived from *Amblystegium*. These results indicate that although *H. comalensis* and *M. pusillus* often occupy the same general habitat areas (Bowles et al. 2003), it appears that these two species are finely partitioning food resources.

The $\delta^{13}C_{AA}$ data for *H. comalensis* and *M. pusillus* were generally in agreement with bulk isotope data for the same consumers. In particular, the FRUITS model outputs using the two consumer species from Comal and the source data from the literature was consistent with the bulk isotope data. In these models, *H. comalensis* derived 67% of its $\delta^{13}C_{AA}$ signature from bacterial sources and 13% from

fungal sources, while deriving 11% of is $\delta^{13}C_{AA}$ signature from algal sources. In contrast, *M. pusillus* derived a much bigger fraction (32%) of its $\delta^{13}C_{AA}$ from algal sources. However, the model results from the *in situ* sources we collected and analyzed (wood biofilms, leaf biofilms, and periphyton) did not clearly align with a singular phylogenetically-identified $\delta^{13}C_{AA}$ source; all three sources were isotopically defined as photoautotroph-derived materials and there was no obvious influence of bacteria or fungi on $\delta^{13}C_{AA}$ signatures. This outcome is likely a methodological issue: we carefully scraped the surfaces of leaves and wood to remove biofilms, but it is likely that we also removed plant material with these biofilms and the $\delta^{13}C_{AA}$ profiles were more representative of photoautotrophic OM. Regardless, the results of the bulk and $\delta^{13}C_{AA}$ analyses support the finding that *H. comalensis* relies more heavily on bacterially-based biofilms on terrestrial CPOM, but that *M. pusillus* exhibits a higher reliance on algal-based resources in the Comal springs system.

In stream ecosystems which receive substantial inputs of terrestrial CPOM, bacteria represent a more predictable and consistent component of the microbial community when compared to fungi (Findlay et al. 2002). The proportion of bacterial to fungal contributions to the microbial community in streams varies with the type of OM and its state of decomposition. Indeed, fungi can be dominant members of the microbial community when there is a large amount of larger-sized, more refractory, and less decomposed OM in streams, whereas bacteria are dominant on more decomposed and less refractory materials (Weyers et al. 1996). In the present study, we found that *H. comalensis* derived a substantial portion of its dietary C from bacterially-based biofilms on terrestrial CPOM. This indicates that H. comalensis is likely using more decomposed wood and leaf material that has undergone the initial phases of decomposition. This finding is consistent with field observations of the most likely places to encounter and collect H. comalensis is on well-rotted and porous woody material and well-conditioned and partially fragmented leaves (W.H. Nowlin and P. Nair, *personal observation*).

The results from the gut content and environmental bacterial sequencing efforts indicated that the microbial composition of the gut contents of *H. comalensis* in the Comal springs system were largely reflective of the composition of the specific substrate they were occupying at the time of collection. Within a particular substrate type, microbial alpha diversity was similar in beetle guts and on the substrate, itself. In addition, most of the major microbial groups and taxa were in similar proportions in the guts as they were in the external grazing environment. However, the phylum Firmicutes was found in substantially higher numbers in the gut contents of H. comalensis than in the external environment (up to 100-fold higher). The presence of high numbers of the Firmicutes (*Bacilli*, in particular) is like because these bacteria are important and abundance members of the internal microbial flora of *H. comalensis*. Previous data for 218 insects found that the Firmicutes (and the Proteobacteria) are some of the most abundant and cosmopolitan microbial flora found in insects (Yun et al. 2014). Furthermore, the same study found that the Proteobacteria were on average 62% of all reads from the diverse selection of insects, a value remarkably similar to our findings (65% of all reads in *H. comalensis*. Thus, sequencing analysis of *H. comalensis* gut contents and the external environment clearly indicates that *H. comalensis* is a non-selective grazer which does not target biofilms composed of specific and unique bacterial groups on the particular substrate upon which they are feeding and that their gut flora is not substantially different from many other insects, globally.

Conservation implications for the EAHCP

A broader conservation implication of this study is that lateral connection to terrestrial OM sources may be particularly important for *H. comalensis*. Lower spring discharge and declining flows can lead to disconnection of the aquatic environment from the bank and to smaller water surface areas of

aquatic habitats, potentially leading to decreased inputs of terrestrial material that support *H. comalensis* populations in the Comal system. In addition, decreased flow rates and water velocities could also lead to lower rates of downstream transport of terrestrial materials from more canopy-covered upstream areas (e.g., the more canopy-covered Spring Runs) to more open areas with lower rates of terrestrial inputs (e.g., Spring Island area).

Results from our study indicate that other spring-associated consumer covered under the EAHCP may also be reliant upon terrestrially-derived CPOM. For example, *S. comalensis* was also largely dependent upon wood- and leaf-based biofilms, and this dependence doesn't appear to change between sites. Therefore, the feeding strategy of these both *H. comalensis* and *S. comalensis* do not appear to respond to spatial changes in the canopy cover and presumably amount of terrestrial detritus inputs. Thus, downstream populations of *H. comalensis* and *S. comalensis* may be particularly sensitive to lower inputs of terrestrial detritus material.

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