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

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Prepared by

Dr. Weston H. Nowlin

Dr. Dittmar Hahn

Parvathi Nair

and

Frank Alfano

601 University Drive
Department of Biology
Aquatic Station
Texas State University
San Marcos, TX 78666
(512) 245-8794
wn11@txstate.edu

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) (**Figure 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 (**Figure 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 in the Comal and San Marcos systems. 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.

We propose to examine 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 will primarily utilize 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 will be supplementary and add to the preliminary stable isotope data that was associated with a previous EAHCP project (BIO-WEST 2015). In addition to using stable isotope analysis, we propose to characterize the microbial communities associated with biofilms in the Comal and compare them to the communities found within the guts of riffle beetles and the biofilms found in different microhabitats within Comal Springs.

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 will collect 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 will allow us to quantify the density of major microbial groups present in various biofilms and in the guts of riffle beetles. Additionally, we will characterize the taxonomic composition and diversity of biofilms through sequencing rRNA genes in the same environmental samples. Collectively, both techniques will help to determine the microbial composition of biofilms (and lures) 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 has become one of the primary means to analyze food webs structure (Layman et al. 2012). Nitrogen and carbon are two most commonly used element 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 has 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 cultures for microbes 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

Stable 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, to determine if riffle beetles are utilizing OM or biofilms associated with OM, we will initially collect several riffle beetle individuals from sites where they are present (Spring Run 3, western shoreline, and Spring Island) for analysis of stable isotopes.

We will collect (by hand picking) at least 10 adult and larval Comal Springs riffle beetle individuals from various sites around the Comal system, allow them to excrete for at least 1 hour in spring water and then will be dried at 60°C for 48 hours in the Nowlin lab at Texas State University. In addition, samples of terrestrial organic matter (leaves, roots, wood, and submerged bark) will be collected from various sites around the Comal system. OM will be gentle wiped with a gloved hand to remove any biofilms and biofilms will be collected onto glass fiber filters. Samples of in-stream algal based biofilms will be collected from rocks will be collected by gently scrubbing rocks with a nylon brush and filtering this material onto glass fiber filters. In addition, a diversity of other aquatic species present in the system (e.g., *Microcoellypus pusillus*, *Hyalella azteca*, snails) will be collected from throughout Comal and analyzed in the same fashion as *H. comalensis* for comparative purposes and to construct the Comal food web.

All isotope samples will be ground into a fine powder and encapsulated in a tin capsule for bulk isotope analysis (for C and N). However, relying solely on bulk isotopes can sometimes cause errors to be made in determining food sources and trophic position (Bowes and Thorp 2015). Therefore, we will also select a smaller group of samples to additionally analyze for $\delta^{13}C$ of specific amino acids. All isotope samples will be sent to the Stable Isotope Analysis Lab at the University of California – Davis.

We will additionally collect riffle beetles from the Comal system with cotton-poly cloth lures and analyze the isotopic composition of the biofilms found in the lures and the isotopic composition of the beetles that have been foraging on the lures. This will allow us to isotopically compare the artificial biofilms that the beetles appear to be attracted to (on the lures) to the actual biofilms they are associated with in the wild (OM biofilms).

Microbial biofilm methods

We will collect information on the microbial composition of the biofilms found on rocks, organic matter, and from 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). Although FISH is not as "current" as metagenomic methods, FISH counts will allow us to quantify the density of major microbial groups present in various biofilms and in the guts of riffle beetles.

Prior to performing FISH counts and the extraction of genetic material, all biofilm samples must be sonicated instead of scraping biofilms from samples, in which scraping bias may inadvertently lead to experimental artifacts. Separate preliminary trials of sonication treatment times will be conducted to determine the optimal sonication treatment. Trial success of sonication treatments will be determined via FISH counts.

Fluorescent in situ hybridization will be used to enumerate total bacteria numerically in biofilms. All microbial cells will be stained with the DNA intercalating dye 40,6-diamidino-2-phenylindole (DAPI). Cy3-labeled probes EUB338, EUB II, and EUB III will be used in combination to detect all bacteria and various probes will be used individually to detect members of various bacterial families that may be present in the biofilms; specific families for targeting have not yet been identified, but will be preliminarily identified in the first several samples that are collected. Slide preparation will be performed via established methods in the Hahn lab at Texas State (Becker et al. 2011). Briefly, for slide application, 1-10 ml of sample will be dispersed sequentially in 0.01%

sodium pyrophosphate to a 1%sample concentration. To improve cell permeability, samples will be treated with 20 ml 10% dimethylsulfoxide for 1 h at room temperature, rinsed with distilled water, allowed to dry, and further treated with 20ml 1% lysozyme for 30 min at room temperature. To hybridize the probes, 9ml hybridization buffer, 1ml of the probe or probe mix (50 ng), and 1 ml of DAPI solution (200 ng) will be applied to each sample and incubated at 42°C for 16 h in a humid chamber. For counting, slides will be mounted with CitifluorTM AF1 solution and examined with a Nikon Eclipse 80i microscope, fitted for epifluorescence microscopy with a mercury lamp (Nikon; X-CiteTM 120) and two filter cubes, UV-2E/C (Nikon; EX340-380, DM400, BA4435-485 for DAPI detection) and Cy3 HYQ (Nikon; EX535/50, DM565, BA610/75 for Cy3 detection). For each biofilm sample, 30 to 50 fields covering 0.01mm^2 will be selected haphazardly from two to five slide wells hybridized with each probe, and cell counts were converted to the average cells per dry gram of biofilm.

Biofilm genetic analysis will be performed on the sonicated material. MO BIO Laboratories PowerBiofilm® DNA Isolation Kit will be used for extraction of genetic material from prepared biofilm samples. The PowerBiofilm® DNA Isolation Kit is the accepted industry standard in biofilm DNA extraction due to the optimized ability of the kit to provide high purity yields in the presence of concentrated inhibitors commonly associated with the extra-cellular matrix produced by biofilm forming bacteria. A blank extraction will be performed with all trials as a control.

Sample collection from riffle beetle guts

Adult and larval *H. comalensis* will be collected *in situ* from sites corresponding to biofilm samples and riffle beetle stable isotope sample collection (Spring Run 3, western shoreline and Spring Island). Sample selection will focus on woody debris, leaves and sticks which are the substrate associated with stable isotope analyses. Samples will be stored in aseptic deionized (DI) water and kept in coolers for transport to Texas State University.

The gut contents of all individuals (n = 5-10 for both larvae and adults) will be individually removed via microscope and suspended separately in vials containing DNA-free DI water. No measures will be taken to sample the foregut, midgut, or hindgut specifically instead the gut contents will be removed entirely.

FISH counts will be performed on isolated gut materials via the methods described above for the biofilms. Extraction of genetic material from beetle gut contents will be performed with the Dneasy Blood and Tissue kit (Qiagen) with modified protocol for both gram- and gram+ bacteria. A blank extraction will be performed with all trials as a control.

Data Analysis

Analysis of stable isotope data can be performed through a variety of analyses, but the current techniques focus on the use of Bayesian modeling methods to estimate the proportional contribution of different food sources to consumers (Layman et al. 2011). For the purposes of this study, the proportional food source contributions to *H. comalensis* will be determined with the use of the Stable Isotope Analysis in R (SIAR) for the "bulk" C and N isotopes and the FRUITS software packages (Parnell et al. 2010; Fernandes et al. 2014) for the AA-specific isotopes. In addition, the functional feeding group of *H. comalensis* (and other consumers in the food web) will be determined by calculating isotopic niche space metrics, such as convex polygon (Layman et al. 2011). All analyses will be conducted in R.

Microbial composition will be determined through FISH and densities (and relative abundances) of major bacterial groups will be determined on a per volume or a per mass basis. Sequence data will be entered into GenBank BLAST to identify the microbial components found in biofilms, beetle guts, and on cotton-poly lures. The justification for a comparative analysis is to determine if gut contents from riffle beetles sampled differ significantly from substrate biofilms in an effort to ascertain if riffle beetles were significantly lacking in components of their gut communities that would suggest that there is an important nutritional component unaccounted for in the riffle beetle diet.

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