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Anchialine Cave Environments:
a novel chemosynthetic ecosystem and its ecology

By

Michal Joey Pakes

Dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Integrative Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Roy L. Caldwell, Co-Chair

Professor David R. Lindberg, Co-Chair

Professor Steven R. Beissinger

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Abstract

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**Professor Roy L. Caldwell, Co-Chair
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It was long thought that dark, nutrient depleted environments, such as the deep sea and subterranean caves, were largely devoid of life and supported low-density assemblages of endemic fauna. The discovery of hydrothermal vents in the 1970s and their subsequent study have revolutionized ecological thinking about lightless, low oxygen ecosystems. Symbiosis between chemosynthetic microbes and their eukaryote hosts has since been demonstrated to fuel a variety of marine foodwebs in extreme environments. We also now know these systems to be highly productive, exhibiting greater macrofaunal biomass than areas devoid of chemosynthetic influx. This dissertation research has revealed chemosynthetic bacteria and crustaceans symbionts that drive another extreme ecosystem - underwater anchialine caves - in which a landlocked, discrete marine layer rests beneath one or more isolated layers of brackish or freshwater.

Most anchialine caves contain low invertebrate abundances, yet some have inexplicably large biomasses of shrimp and remipedes, a rare crustacean class endemic to caves. Since only anecdotal evidence of remipede feeding in the field has been published and little ecological information about anchialine organisms is known, *in situ* studies are crucial to understanding whether there are direct or indirect links between chemosynthetic input into anchialine foodwebs and remipede abundance. *This dissertation research integrates studies of geochemistry, microbiology, invertebrate food web dynamics, and behavior in these extreme ecosystems.* Findings include the following: 1) highly stratified anchialine caves support communities of freeliving chemosynthetic bacteria, 2) *Typhlatya pearsi*, an anchialine shrimp, harbors chemosynthetic endosymbiotic bacteria, 3) the remipede, *S. tulumensis,* may also harbor chemosynthetic symbionts ectobiotically 4) food webs in the cave are complex and vary spatially, driving community structure changes 5) remipedes likely use chemical gradients and specialized chemosensory receptors to navigate in their lightless environment.

This combined body of research provides the first systematic study into anchialine ecology and has found them to be rich in taxa for comparative study with other chemosynthetic systems. For example, the finding of the first chemosynthetic

endosymbiosis in a crustacean, *T. pearsi*, and the first chemosynthetic epibiosis in a remipede, *S. tulumensis*, allows for a variety of comparative coevolutionary studies. *Typhlatya*, a genus of troglobytic shrimp, are found in anchialine systems across the Caribbean and Bermuda and likely all contain chemoendosymbiotic bacteria. As such this discovery provides a springboard for comparative research into the transmission and evolution of chemosynthetic symbiotic associatons. The most interesting outcomes, however, may result from comparing ecological and ecosystem function patterns described in caves to those known for deepwater as well as molluscan and annelid systems. Furthermore, anchialine systems, a primary source for agricultural, domestic, and industrial water use, are especially susceptible to climate change. Therefore, not only are studies of ecosystem function such as this imperative to conserving endemic cave fauna and their suitable habitat, but they may also help in the preservation of clean water caches for future generations.

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DEDICATION

I dedicate this work to three mentors
from whom I have been fortunate
to learn and take inspiration.

C. Cavanaugh

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T. Thomsen

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J. Yager

ACKNOWLEDGEMENTS

A dissertation is in many ways a study in learning with the student as the central experiment. Throughout my time at UC Berkeley, people at the university and beyond have carefully observed my progression, making adjustments where needed and providing the materials and support necessary for this experiment to succeed. There are many institutions, agencies, mentors, and peers without whom this project would not have progressed. Their generosity can not only be seen through the written words and figures that follow, but also in the way that I now view teaching and learning.

I have trouble finding words to thank my co-advisors, Roy Caldwell and David Lindberg. Together they have made me an academic in a way I didn't realize I could be. They have allowed me to test, fail, and succeed with help and on my own. Early on, they allowed me to gamble on a largely unknown system and fostered my questioning with both interest and realism. I could not have wished for better academic family.

The way that Roy has drawn from his knowledge and experiences to advise me over the past 7 years has shaped this dissertation research. While some would say that Roy's academic love is stomatopods, I would counter that it is his students. He is always willing to meet with us, whether at his desk or while photographing a new Pseudosquilla egg clutch. During this time, he prepared me for fieldwork in a complicated underwater system, and with teams of researchers. He advised me to plan carefully, all the while telling me about his own difficulties in the field (lots of sharks, lots of decompression). He similarly could joke with me while expecting my rigor in experimental design and focus in writing style. Most importantly, he has asked me questions that are so relevant about my system that have changed the way that I look at it. I frequently ask myself: When I leave here, how will I know what I have left out? Thank you Roy for all of your guidance.

Dave Lindberg has instilled in me an evolutionary and deep time perspective that was only burgeoning when I arrived at Berkeley. "Things Change" may be a mantra of the lab, but we will always (past and future) benefit from your encouragement that we think outside of our organism, outside of our system, and outside of our timescale. This depth of perspective has affected me in my research, in my mentorship, in my teaching, in my outreach, and in my general worldview. In these ways, Dave has expanded the toolbox I will use to tackle academic, interpersonal or canine problems. For this, I will always be grateful.

Several other mentors have generously advised me throughout my time at Berkeley. Steve Biessinger was a fantastic outside dissertation committee member, aiding me with data interpretation and improving my writing immensely. His integrative work inspires me to both broaden my reaches and to focus my questions. C. Marshall has been an informal mentor, taking time from his directorship to provide writing, research, public presentation, and career advice. His words have brought me both encouragement and warnings– sometimes at once, reminding me that "success begets success." I have also carried with me the advice granted from- R. Woollacott, C. Cavanaugh and G. Giribet and have returned to Cambridge to get tune ups on occasion.

I would additionally like to thank my qualifying exam committee. J. Coates, T. Dawson, M. Power, and J. Lipps have mentored me into a new field through multiple conversations leading up to my exam and after it. There are few times when I have felt more lucky or stretched than during my qualifying exam. Their support for my project and ability to mentor me into different ways of thinking about cave ecosystem function and the evolution of karst systems has been integral in forming my dissertation ideas. Additionally, the training, space, and support I received in J. Coates' laboratory were imperative to completing my microbial work. T. Dawson has been similarly generous with his stable isotope facilities and expertise.

Who knew I would be so attached to an institution of paleontology? For me and for many other affiliates of the UC, the Museum of Paleontology is a place of mental stimulation, as well as financial and academic support. Coming to Berkeley, I imagined that I would gain a home for my laboratory and specimen based research behind the T-rex, but had no idea that this space also housed the jumping off point for a collection of educational and outreach initiatives. I feel so fortunate to have been a part of this organization over the last six and a half years and to have learned much from its many members in seminars, meetings, and hallways.

There were several other people and places where I received expertise for field and laboratory research while at Berkeley. J. Hayward, thank you for supporting scientific cave diving at the UC. Your training and advice has not only been integral in keeping me and my team safe during my doctoral work, but has also been necessary to the completion of this research. Few DSOs are so dedicated to their student's research and I appreciate your guidance. A similarly dedicated research facility is the Center for Stable Isotope Biogeochemistry at Berkeley. Here, I had the good fortunate to learn from P. Brooks and S. Mambelli. The time I spent with you both testing stable isotope analyses was not only imperative to the completion of this dissertation, but also incredibly rewarding. Thank you.

Much of what I learned in the UCMP and in the Department Integrative Biology centered on teaching and would not have been possible without the guidance of several key educators. T. Herlinger and J. Marshant have vast knowledge of teaching in the sciences and are always willing to lend a hand with course instruction. B. Mitchell and J. Scotchmoor introduced me to educational outreach at an organized level through the CALBLAST program. Through them I was able to meet Oakland city teachers and administrators as well as learn techniques to enhance my teaching at every level. Their mentorship helped me not only to teach, but also to work in large and diverse teams and for that I will also be grateful.

In addition, I have had the pleasure of meeting a number of bright UC Berkeley undergraduates during my dissertation research. Mentoring these undergraduates has been important for my understanding of the positive feedback loop that is academia. I appreciate not only their help in my research, but also their willingness to work with me as a team. In particular, I would like to mention several students who have made impressive independent contributions to science and to my own development as a scientist. D. Glenn, how lucky I feel to have fumbled into a working relationship with such a dedicated and creative scientist. M. Moritsch, thank you for taking our cave studies in new and exciting directions. A. Weiss, your breadth of interests, dedication, and enthusiasm are inspiring to me. V. Lovenburg, wrangling 6 tired cave divers is not an

easy task. Your involvement in my work came at a pivotal time in my progression and I appreciate you for your person as much as your research.

Which brings me to the following: my field work would not have been possible without the advice of Tom Iliffe and the dive assistance of his lab. Thank you E. Brodsky, J. Neisch, S. Haukebo, and especially B. Gonzalez for your underwater help and humor. K. Maverdeen and C. Richards your map of Cenote Crustacea is helpful and beautiful. T. Thomsen I look forward to many more research expeditions with you and cannot imagine a better mentor, videographer or partner during cave diving research.

Labmates may be the best buffer against over-betting or underselling your hand. Here, I have been fortunate to have two labs from which to draw advice and support. Thank you J. Alupay, J. Atterholt, S. Bush, D. Cotoras, M. deVries, L. Dougherty, J. Hofmeister, J. Judge, R. Romero, and M. Wright for grounding for me at every swing of this research and learning process. Each of you has improved my work through editing and critiquing my applications, papers and presentations. Each of you has improved my mood through hugs, smiles, snacks, and laughter.

During my second year at Berkeley, I had an adopted home in the Coates lab. I would like to thank this laboratory for introducing me to the wonders of microbiology, the truth that "phylogeny does not determine physiology," and the tools for enriching my own microbial cave bugs. Thank you I. Van Trump, C. Thrash, and K. Wrighton for your training and your conversation. J. Coates I appreciate that you lent me your home away from home and sometimes invited me into your real one. I would be a different person without the eye-opening experience I had in PMB and for that I am indebted to you all.

A crew as solidly able to talk science as they are to chat sox, pop popcorn, or lament lamentation will not be found again. As colleagues you will be respected and perhaps one day earn your gold stars, I may not be able to promise the same height of reputation as my friends. Thank you for your humor and thank you for your badly drawn graphs and diagrams on restaurant napkins: S. Fay, E. Robinson, J. Shade, G. Goldsmith, B. Carter, K. Simonin, K.Maguire, J. McEntee, C. DiVittorio, S. Rovito B. Williams, M. Wright.

Sometimes a trip, wine, and childhood memory has been equally helpful in re-igniting my mental fires or settling the flame down a bit. I admire P. Nelson's perspective and strength with all my heart. I feel fortunate that he is willing to share them with me (along with his computer) in times of rapid personal growth. If I could work as smart as N. Mutyala, I believe I would have a research station in a cave somewhere (this might be a venture to think about, actually). C. McKay, I will keep the tortes coming, if we promise to keep following each other in life. And who could have thought that a bridge club could provide me with so many tips and so much mental exercise? (This of course, before we deal our first hand). Lastly, J. and T. Veazey, I love your family as if it were my own and will suffer a few stitches myself for another 15 years of dinners and adventure.

My family has been a committee in itself, advising me when I ask and giving me solutions and perspective when I do not. My mother's ecological intuition and statistical knowledge has been training me as a scientist for years. My brother by example and advice encourages my risks (sometimes calculated) and inspires me in my teaching. It has taken me until now to understand my father's joy in mentorship and also to fully appreciate how well he has motivated and mentored me throughout my studies. I am

CHAPTER 1

Anchialine cave geomicrobiology: A multi-layered perspective

Abstract

The highly stratified water layers typical of anchialine caves likely house structured and potentially chemosynthetic microbial communities. Yet, the presence of chemosynthetic taxa in these extreme environments has only been inferred from a few geochemical and phylogenetic studies. Here, I describe the geochemistry and beta diversity of microbes across microhabitats of Cenote Crustacea, an anchialine cave in Quintana Roo, Mexico. This cave is known for its relatively high densities of crustaceans and located in a region of dense cave networks. Geochemical description of the water column and sediment of a section of Cenote Crustacea was used to infer the microbial habitat and biogeochemical processes at the time of sampling. Anaerobic enrichments inoculated with mat-containing cave sediment collected at the time of geochemical analyses and incubated without light have resulted in the isolation of nine sulfate-reducing chemolithoautotrophic bacteria and over 20 other strains of bacteria. 16S rRNA gene sequencing of isolated bacteria and subsequent phylogenetic analyses has revealed dominance of the Gammaproteobacteria and Deltaproteobacteria. The use of oligonucleotide phylogenetic microarrays (PhyloChips) has shown differences in active and persistent members of the microbial community amongst and between these samples as well as communities structured by microhabitat. Integration of geochemistry, enrichment, and microbial community structure studies begins to elucidate the role of chemosynthetic microorganisms in anchialine systems and illustrates their potential impact on ecosystem function.

Introduction

The availability of a variety of physicochemical microhabitats in anchialine caves make them ideal systems in which to study microbial community structure and discover novel chemosynthetic metabolisms (Seymour et al., 2007). These extreme environments are formed in porous rocks, such as limestone, and are often devoid of light. Here, water exchange with the landlocked marine habitat is severely restricted (Iliffe and Kornicker, 2009), resulting in stable gradients often including anoxic and sulfidic marine layers, underlying brackish water layers [Pohlman et al., 1997; Cangenella et al., 2007; Iliffe and Kornicker, 2009) (Figure 1). The distinct halocline at which these marine and brackish waters meet is often called the zone of mixing (ZOM). Methane may also be present where anoxic marine water sulfates are biogenically reduced in anoxic sediments (Seymour et al., 2007), making these interfaces potentially viable habitats for chemosynthetic microbes. Anchialine habitats thus contain both the highly structured environment and redox gradients necessary for the persistence of highly structured chemosynthetic communities. These metabolisms are especially important in cave ecosystems, which are historically characterized by little nutrient supply (Engel, 2013).

As subterranean environments are difficult to access and sample, there are few systematic studies of the ecological processes governing microbial community structure

and function within them. Initial theories regarding dark, oligotrophic cave environments made the following assumptions which lead to the hypothesis of low cave microbial diversity and biomass: 1) Inability of bacteria to harness energy within subterranean systems, 2) necessity of microbial transport into caves by abiotic or biotic vectors (Barton and Northup, 2007) and 3) the assumption that few microbial species could survive these extremely nutrient-limited environments (Palmer et al., 1991). Yet, these assumptions appear to be unfounded. Chemosynthesis occurs in geothermal submerged limestone (karst) caves (e.g., Hose et al., 2000; Dattagupta et al., 2007) as well as dry caves (Barton Northup, 2007). In addition, novel microbial diversity has been reported in sunlit portions of anchialine caves (Gonzalez et al., 2011). Microbes structured by depth in an Australian dark anchialine sinkhole provide further evidence of complex and thriving anchialine communities (Humphreys et al., 2012).

Phylogenetic beta diversity approaches allow for the incorporation of evolutionary history into investigation of community structure differences across microhabitats (Lozupone and Knight, 2008). While alpha diversity measures the number of taxa in a single community, beta diversity measures how the structure of these communities vary over time or space, taking similarities between taxa into consideration when comparing the presence or abundance of operational taxon units between samples (e.g., Shawkey et al., 2009). Phylogenetic microarray (PhyloChip) analysis may uncover 35 times the beta diversity of lower resolution techniques such as clone library analyses (Wrighton et al., 2008). There is great variation in microbial communities from similar environment types (Lozupone and Knight, 2007). Therefore, in order to uncover the ecological processes shaping anchialine microbial communities, studies relating abundance and diversity to geochemistry are needed at multiple spatial and temporal timescales.

The many microbial ecology studies aimed at molecular identification of 16S rRNA sequences in the environment leave major questions unanswered about microbial community function (Manefield et al., 2002; Wrighton et al., 2008). Inferring metabolic (e.g., chemolithoautotrophic) presence or capacity through phylogenetic affinity is impossible. As such, while there is evidence for chemosynthetic taxa in anchialine systems without geothermal input, more study remains to confirm their presence. Stable isotope food web analyses of cave animals (Pohlman, et al., 2007) along with studies of water geochemistry (Stoessell et al., 1993; Socki et al., 2002) indicate that these caves also support dynamic and potentially chemosynthetic bacteria. For example, the low $^{34}S:^{32}S$ ratio of sulfide relative to sulfate in Cenote Xcolac in the Yucatan Peninsula suggests fractionation caused by bacterially mediated sulfate reduction to sulfide (Socki et al., 2002). In addition, stratified concentrations of sulfate, hydrogen sulfide, bicarbonate, and nitrate suggest microbially mediated oxidation and reduction reactions in various microhabitats of Maya Blue and Angelita Yucatan cenotes (Stoessell et al., 1993).

The present study uses both cultivation-dependent and cultivation-independent approaches to examine the microbial community function of both water column and sediment communities in a macrofauna-rich anchialine system in Mexico, a region of high cave density. Phyogenetic microarray (PhyloChip) analysis provided greater understanding of the drivers of beta diversity in this cave and allows correlation with geochemical parameters. Enrichment of microbes in cave sediment yielded the first known cultured bacterial strains isolated from anchialine systems. Anaerobic enrichments

inoculated with mat-containing cave sediment resulted in the isolation of nine sulfate-reducing chemolithoautotrophic bacteria and over 20 other strains of bacteria. These Gammaproteobacteria and Deltaproteobacteria confirm the identity of chemosynthetically capable strains of microbes in anchialine caves.

Materials and Methods

Study Site

Divers collected samples from Cenote Crustacea, a Quintana Roo cave system in Mexico's Yucatan Peninsula (Figure 2). This site was chosen due to the high numbers of crustaceans in sections of Cenote Crustacea as compared with surrounding caves (Koenemann et al., 2007) and variation in geochemistry and macrofaunal densities throughout the cave (Haukebo, 2013; pers. observ.). In addition, Cenote Crustacea contains many blackened areas of the sediment, potentially thickened with extracellular polymeric secretions, in areas of high remipede densities (pers. observ. 2008). Blackened sediment may be microbial mat and may be correlated with high local macro-crustacean densities.

Fieldwork and Sample Collection

All fieldwork was performed on SCUBA approximately 400m from the cenote entrance in October 2008. Salinity-, temperature-, pH-, dissolved oxygen- (DO), and oxidative reductive potential- (ORP) depth profiles were collected in situ with a SONDE multiprobe. Water samples collected from above, in, and below the zone of mixing and from sediment samples were collected in triplicate for geochemical analysis. 1M HCL was added to a subset of each of these samples until pH fell below 2 for subsequent ferrous iron and anion quantification. All water and sediment samples preserved for geochemical analysis were kept at 4^oC for transport to UC Berkeley. Three additional black sediment samples were kept in the dark at 4^oC for bacterial enrichment until UC Berkeley, as well. SCUBA divers also collected two black and one non-black sediment sample in addition to one-liter water samples from the following depths: 1m above, at, and 1m below the zone of mixing. Water samples were subsequently vacuum pump filtered with .22micron Millipore filters. These sediment and filter samples were preserved in RNAlater for transport back to UC Berkeley and subsequent PhyloChip preparation and analysis.

Geochemical description of Cenote Crustacea

Geochemical data collected across a vertical section of Cenote Crustacea provided data for description of microhabitats and subsequent correlation between specific abiotic factors and microbial communities. Anions present in sediment water samples, as well as water samples collected above, in and below the halocline in Cenote Crustacea were quantified by ion chromatography on a Dionex DX500 employing a CD20 conductivity detector suppressed with an ASRS®-ULTRA II 4-mm system. In addition, we quantified Chemical Oxygen Demand in samples from these microhabitats as previously described (Gaudy, 1964). Organic acid concentrations in the same samples were quantified by HPLC with UV detection using an HL-75H+ a cation exchange column (Hamilton

#79476). Fe(II) was quantified in these samples as well as in sediment samples in triplicate as previously described (Stookey, 1970).

Enrichment and Isolation

To definitively associate taxonomic identification of anchialine microbes with their physiological function, bacteria from anchialine sediments were isolated and their 16S rDNA fragments were sequenced. This strategy allowed me to determine 1) what microbes were present in Cenote Crustacea and 2) what metabolic capabilities these microbes have. Sediment was enriched in the dark in presterilized, anoxic, bicarbonate buffered (pH 6.8-7.0), marine artificial poor water or basal media. Media was prepared anaerobically under 80:20 N_2/CO_2 headspace as previously described (Achenbach et al., 2001) and one of the following electron donors: 10mM formate, 10mM lactate, and 10mM Hydrogen, as well as one of the following electron acceptors: 10mM sulfate and 10mM AQDS (a humic substance analogue) were added after sterilization and before the addition of sediment samples. Bacteria were transferred at least three times using each electron donor acceptor media condition before isolation and isolated using the agar shake-tube technique (Coates et al., 1996). Pure cultures were confirmed using DNA extraction and subsequent 16S rRNA Gene Amplification.

Nucleic acid isolation

For Phylochip– Nucleic acids were extracted from RNAlater-preserved Millipore filters and sediment samples using a modified CTAB extraction buffer (equal volumes of 10% CTAB in .7M NaCl and 240mM potassium phosphate buffer, pH 8). First, 1ml of liquid nitrogen was first poured on filters in order to make them brittle enough to break them into pieces with forceps. One half of each filter or a 1g subsample of sediment sample was then added to Lysing Matrix E tubes (Bio101 Systems, CA, USA) containing 0.5 ml CTAB buffer, 0.1 mg ml^{-1} proteinase K (AMbion, TX, USA), which was then dipped in liquid nitrogen. Samples were then mechanically lysed by beadbeating at 550 r.p.m. for 20 s. We extracted nucleic acids first with phenol: chloroform:isoamyl alcohol (25:24:2), followed by a chloroform:isoamyl alcohol extraction (24:1). An overnight precipitation of nucleic acids with isopropanol at -20 degrees followed and pellets were subsequently rinsed with 70% ethanol. Pellets were resuspended in 50 μl TE buffer. RNA and DNA were purified using the All Prep DNA/RNA kit (Qiagen, CA, USA). On column DNAse digestion with DNAse-free RNAse set (Qiagen) was used to purify RNA, removing any potentially contaminating DNA. Absence of DNA contamination was confirmed with PCR amplifications using non-reverse transcribed DNAse treated RNA as a control. Only samples that did not PCR amplify were reverse transcribed into cDNA using Superscript II reverse transcriptase per the manufacturers protocol (Invitrogen, CA, USA).
For isolates– Genomic DNA (gDNA) was extracted from isolate growth cultures by adding 1ml of culture containing media to MoBio® Power Soil DNA kit (MoBio® Laboratories Inc., Solana Beach, CA) and subsequently following the manufacturer's protocol.

16S rRNA Gene Amplification for PhyloChip and Isolate Identification

The 16S rRNA gene was amplified from gDNA and cDNA using universal primers for bacteria and Achaea:

Bacteria: 27F (5' -AGAGTTTGATCCTGGCTCAG)
            1492R (5' -GGTTACCTTGTTACGACTT)
Archaea: 4Fa (5' -TCCGGTTGATCCTGCCRG-3')
            1492R

PCR amplifications were set up according to standard protocols for Phylochip preparation (Flanagan et al., 2007). All samples underwent equivalent PCR conditions. This pooled sample was concentrated by precipitation and resuspended in 50 μl sterile nuclease-free water.

Sequence Alignment and phylogenetic analysis of isolates

16S rRNA gene fragments of isolates were assembled using Sequencher (www.genecodes.com). Concatenated sequences were then compared to known cultured and uncultured bacteria retrieved from GENBANK. These additional 16S rRNA gene sequences always included the closest hit for each isolate's 16SrRNA sequence and were used for future phylogenetic analyses. Primers were trimmed from all fragments, which were subsequently aligned using MUSCLE 3.6 (Edgar, 2004). A Maximum Likelihood Analysis of 16S rRNA gene phylogeny was constructed using RaxML (Stamatakis, 2008) in order to illustrate the phylogenetic position of isolates procured from sediment in Cenote Crustacea.

16S rRNA amplicon analysis by PhyloChip hybridization

Taxonomic identity of microorganisms present in Cenote Crustacea in October, 2008 was inferred by running amplified 16S rRNA community samples on high-density phylogenetic microarrays (Phylochips), containing ribosomal 16S sequences of bacterial representatives (Wrighton et al., 2008). A total of 12 PhyloChips were examined, including DNA arrays for each of the six microhabitats (above, in and below the zone of mixing (ZOM), two black, and one non-black sediment) and cDNA arrays for the same microhabitat samples. DNA arrays were loaded with 400ng of archaeal and bacterial combined PCR product that resulted from the gDNA template. cDNA arrays were loaded with 60ng of bacterial PCR and 10μl of PCR product resulting from cDNA template. Samples were spiked with known concentrations of synthetic 16S rRNA gene fragments and non-16S rRNA gene fragments as internal standards for normalization, with quantities ranging from 5.02 x10^8 to 7.29 x 10^{10} molecules applied to the final hybridization mix. Target fragmentation, biotin labeling, PhyloChip hybridization, scanning and staining, as well as background subtraction, noise calculation and detection and quantification criteria were described by Flanagan et al., 2007. A taxon was considered present in the sample when 90% or more of its assigned probe pairs for its corresponding probe set were positive (positive fraction ≥ 0.90).

Comparing anchialine microhabitat community membership and dynamic populations by PhyloChip

To compare organisms that occur in the various microenvironments (above, in, and below the ZOM, as well as black and non black sediment) in Cenote Crustacea to known microorganism 16S sequences on Phylochips, cave microorganism genomic were hybridized and resultant data was assessed. Both cDNA and DNA chips from the aforementioned microhabitats were hybridized and analyzed to determine whether

differences occurred between active and persistent communities, respectively, across samples. 16S rRNA and 16S rDNA (synthesized to cDNA prior to hybridization) PhyloChip arrays were performed on the following samples: three sediment arrays (two black and one non-black) and arrays from above, in, and below the halocline 400 m from the cenote entrance in Crustacea. Hierarchical cluster analyses were performed on PhyloChip outputs from 16S rRNA and 16S rDNA arrays in fast unifrac (Hamaday et al., 2010). These analyses were weighted in order to incorporate abundance of OTUs present and data was normalized. Principal components analysis performed in Fast Unifrac allowed for the further incorporation of environmental data on microhabitats in order to explain variation in both active and persistent community structure between samples. Since DNA sediment and water samples clustered separately, these sets of samples were later pooled to examine which taxa were more abundant (or enriched) in the water column than in the sediment and vice versa. In order to determine whether enrichment between microhabitats occurs, hybridization intensity score (HybScore) differences between samples were assessed for focal taxa. HybScore differences were used as a direct measurement of changes in gene copy number; 1000-fold change in HybScore is proportional to a single log change in relative abundance (Brodie et al., 2007).

Results

Geochemistry of anchialine microhabitats

A vertical profile of a section of Cenote Crustacea showed that temperature, dissolved oxygen, pH, and oxidative reductive potential (ORP) varied with depth (Figure 3). Specifically, decreases in temperature and ORP were measured at the halocline, whereas decreases in DO and pH occurred at depths slightly greater than the halocline (Figure 3, 4). Sulfate (30.06 +/- 4.54) and Iron (II) (76.92 +/-69.82) concentrations were greatest in the marine layer, while nitrate was most concentrated in brackish waters above the halocline (2.97 +-0.39) (Figure 4, Table 1).

PhyloChip analysis of microbial communities

DNA and cDNA PhyloChip analyses, representing the persistent (via 16S DNA) and Active (via 16S rRNA) hybridization illustrated community structure processes (Figure 5, 6). For example, rank abundance curves for samples were similar when considering 16S rDNA outputs (Figure 5a). In contrast, trajectories of these curves varied by microhabitat for 16S rRNA hybridization data with more even communities in water column samples than in sediment samples (Figure 5b). Fastunifrac principle component analyses of community structure PhyloChip datasets weighted for abundance showed grouping of water column samples versus sediment samples for both 16S rDNA and 16S rRNA outputs (Figure 6). However, the relationships within these groups differed for 16S rDNA and 16S rRNA hybridization intensity data. For instance, within the water column, communities in the zone of mixing and below it were most similar when considering 16S rDNA outputs (Figure 6a). Yet, 16S rRNA datasets showed a more close relationship between communities in the zone of mixing and above it (Figure 6b). A hierarchical cluster analysis of PhyloChip intensity data comparing the overall community structure within and between water column and sediment samples showed detected three clusters: sediment 16S rDNA communities, sediment 16S rRNA communities, and a combined

cluster of water column communities derived from 16S rDNA and 16S rRNA hybridized chips (Figure 6c). Within the water column cluster, 16S rDNA and 16S rRNA profiles did not cluster (Figure 6c).

There were 145 taxa enriched in water column samples versus sediment samples (1000x log difference in hybridization intensity) as opposed to 59 taxa that were enriched in the sediment (Figure 7). The majority of both of these sets of enriched persistent taxa were Proteobacteria (Figure 7). However, Proteobacteria that were relatively abundant in the water column were mostly Betaproteobacteria (Figure 7). For example, the order Burkholderiales was commonly abundant (67%) taxa in this subset (Appendix 1). In contrast, those taxa enriched in the sediment, were largely Deltaproteobacteria (Figure 7) comprised of 62% Desulfobacteriales (Appendix 1).

Isolation of bacteria from anchialine sediments

Phylogenetic analyses of placement of 16S rRNA sequences of bacteria isolated anaerobically from Cenote Crustacea sediment fell out among the Proteobacteria (Figure 8). Gammaproteobacterial and deltaproteobacterial chemolithoautotrophic strains were discovered to use sulfate as an electron acceptor and hydrogen as an electron donor (Figure 8). Metabolisms of isolated deltaproteobacterial chemosynthetic strains also included the use of AQDS as an electron acceptor and hydrogen as an electron donor (Figure 8). Gamma- and deltaproteobacterial chemosynthetic strains were also isolated using formate as an electron donor and sulfate as an electron acceptor (Figure 8). Enrichments that provided lactate as an electron donor and sulfate as an electron donor also led to the isolation of a representative of the Gammaproteobacteria (Figure 8).

Discussion

The integrated culture independent and culture-dependent design of this study has illustrated the dominant role of Proteobacteria with chemosynthetic metabolisms in dark, anchialine systems. These results not only confirm the ability of these microbes to fix carbon endogenously within these extreme systems, but also allow inference of microbial community metabolism through the correlation of taxonomic abundance data with geochemical variation. Findings reported here give basis to evidence suggesting that macrofauna in anchialine systems are dependent on a chemosynthetic source of carbon (Pohlman et al, 1997). In addition, here we allow comparison between microbes present in the system and those found in deep (Humphreys et al., 2013) or lit (Gonzalez et al., 2013) anchialine habitats. More recently, subterranean ecosystems have been described as a continuum of chemosynthetically-dependent ecosystems rather than discrete environment types that extend from near shore anchialine "continental estuaries" to the deep substrata beneath hydrothermal vents (Dov Por, 2008). Further comparisons of anchialine data with those collected in cave systems with geothermal input (e.g., Movile Cave, Romania, Sarbu et al., 1996; Frasassi cave system, Italy, Macalady et al., 2008) may also inform our understanding of chemosynthetic energy production and nutrient flow in the substrata.

Significance of anchialine chemosynthetic Proteobacteria

Together the 16S rDNA and rRNA PhyloChip results imply that Proteobacteria play a

major role in the anchialine community. 16S rDNA sequences belonging to the Proteobacteria have previously been detected in anchialine cave wall microfilms (Gonzalez et al., 2011; Humphreys et al., 2013). However, these studies did not include investigations of the sediment. In addition, only one study has reported 16S rDNA data from a dark anchialine system (Humphreys et al., 2013) that may be comparable to the Cenote Crustacea system.

Furthermore, this study marks the first report of a culture-based approach to describe members of the anchialine bacterial community, linking taxonomic identity to physiology. We isolated several bacteria representing three genera (Shewanella, Pseudomonas, Marinobacter) of Gammaproteobacteria and several representatives of the Deltaproteobacteria (e.g., Geobacter, Desulfovibrio species). PhyloChip analyses indicated that Deltaproteobacteria represent the most dominant clade enriched in the sediment community. Isolates were capable of chemosynthetic metabolisms using sulfate or AQDS as an electron acceptor.

Stratified microhabitats drive microbial community structure

Geochemical data combined with beta diversity analyses shed light on the links between geochemical differentiation and beta diversity amongst these highly stratified systems. Understanding microbial resource use will also provide insight into nutrient sources available to macrofauna endemic to these extreme habitats (e.g., Remipedia Yager, 1981). Most directly, further study of the suite of bacteria living freely in these environments will give insight into transmission strategies of anchialine symbioses (see Chapter 2, 3).

The microhabitats present in anchialine systems (sediment, below ZOM, above ZOM, and in ZOM) are physiochemically distinct and provide varying substrate for microbial metabolism likely driving community structure variation. For example, Burkholderiales (Betaproteobacteria) were enriched in the water column with respect to the sediment communities. The zone of mixing which is oxygen poor was found to contain depleted concentrations of nitrate and increased $Fe(II)$ with respect to the brackish water above the zone of mixing. These data suggest that microbial nitrate reduction coupled to iron oxidation may occur at this redox zone. As members of the Burkholderiales found in this study are involved with these annamox reactions, further study is recommended to confirm the presence of these physiologies in situ or in culture. Nitrification has also been suggested in Australian anchialine systems at this type of halocline (Seymour et al., 2007) pointing to this zone as an important for the nitrogen cycle.

The anchialine marine sediment water interface likely represents another microhabitat of microbially-mediated oxidation-reduction reactions. Here, increased $Fe(II)$ concentrations in the sediment pore water as compared to waters directly above the sediment imply anoxic reducing sediment. Additionally, the depleted concentrations of sulfate in the sediment pore-water indicate sulfate reduction. As communities enriched in the sediment were dominantly Proteobacteria, and specifically Deltaproteobacteria known to reduce sulfate anaerobically (e.g., Desulfovibrio spp. and Desulfobacteraceae) (Miyatake et al., 2009). Geochemical and stable isotope data has suggested redox cycling of sulfate and nitrates in similar anchialine systems (Socki et al., 2002). Therefore, this zone is likely a region of great microbial activity and metabolic diversity.

Active versus persistent community structure

PhyloChip data supported discrepancies between persistent (16S rDNA) and active (16S rRNA) communities that have been noted in other systems (e.g., Manefield et al., 2002, Wrighton et al., 2008). These studies emphasize the importance of examining RNA in functional diversity studies. As the first report comparing persistent and active communities in anchialine systems, whether these differences exist in other caves and blueholes remains to be seen. The current study noted a high similarity among 16S rDNA and 16S rRNA communities from anchialine sediments, but variation between them. RNA, which is susceptible to enzymatic and environmental degradation, is more likely to reflect the phylogenetic composition of metabolically active communities than DNA (Kerkoff and Kemp, 1999). Therefore, these data indicate that there may be great turnover in the microbial communities dominating the substrate. It is possible that molted crustacean carapaces seen on occasion on dives, carcasses of macrofauna or excrement may be providing resources for bursts of microbial activity and growth. These data suggest that communities may greatly vary across seasonal and finescale time periods. Such findings indicate a need for future temporal and successional studies into anchialine microbial community structure.

In contrast, the phylogenetic distance between active and persistent communities in the water column was not as marked. However, evenness of the persistent brackish water (above ZOM) community was generally greater than that of the corresponding active one. These differences in community structure based on 16S rDNA and 16S rRNA hybridization data suggest that turnover of dominant members may be higher in this oxygenated, brackish water region than in areas surrounding it. Findings such as these support early hypotheses that anchialine systems are both highly stratified and dynamic (Seymour et al., 2007).

Future research into anchialine microbial ecology will have implications for a variety of fields. New clades of microbes isolated from these environments, such as those reported here, may exhibit novel metabolisms. Furthermore, investigating the microbial function in the laboratory and in situ will inform our understanding of the physiochemical processes governing anchialine communities. These habitats are important inputs into the industrial and agricultural water supply in Mexico (Humphreys, 2006) and subterranean freshwater caches may impact future water supply (Post et al., 2013).

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Table Caption

Table 1. *Ex-situ* geochemistry of Cenote Crustacea. Ion chromotography and ferrozine assays of samples taken from above, in and below the halocline, as well as from blackened areas of the sediment reveals that the marine layer of Cenote Crustacea is low in fatty acids (data not shown) and nitrates. Ferrous Iron and Sulfate concentrations suggest anaerobic conditions in the sediment typical of anoxic marine sediment.

Table

Table 1.

	SO_4^{2-} (mM)	Fe (II) (nM)	NO_3^- (µM)
Above ZOM	8.19 +/- 0.99	39.05 +/-8.93	2.97 +-0.39
In ZOM	12.49 +/- 7.89	3.55 +/-3.55	2.69 +/-0.49
Below ZOM	30.06 +/- 4.54	76.92 +/-69.82	0.49 +/-0.17
H_2O of Black Sediment	22.30 +/- 2.42	91.12 +/-30.83	2.68 +/-0.87
Black Sediment	N/A	198.46 +/- 83.43	N/A

Figure Captions

Figure 1. (above) Anchialine Cave. A. Pakes collecting in Mexican system. Photo by T. Thomsen. B. Schematic of anchialine cave, illustrating meteoric brackish water lens overlaying marine layer as well as access points (adapted from Moritsch, Pakes, and Lindberg (accepted, Org. Divers. Evol.)

Figure 2. Map of Yucatan Peninsula, illustrating anchialine localities known for their endemic macrofauna (at circles) with sea level dropped to less than 20. Closed circle and arrow illustrates Cenote Crustacea, the site used in the current study. Map adapted from Moritsch, Pakes, and Lindberg (accepted, Org. Divers. Evol.)

Figure 3. In-situ geochemistry of Cenote Crustacea. October, 2008 Temperature-(^{o}C), Salinity- (ppt), DO- (mg/L), pH, ORP- (V) depth (m) SONDE profiles at collection site of Cenote Crustacea, illustrating changes in geochemistry with depth. This profile suggests that the marine layer in Cenote Crustacea can support the sulfate reducing metabolisms exhibited in isolates.

Figure 4. Principle components analysis of Cenote Crustacea microhabitats (In, Above, and Below Zone of Mixing as well as Sediment pore water) in October, 2008. Temperature-(^{o}C), Salinity- (ppt), DO- (mg/L), pH, ORP- (V) were collected with a SONDE profiler and averaged across microhabitat depths. Sediment pore water approximation for these parameters is for readings near sediment. Chemical Oxygen Demand (COD), $Fe(II)$, NO_{34}^-, PO_4^-, SO_4, depth (m) SONDE profiles at collection site of Cenote Crustacea, also illustrate distinct microhabitats. Not that Nitrates and Phosphates are characteristic of the upper water layer ans zone of mixing, respectively.

Figure 5. Rank abundance curves for bacterial communities within microhabitats of Cenote Crustacea, Mexico. (A) 16S rDNA rank- abundance curves illustrate similar richness and evenness between samples when examinging persistent communities. (B) Active communities (16S rRNA data) examined revealed differences in community structure between samples with black sediment samples showing less evenness than water column samples.

Figure 6 UniFrac analyses showing community relationships between microhabitats in Cenote Crustacea, Mexico. (A) Principle component analysis of PhyloChip DNA hybridization data reflect similarity of sediment samples as well as in and below zone of mixing samples B) Principle component analysis of PhyloChip cDNA hybridization data reflect similarity of sediment samples as well as above and below zone of mixing samples (C) Bacterial communities measured by PhyloChip hybridization intensity cluster into three distinct groups: sediment DNA, sediment cDNA and water column samples. All analyses incorporated normalized PhyloChip hybridization intensity as a measure of abundance.

Figure 7. Relative abundance of bacteria in water column versus sediment. (A) Identity of bacterial clades found in 1xlog greater abundance in the water column than in the

14

sediment shown at top. (B) Identity of bacterial clades found at 1xlog greater abundance in the sediment than in the water column shown at bottom. Both subsets were most likely to occur in the Proteobacteria. Those proteobacterial OTUs that were more abundant in the 1xlog water column or the sediment are further broken down by family at right of (A) and (B) with Betaprotebacteria enriched in the water column and Deltaproteobacteria enriched in the sediment.

Figure 8. Phylogenetic placement of bacteria isolated anaerobically from Cenote Crustacea sediment revealed that chemosynthetic isolates were Proteobacteria according to 16S rRNA gene analysis. All isolates were grown anaerobically and in the dark. Tree was constructed using Maximum Likelihood analysis. Bootstrap values (1000 replicates) are shown at nodes. Gammaproteobacterial and deltaproteobacterial chemolithoautotrophic strains reducing sulfate and using hydrogen as an electron donor (closed stars) were isolated. Deltaproteobacterial chemosynthetic strains reducing AQDS and using hydrogen as an electron donor (open stars) were also isolated. Other isolated strains used formate (closed circle) or lactate (open circle) as an electron donor and sulfate as an electron acceptor and when sequenced were found to represent the Gammaprotebacteria and Deltaproteobacteria. The outgroup consists of *Acidobacterium capsulatum* and *Holophaga foetida* strain TMBS4-T in the Acidobacteria.
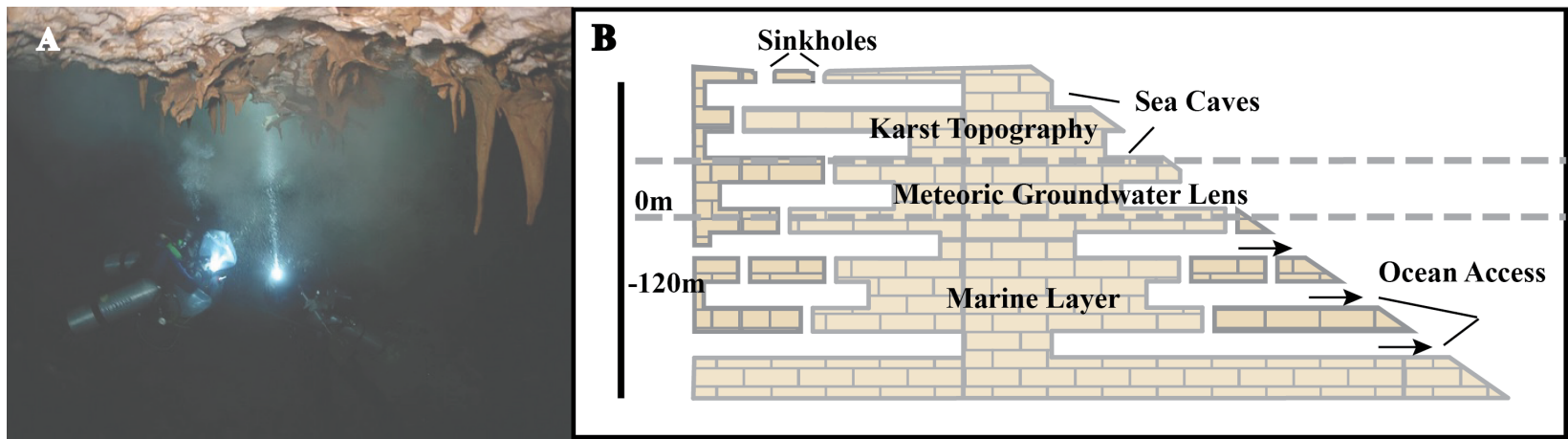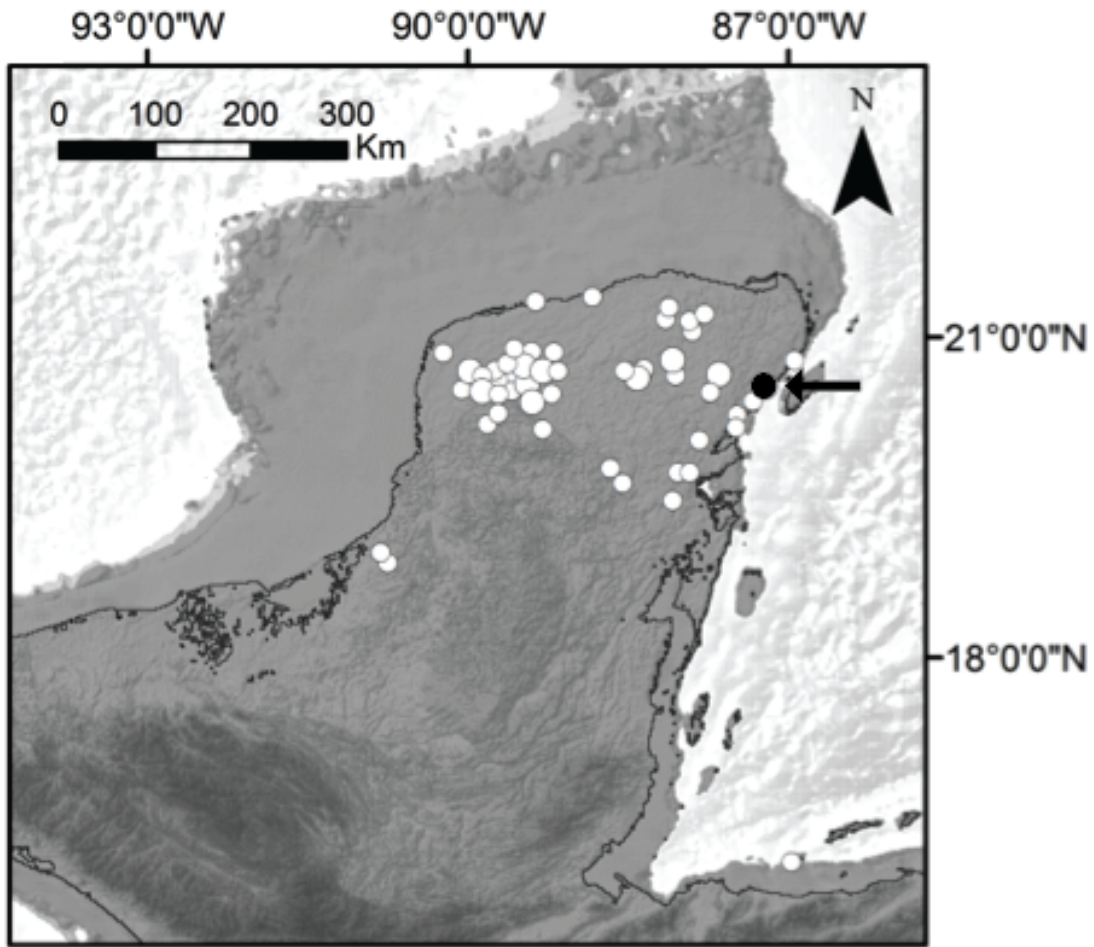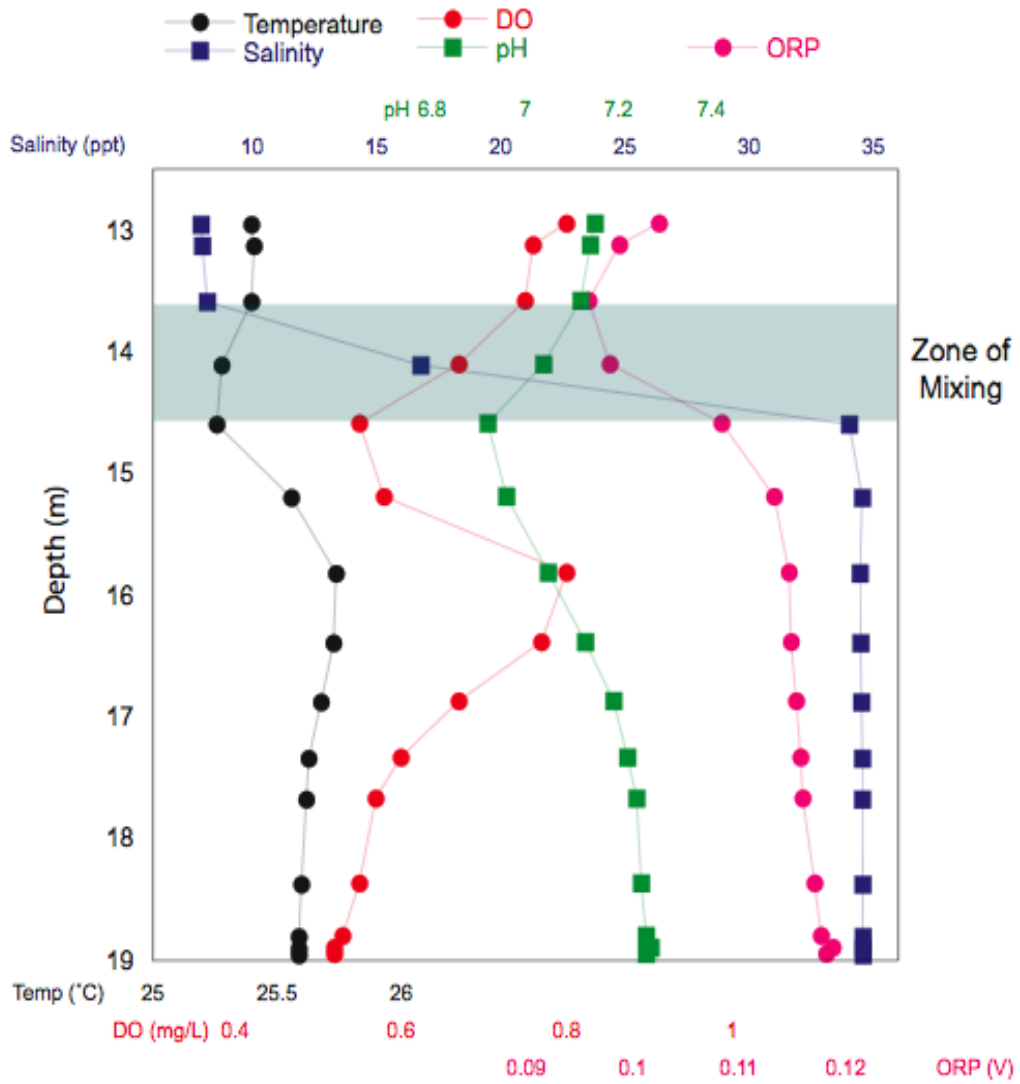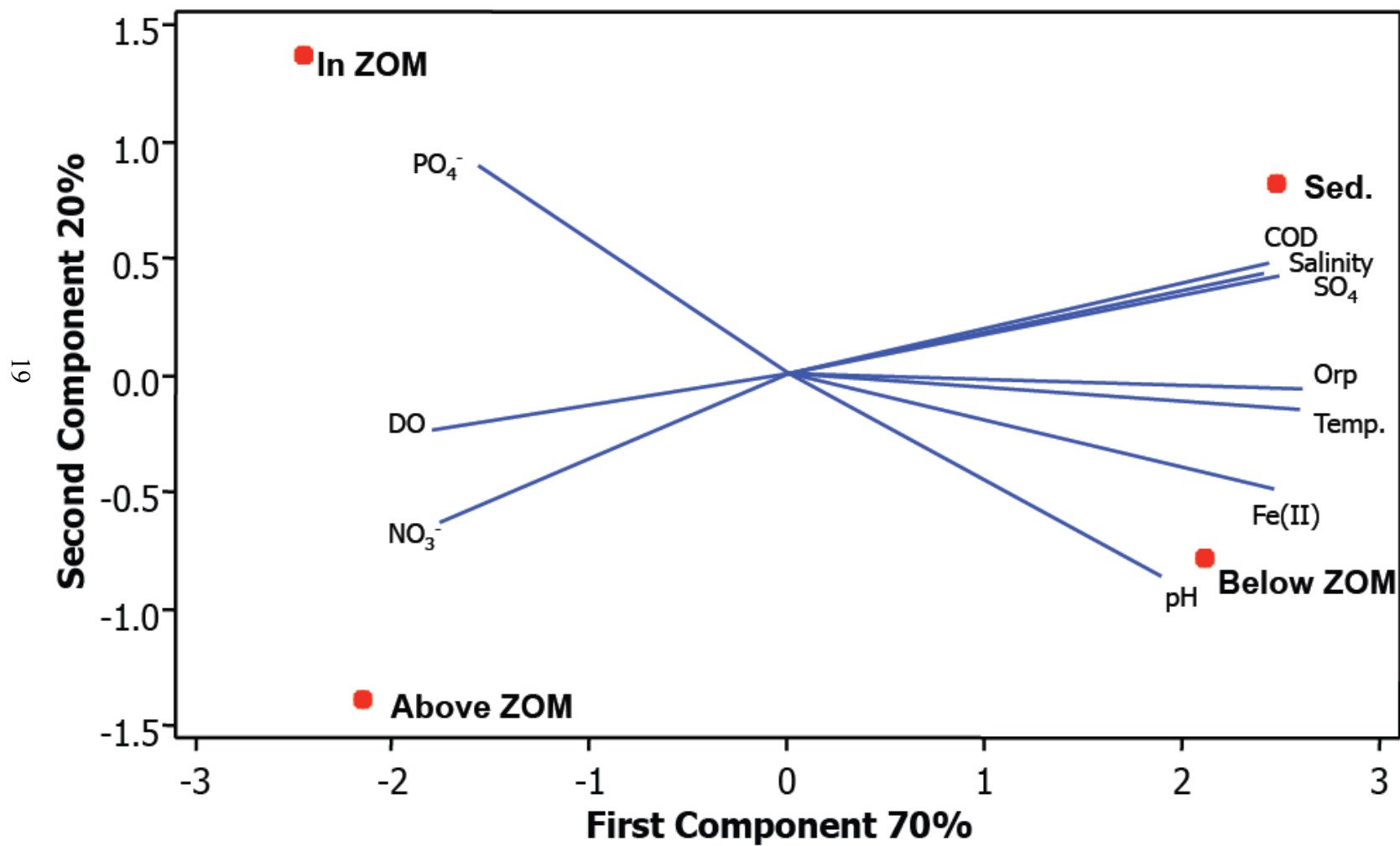
Figures

Figure 1.

Figure 2.

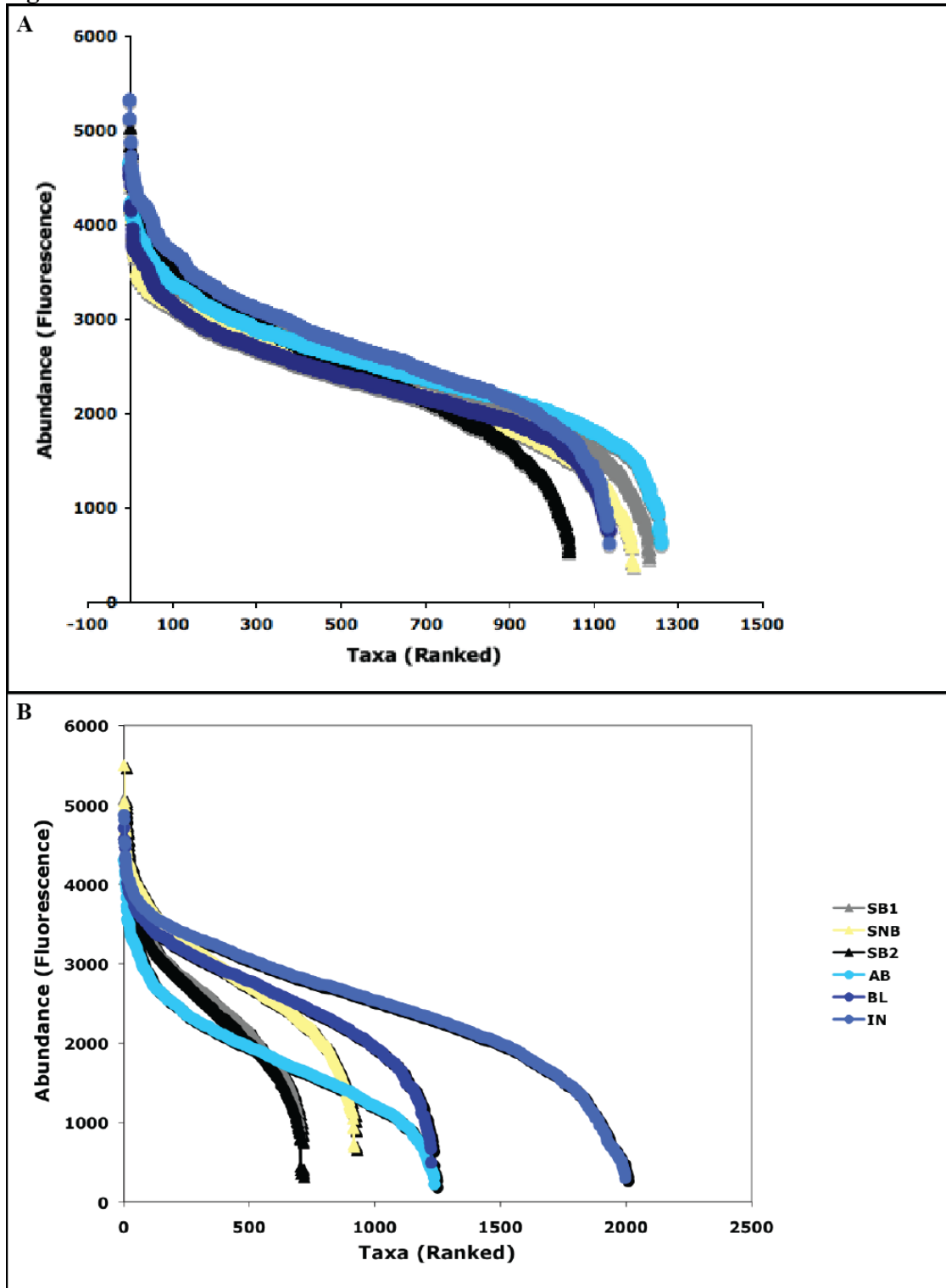**Figure 3.**

Figure 4.

Figure 5.

Figure 6.

Figure 7.

22

Legend:

- Acidobacteria
- Chloroflexi
- Firmicutes
- Natronoanaerobium
- NC10
- Nitrospira
- OS-K
- Planctomycetes
- Proteobacteria
- Spirochetes
- Actinobacteria
- Bacteriodetes
- Cyanobacteria

- Alphaproteobacteria
- Betaproteobacteria
- Deltaproteobacteria
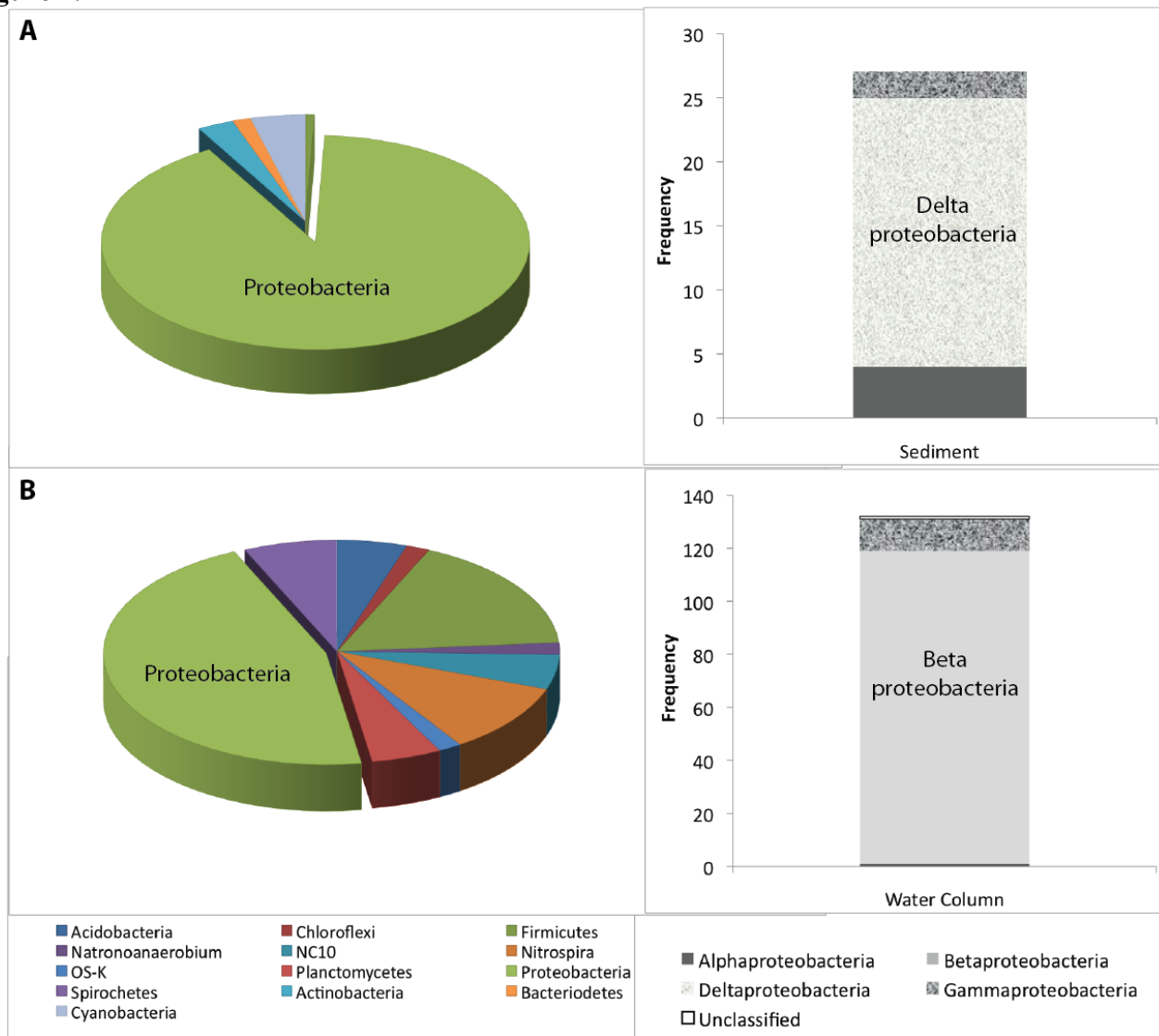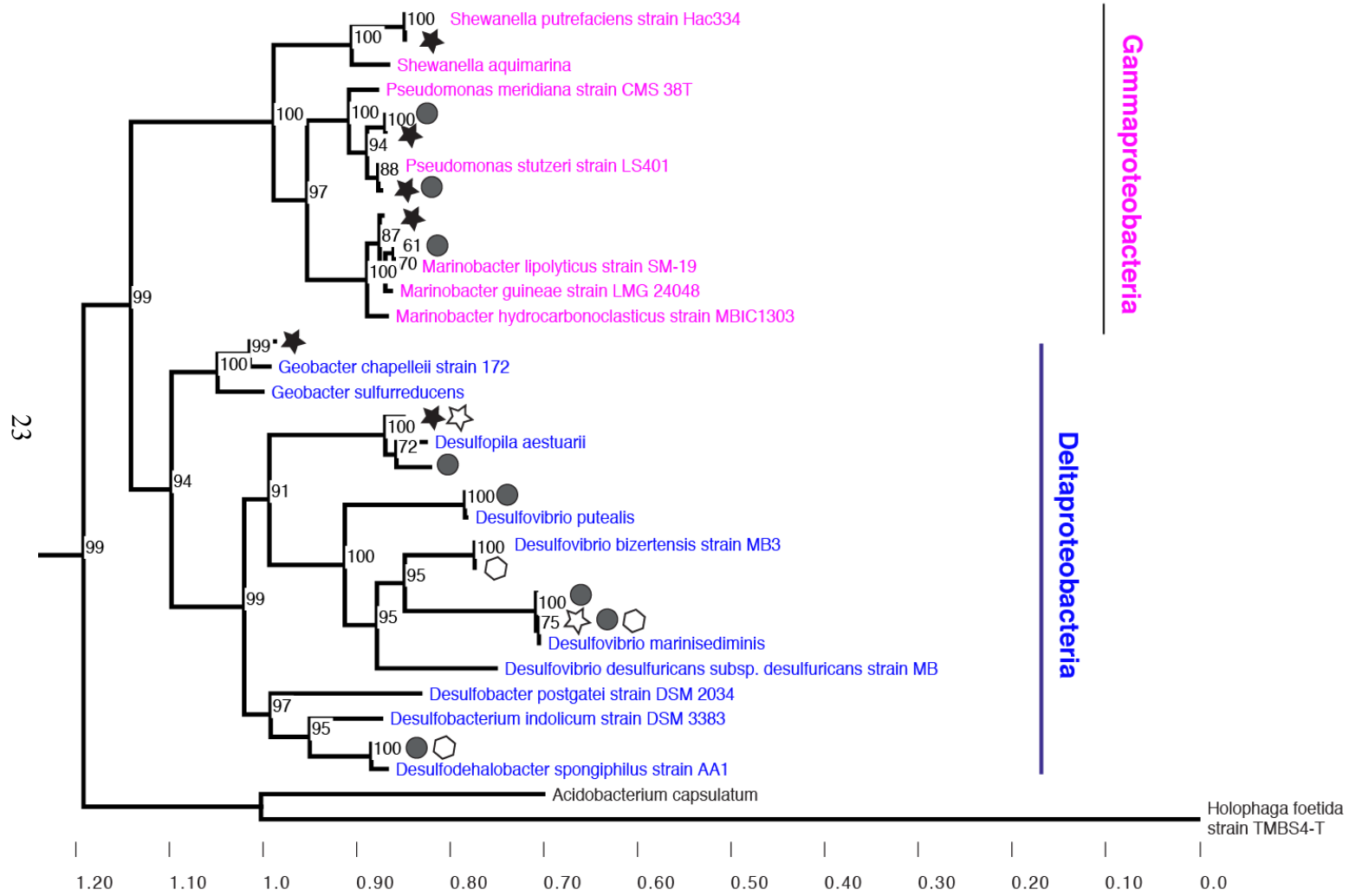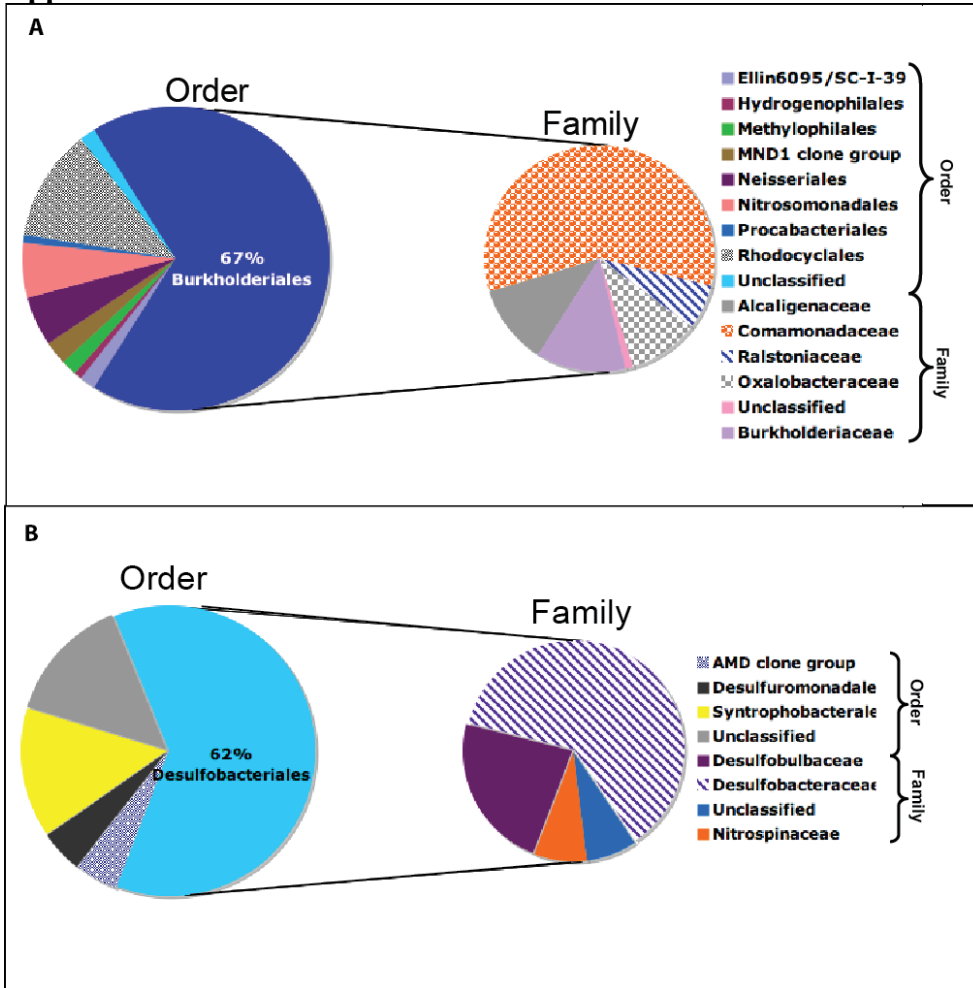- Gammaproteobacteria
- Unclassified
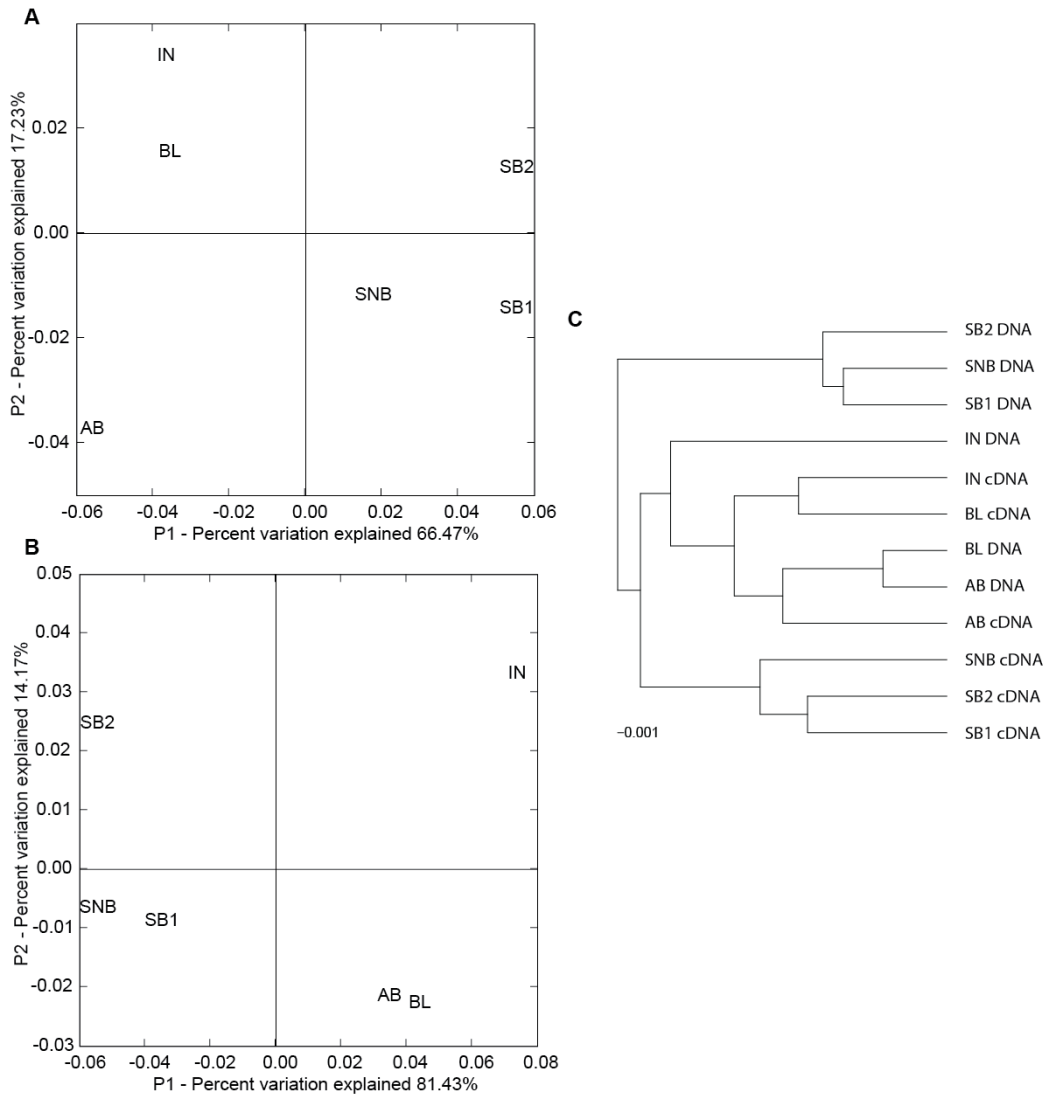
Figure 8.

Appendix

Appendix 1.

Appendix 1. Enriched persistent taxa in water column versus sediment samples (A) Betaproteobacteria more Abundant in Persistent Water Column Community (B) Deltaproteobacteria More Abundant in Persistent Sediment Community.

CHAPTER 2

An unusual symbiosis in discovered in anchialine shrimp, *Typhlatya pearsei*

Abstract

Mutualisms with chemosynthetic microbes are surprisingly rare in arthropods and their relatives. Despite the evolutionary benefit assumed in retaining symbionts through growth cycles, this molting group also lacks intracellular mutualisms. We report our findings of the first chemosynthetic intracellular symbiosis in the Arthropoda, discovered in extreme anchialine caves. These unusual groundwater systems enclose a landlocked marine layer, are typified by sharp salinity and oxygen gradients and are analogous to better studied extreme environments (e.g., hydrothermal vents, methane seeps). Our investigations via electron microscopy, stable isotope analysis and an enrichment experiment reveal that shrimp harbor endosymbiotic mutualistic bacteria. As intracellular mutualisms require more morphological and genetic adaptations than their extracellular counterparts, our findings shed light on the selective pressures towards endosymbiosis in extreme ecosystems. Intracellular symbiosis may serve as an innovation for maintenance of mutualistic bacteria through molt cycles in systems that are sparsely populated with low concentrations of chemosynthetic substrates.

Introduction

While arthropods and their molting relatives (Ecdysozoa) comprise over 1.2 million species (Chapman, 2009) only 21 genera host chemosynthetic symbioses, the majority of which are extracellular (ectosymbiosis). Only three known Ecdysozoan genera host intracellular (endosymbiosis) chemosynthetic symbionts Nematode genera (Ott et al., 1982; Miljutin et al., 2006). In contrast, in the much less taxonomically diverse (<130,000 species) Lophotrochozoa (i.e., brachiopods, annelids, mollusks, etc.) (Chapman, 2009), more than 60 genera are known to host chemosynthetic symbionts and the majority (> 45) of these associations are intracellular (endosymbiosis). This phylogenetic pattern is not habitat driven: endosymbiotic lophotrochozoa and ectosymbiotic Arthropoda co-occur in a diversity of habitats, including intertidal, hydrothermal vent, methane seep, sulfur spring cave, whale and wood fall ecosystems (Cavanaugh et al., 2006). The rarity of ectosymbiosis in molting organisms, such as arthropods, is expected as the microbes are thought to be lost with every molt cycle and must be maintained through replacement from a microbe population in close proximity (e.g., discarded molts) (Gebruk et al., 2000). Given the nutritional benefits of symbiosis, it is therefore surprising that intracellular modified cells (bacteriocytes) to host endosymbionts are nearly absent in ecdysozoans as this morphological adaptation would eliminate the need to repopulate symbionts after molts.

Symbioses (Joy, 2013), and particularly the association of eukaryotes with microbes capable of harnessing energy from chemical gradients in dark, extreme environments have resulted in the diversification of symbiotic partners [Macalady et al,

2008; Dattagupta et al., 2009; Wernegreen , 2012). These pairs live at steep chemoclines (sharp chemical gradients) between the high-energy substrates necessary for chemosynthetic metabolism (e.g. methane or sulfides) present in anoxic environments and the oxygen used for oxidation of these compounds (Gebruk et al., 2000; Polz et al., 2000; Dubilier et al., 2008). These chemoclines, found in hydrothermal vents, methane seeps, and sulfidic groundwater systems are also typical in anchialine caves. Here, water exchange with the landlocked marine habitat is severely restricted, creating stable gradients between anoxic and sulfidic marine layers and overlying brackish layers (Iliffe and Kornicker, 2009; Cangenella et al., 2007; Seymour et al., 2007) (Figure 1D). Toxic to many organisms, these caves are populated by a low-density, endemic crustacean fauna (Neiber et al., 2011). The increase in methane at marine-brackish interfaces and in sulfides at marine-sediment interfaces (Pohlman et al., 1997), make these habitats ideal for chemosynthetic mutualisms (Figure 1A-B).

Our investigation of anchialine cave organisms has revealed the first occurrence of chemosynthetic, intracellular endosymbionts in an arthropod host - the shrimp *Typhlatya pearsei* (Atyidae; Crustacea). Electron microscopic examination of *T. pearsei* showed evidence of: 1) chemosynthetic microbial symbioses, 2) host-mediated adaptations to their symbionts, and 3) host-mediated adaptations to sulfide toxicity. In addition, carbon stable isotope values of these crustaceans are similar to those of invertebrates known to harbor chemosynthetic symbionts. To illustrate that there is likely transfer of dissolved inorganic carbon from the water column to the host-symbiont system, we also report the results of stable isotope enrichment experiments. Our findings provide the foundation for understanding the ecosystem function of these unique environments and implications for understanding evolution of endosymbioses in molting organisms.

Material and Methods

Collection
Typhlatya pearsei were collected from Cenote Crustacea, in Quintana Roo, Mexico, both for microscopic investigation of symbionts location or freezing for stable isotope analysis. All specimens were collected on SCUBA and kept alive until dissection or freezing.

Scanning and Transmission electron microscopy
Most subsamples for both TEM and SEM were immediately fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate (pH7.2). Some subsamples for SEM were fixed in 5% formalin in methanol. All samples were then stored at 4°C until post fixation at UC Berkeley Electron Microscopy Laboratory. Antennule, antennae, and eye complexes, maxillipeds and mandibles, legs, and carapace were dissected from *T. pearsei* after 1-6 months in initial fixative. The tissues were then rinsed in the 0.1 M sodium phosphate buffer and postfixed for 1 h in 1% osmium tetroxide in a 0.1 M sodium phosphate buffer.
    After fixation, samples were rinsed several times with this buffer and then distilled water. Tissues were then incubated in the dark at 4°C in 0.5% aqueous Uranyl Acetate for one hour (as in Kenicutt et al., 1992). Following rinsing with distilled water,

samples were dehydrated in an ethanol gradient (as in Kenicutt et al., 1992). Samples for SEM were critical point dried before viewing on a Hitachi TM-1000 Scanning Electron Microscope. Ultra thin (70nm) and semi-thin (100μm) sections of TEM samples embedded in Epon resin (Eponate 12, Ted Pella, Inc.) were prepared on a Reichert Jung-Ultracut E microtome with a Diatome ultracut 45° knife. After staining with uranyl acetate and lead citrate, sections were viewed using JEOL JEM-1200ex and FEI Tecnai 12 Transmission electron microscopes.

Stable Isotope Enrichment

Ten *T. pearsei* individuals were collected from the meteoric layer of Cenote Crustacea to determine whether inorganic carbon uptake occurs in shrimp-microbe pairs . These individuals were separated into closed 50ml BD falcon tubes filled with 25ml of meteoric cave water pre-sterilized by vacuum pump filtration using Millipore GS filters (0.22 μm pore size). Six mg of NaH^{13}CO3, an enriched inorganic carbon source, was added to the water of five individuals prior to incubation for 24 hours in the dark at ambient temperature. After incubation, specimens were stored frozen for transport to UC Berkeley, where scaphognathites of shrimp were dissected and prepared for stable isotope analysis. Samples were then analyzed for carbon stable isotope analysis at UC Berkeley.

Results

Location of symbionts

Filamentous bacteria and polysaccharide excretions were found on the exterior surfaces of *T. pearsei* (Figures 2A,E–G). In addition, the lamellar surfaces of *T. pearsei* gills (Figure 2A) are covered with nodules (Figures 2B-C), which are packed with dividing rod shaped bacteria (Figure 2D). Such nodules were also found with SEM at the base of appendages, such as the maxilliped mouthparts (Figure 2*f*). Actively growing bacterial colonies were also seen on the underside of exoskeleton (Figure 2E). Frequently, plumose setae, found on shrimp, were covered with polysacharide excretions and a hardened matrix typical of biofilms (Figure 2E). TEM revealed endosymbiotic bacteria in numerous *T. pearsei* tissues (Figure 2H-J). These rod-shaped bacteria are housed in densely colonized nucleated bacterioctyes located in the gills and maxillipeds (Figure 2H-J). Bacteriocytes were also found near the thinner cuticle of maxillipeds as well as adjacent to carapace exoskeleton (Figure 2H,I).

T. pearsei gill lacuna, which contain oxygen transporting hemolymph, are often surrounded by mineral deposits and cocci bacteria (see Appendix 1). In addition, electron dense deposits as well as numerous mitochondria packed with electron dense granules always surround bacteriocytes (see Appendix 1). These electron dense are also housed in mitochondrial cristae and resemble minerals (see Appendix 1). They may be sulfide deposits Cells containing mitochondrial aggregations generally had electron dense deposits outside of their cell membrane, as well (see Appendix 1).

Stable isotope analysis and enrichment experiments

Mean natural stable isotope values for *T. pearsei* were ∂C -37.2 (±SD 0.5), ∂N 2.1 (±SD 0.6), and ∂S -9.5 (±SD 0.9) (N = 54 with individuals ranging from 3-20mm; Figure 3a).

T. pearsei gills had a similar value of -37.1 (±SD 3.0; N=5) with individuals ranging from 4.5-8.5mm; Figure 3a).

$\partial13C$ values of shrimp gills from *T. pearsei* and bacterial pairs (-10.62±8.38) kept in $\partial13C$ enriched meteoric cave water after 24-hour dark incubations were more enriched in $\partial13C$ than values of those pairs kept in control conditions (-37.12±1.50) (Figure 3B). This significant difference in means (p<0.05) suggests inorganic carbon uptake by shrimp and microbe pairs.

Phylogenetic distribution of chemosynthetic symbiont hosts

Chemosynthetic symbioses are more common in the Lophotrochozoa (i.e., mollusks and annelids) than in the Ecdysozoa, a clade encompassing crustaceans and hexapods (Figure 4). In addition, while most lophotrochozoan chemosynthetic symbionts are intracellular, endosymbiosis is rare in the Ecdysozoa and *T. pearsei* is the first reported arthropod harboring a chemosynthetic endosymbiont (Figure 4).

Discussion

Our report provides evidence that while mutualism facilitates the colonization and occupation of extreme habitats in a diversity of invertebrate taxa, symbiont presence and type is unevenly distributed across host phylogeny. Dark, geologically isolated, oxygen-poor habitats, such as caves and the deep sea, were long thought to be devoid of life due to low nutrient influx (Barton and Northup, 2007). Yet the biogenically and geochemically produced sulfides and methane often associated with these systems (i.e., deep-sea vents and seeps) that are toxic to eukaryotic aerobic respiration also provide ecological opportunities for the diversification of eukaryotes that form chemosynthetic mutualisms with bacteria (Macalady et al., 2008). The ecosystem and life history of anchialine shrimp has resulted in the evolution of not only chemosynthetic ectosymbionts, but also endosymbionts- not previously known to occur in the Arthropoda (Figure 4). The evolution of such mutualisms in anchialine shrimp shed light on conditions resulting in selection of intracellular symbioses in extreme systems.

Anchialine crustacean symbionts

The location and morphology of the cave shrimp endosymbionts show greater similarity to chemosynthetic annelid and mollusk (Lophotrochozoa) symbionts than to previously observed crustacean chemosynthetic symbionts. As in other endosymbiotic taxa (e.g., the bivalve mollusks *Bathymodiolus* spp.) (Dubilier et al., 2008), *T. pearsei* harbors two morphotypes of symbionts, which are segregated in different parts of the shrimp tissue. Crustacean ectosymbiotic symbionts are typically reported to be filamentous long, rod-shaped bacteria of 10μm of greater (Cavanaugh et al., 2006; Dattagupta et al., 2009), and are morphologically distinct from the cocci and rod shape bacteria reported here. While morphological plasticity of microbes prevents definitive phylogenetic assignment, the overall similarity of the shrimp endosymbionts with those previously reported in Lophotrochozoa (Dubilier et al., 2008) is striking and may indicate a shared bacterial lineage or convergence.

We found mitochondria and bacterial aggregations near lacuna and electron dense granules suggesting host or symbiont-mediated positioning at metabolically optimal

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gradients. Lacuna contain hemolymph that carries the oxygen necessary for both host and sulfide-oxidizing symbiont metabolisms. Electron dense granules, which were not identified by crystallographic methods, resembled minerals (i.e., sulfide mineralization) (Compère et al., 2002), and may serve as substrates for symbiont metabolism. This orientation has been described in other chemosynthetic endosymbiotic organisms (i.e., lophotrochozoan bivalves and annelids (Compère et al., 2002; Hourdez and Lallier, 2007; Tresguerres et al., 2013). In lophotrochozoans (i.e., *R. pachyptila*), sulfur-binding proteins may also serve to detoxifying body fluids of sulfides as well as transporting sulfides to chemosymbiont rich tissues (Hourdez and Lallier, 2007). In addition, sulfides may be directly used in mitochondria to facilitate the production of ATP in both invertebrates (Parrino et al., 2000) and vertebrates (Fu et al., 2012). The presence of these putative mineral accumulations and symbionts concentrated around mitochondria may indicate sulfide- detoxification or facilitated ATP production in anchialine shrimp. These granules were found localized at the edges of bacteriocytes and mitochondrial aggregations, further indicating host-mediated transport and concentration of chemosynthetic substrates.

Stable isotope analysis provides further evidence that anchialine shrimp host chemosynthetic symbionts. *Typhlatya* shrimp have ∂C values (-37.2 ± 3.8) that are depleted in ^{13}C relative to photosynthetically fixed carbon (-18 to -28) (Cavanaugh et al., 2006). These ∂C values are similar to vent and seep bivalve mollusks with sulfide oxidizing endosymbionts (-27 to -35) than to vent crustaceans with ectobionts or annelid worms with endosymbionts (both -9 to -16) (Figure 3A). These values can also vary because of carbon selectivity by fixation enzymes or different sources of available carbon (e.g., dissolved CO_2 versus methane). Based on these comparisons, it appears that *Typhlatya* symbionts are using Form II RubisCo for sulfide oxidation or are methanogens (Figure 3A). In contrast, vent and seep tube worms and vent rimicarid shrimp have bacterial symbionts that use Form I RubisCo thereby producing a more enriched $\partial 13C$ value. The enrichment experiments reported above also suggest that bacteria living in shrimp are capable of fixing carbon chemoautotrophically (Figure 3B).

The evolution of chemosynthetic endosymbiosis

Endosymbiosis is thought to be more derived than ectosymbiosis (Smith, 1979). This theory stems from the increased necessity for host-symbiont recognition and host specificity as well as reduction of bacterial symbiont genomes and morphological adaptation associated with intracellular associations (see Cavanaugh et al., 2006; Wernegreen, 2012). Selective pressures for the evolution of chemosynthetic endosymbiotic relationships may include: 1) a dependence on chemosynthetic microbes for nutrition, 2) low environmental concentrations of substrates (e.g., sulfide) available for symbiont chemosynthetic metabolisms and 3) the absence of a mode to repopulate symbionts that are susceptible to cyclic loss (e.g., molting of exoskeletons) (Gebruk et al., 2000).

Like hydrothermal vent alvinocarid shrimp (Gebruk et al., 2000), *Typhlatya* likely gain most of their nutrition from their chemosymbioses as adults but may also scrape microbial biofilms from surfaces (De Grave et al., 2008; pers. observ. MJP). Both groups shed their epibionts at each molt stage. However, there are several differences between these systems. At hydrothermal vents, both microbes and hosts (shrimp) occur at much

higher densities than in caves. For example, *Rimicaris exoculata* grazes in aggregations of up to 3000 individuals m^{-2} on the dense bacterial mats that cover sulfide black smoker chimneys (Gebruk et al., 2000). In contrast, meager microbial growth in most anchialine caves (Gonzalez et al., 2011; pers. observ. MJP) suggest a reduced environmental food source alternative for shrimp. Furthermore, densities of cave crustacean fauna in are low (Pohlman et al., 2000) and shrimp residing in upper water layers of the cave lose access to sinking exoskeletons. Without localized shedding of symbiont covered exoskeletons, the chances of epibiont reinnoculation facilitated by host swarms are reduced.

Lastly, there is lower chemosynthetic substrate availability for chemosynthetic bacteria in anchialine environments than analogous chemosymbiotic systems. In contrast to the high sulfide levels present in hydrothermal vents (i.e., 4-8 mmol H$_2$S in vent fluid) (Hourdez and Lallier, 2007) or sulfidic groundwater systems (i.e., up to 1.2mmol in Frasassi cave water) (Macalady et al., 2008) where we find epibiotic mutualisms, anchialine caves with crustacean fauna lack the mineral rich groundwater. Sulfides in these systems are produced geomicrobially (Socki et al., 2002) and occur at low levels (i.e. <2mmol H$_2$S L^{-1}) (Pohlman et al., 2000) Likewise, methane at a nearby anchialine cave was measured at less than 200nM (Pohlman et al., 1997). Low environmental sulfide and methane abundance may favor the establishment of endosymbiosis in order to allow for the concentration of this substrate internally in host tissues packed with symbionts. Our findings of chemosynthetic symbioses in anchialine systems (less densely populated than vents) present the opportunity to further examine evolutionary hypotheses surrounding the drivers selecting for endosymbioses in chemosynthetic systems.

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Figure Captions

Figure 1. A representative limestone anchialine cave with endemic crustacean inhabitants. (A) Photo of dark anchialine cave, Cenote Crustacea, with two divers and stalactites hanging from ceiling. (B) A *Typhlatya* shrimp at arrow near the ceiling and above the halocline of Cenote Crustacea. (C) Physio-chemical profile of a section of Cenote Crustacea showing salinity (red), temperature (blue), and dissolved oxygen (green) changes with depth. Note the marked change in all three at the halocline and the decrease in dissolved oxygen and temperature near the sediment. Photographs by T. Thomsen.

Figure 2. Location of symbionts of shrimp *Typhlatya pearsei* from Cenote Crustacea, an anchialine cave in the Yucatan peninsula. (A) *T. pearsei* with locations of carapace (c), gill (g) and maxillipeds (m) noted. Scanning Electron Micrographs (SEM) of (B) lammelar surface of *T. pearsei* gills, (C) nodules on gill surface that likely contain bacteria, and (D) dividing rod shaped bacteria (db) inside of gill tissue. (E) Rod shaped bacteria can be seen on the exterior of the carapace via SEM. (F) SEMs of the base of the maxillipeds, such as the 1st maxilliped reveal many nodules likely filled with bacteria. (G) The setae of these endopods, such as the plumose setae of the 2nd maxilliped shown here in SEM contains communities of cocci with what appears to be polysacharide excretions and a hardened matrix typical of biofilms. (H-J) Bacteria are also located intracellularly in bacteriocytes in the gills and maxillipeds, such as near the cuticle (cu) of the second maxilliped. (I) Nucleus (nu) of bacteriocyte shown. (J) illustrates bacteria in bacteriocytes. Single arrows indicate mineral deposits lining the exterior membrane of bacteriocytes or membranes, double arrows illustrate inner and outer membranes of bacteria. Scale bars: A= 5mm; J= 100nm; I= 0.5 μm; B= 100μm; C, F, G= 30μm; H = 5μm; 10μm= D, E.

Figure 3. Carbon Stable Isotopes indicating chemosynthetic activity of Shrimp symbionts. (A) Naturally occurring carbon stable isotope values of anchialine and deep-sea specimens. *T. pearsei* shrimp ∂13C values are more similar to vent and seep bivalve values than to that of environmental samples from anchialine caves (filtered water from above in and below the halocline or microbial mat) or to crustaceans and annelids from deep-sea chemosynthetic communities. Dotted Line separates anchialine samples collected in this study (upper) from published vent and seep studies (lower). Individual ∂13C values are shown where available (back circles). Averages (open circles) and standard deviations are also shown. Subscript key is as follows. Numbers indicate sample size, letters indicate habitat for non-cave samples (V = vent, S = seep), and symbols indicate references for data acquired from other studies: *=data from Gebruk et al., (2000), †=data from Rau (1981), and ††= data from Kennicutt et al., (1992). (B) Enrichment of ∂13C of *Typhlatya pearsei* -symbiont pairs from Cenote Crustacea, Quintana Roo, MX. Uptake of ∂13C labeled inorganic carbon in filtered cave water by symbiont and crustacean pairs after 24-hour dark incubations, indicating the presence of chemosynthetic activity in *T. pearsei* host-symbiont pairs (n=5).

Figure 4. Phylogeny of Bilateria with chemo-endosymbiotic and ectosymbiotic genera mapped onto it. Numbers on branches at left are genera with chemoendosymbiotic associations. Those at right are chemosynthetic ectobiotic associations. Number in red with asterix indicates new endosymbiotic association reported by this study. Tree adapted from Hejnol et al., (2009). References for symbiotic taxa as follows: Ott et al., 1982; Polz et al., 2000; Macalady et al., 2008; Dattagupta et al., 2009. Also see Appendix 2 for references supporting this figure.
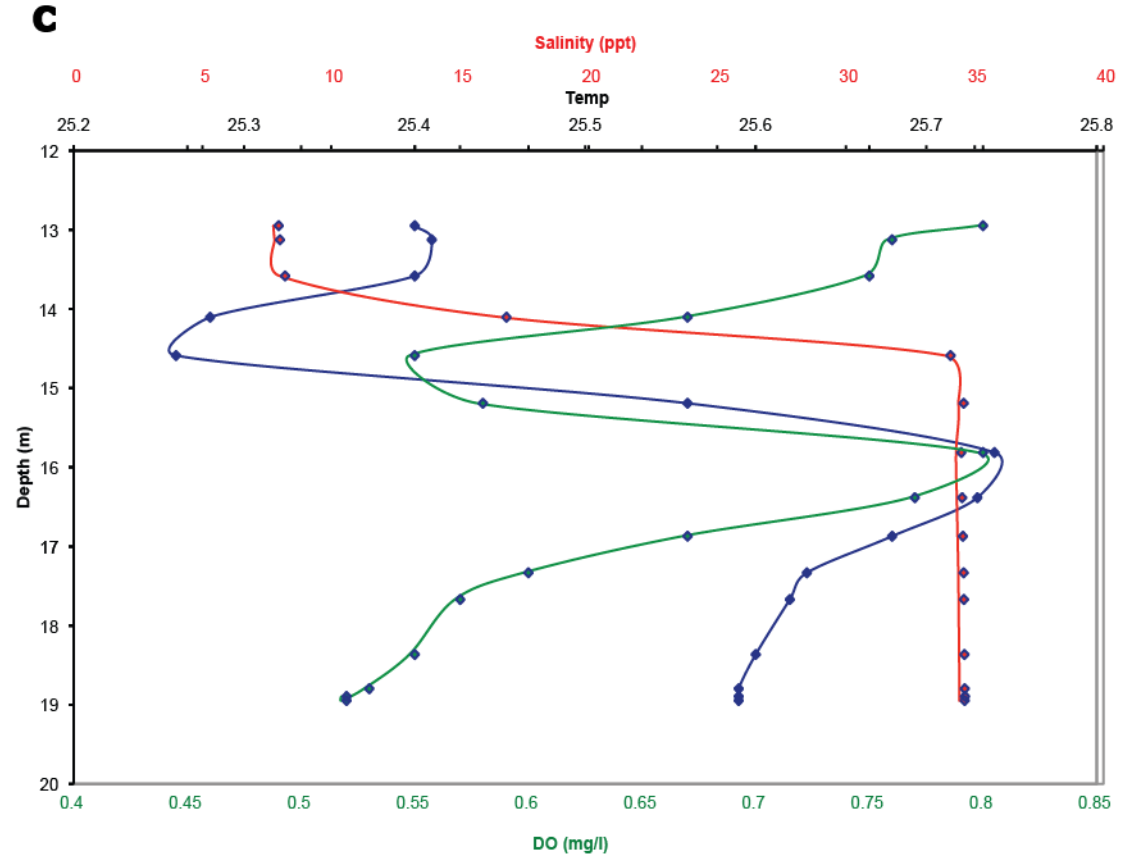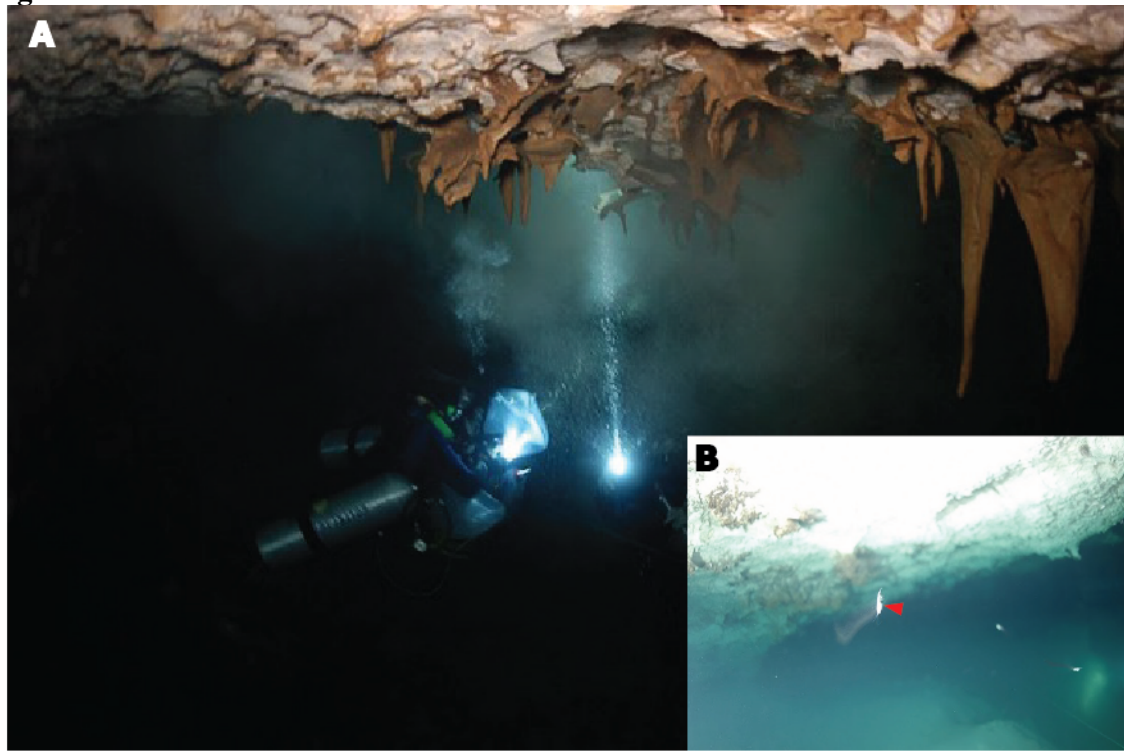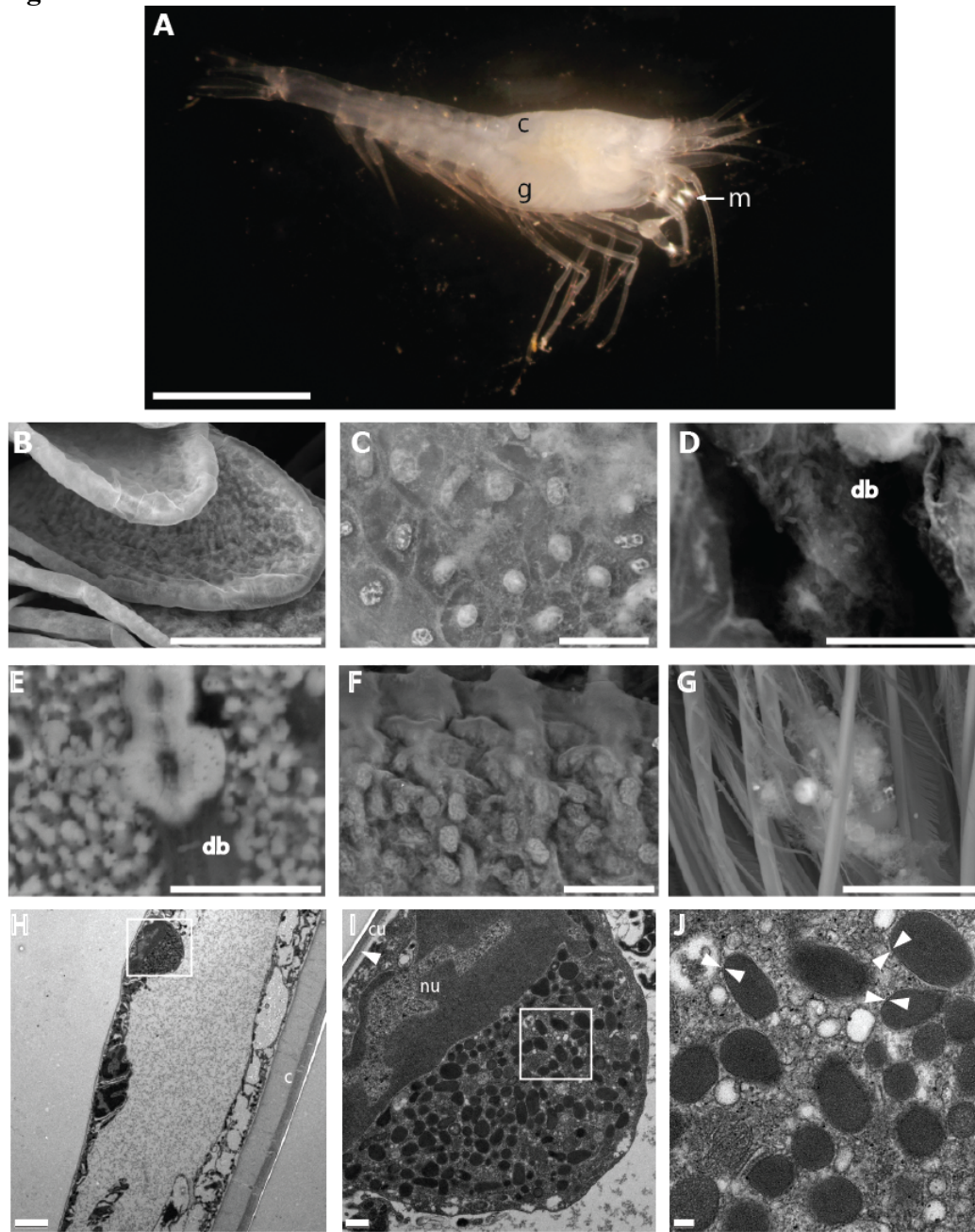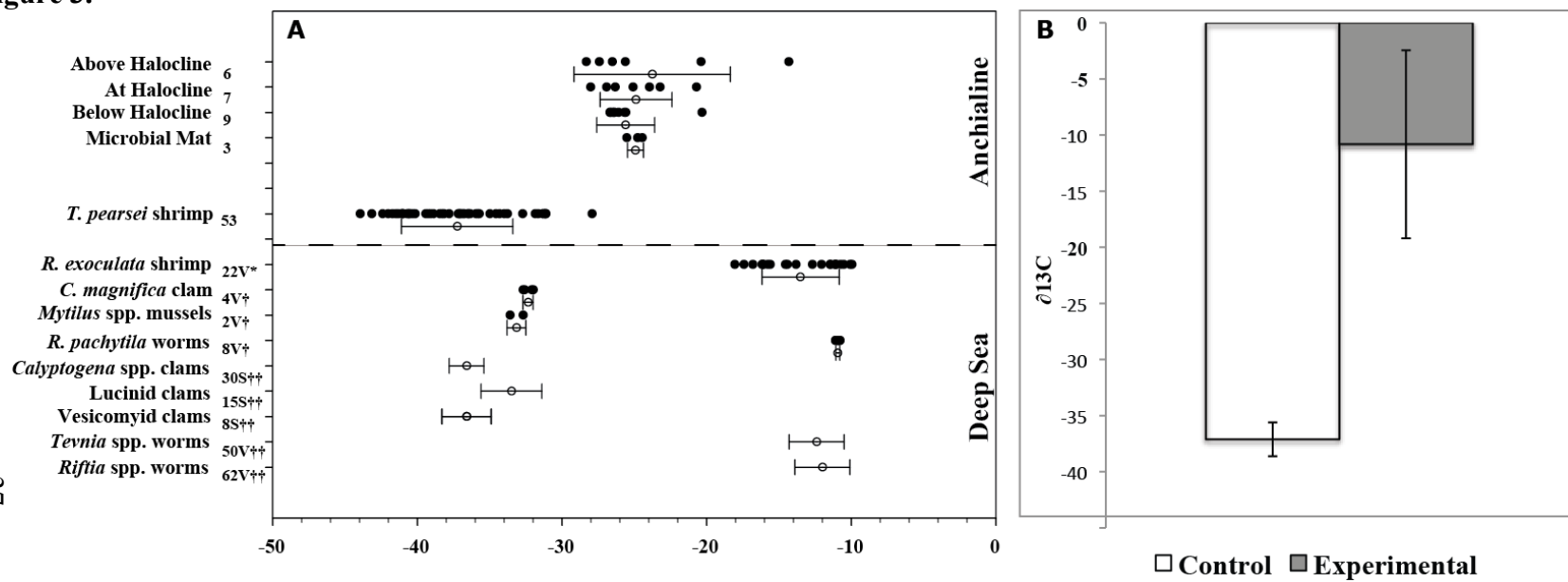
Figures

Figure 1.

Figure 2.

Figure 3.

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Figure 4.
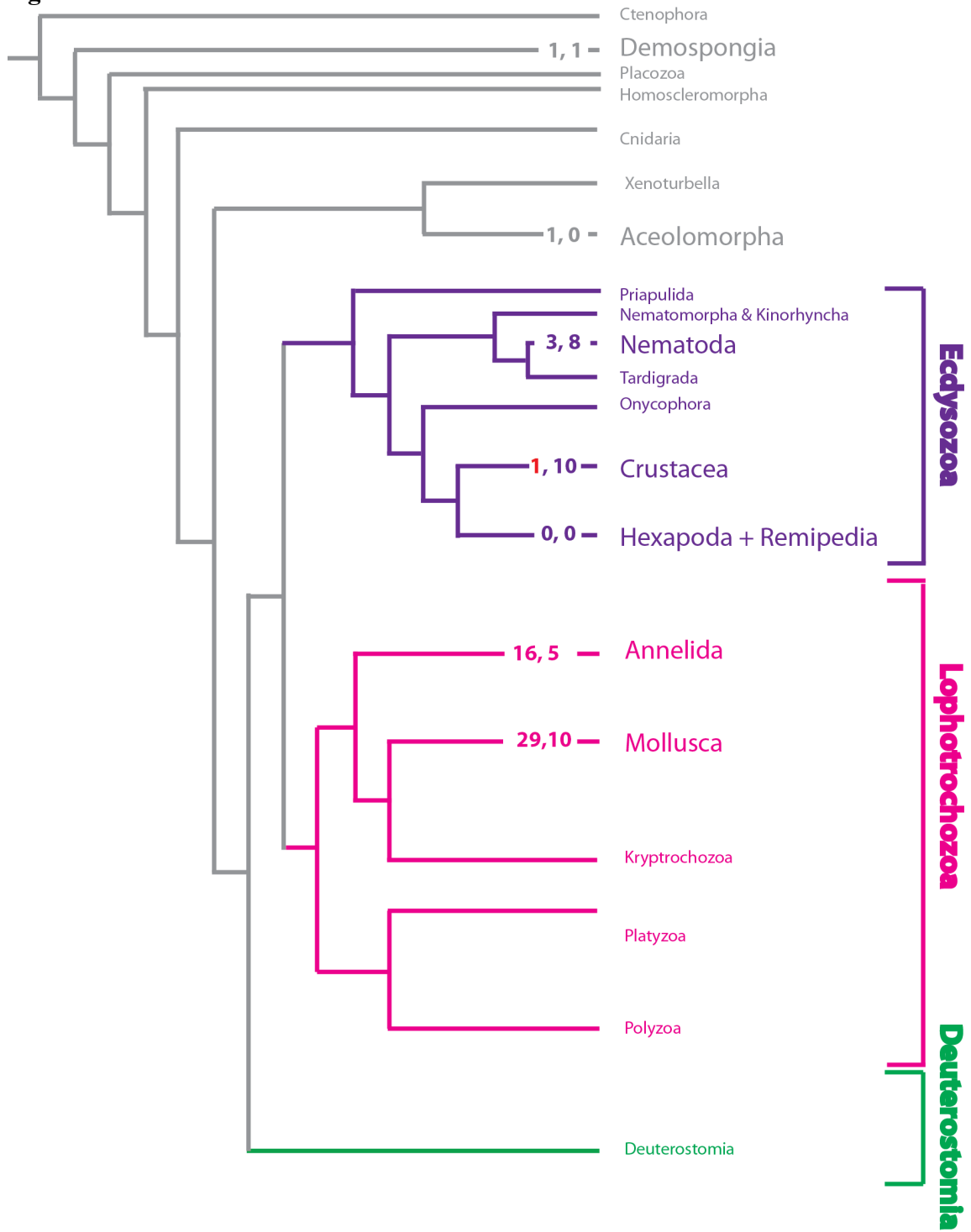
APPENDICES

Appendix 1.
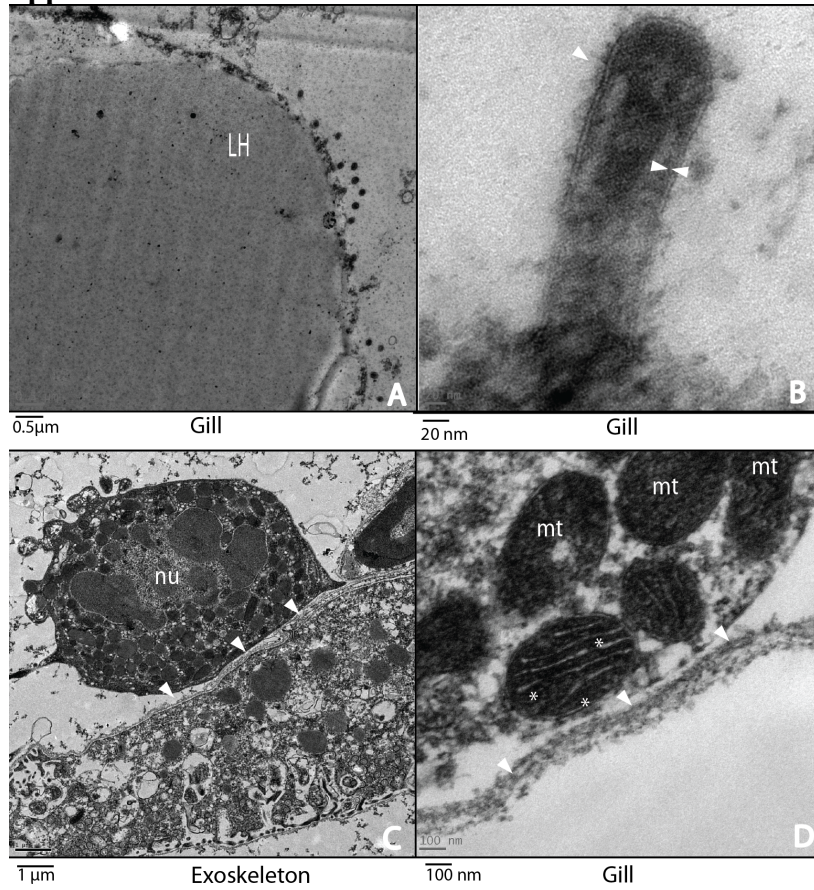
Appendix 1. Bacteria-rich *Typhlatya pearsei* tissues are located in close proximity of oxygen-containing hemolymph and electron dense particles resembling sulfides. (A) Cocci bacteria line the exterior edge of gill lacuna containing hemolymph (LH), which transports oxygen. The arrow indicates mineral deposits in close proximity to the bacteria. Both these cocci bacteria and rod-shaped bacteria, shown in (B) have clearly visible inner and outer bacterial membranes, indicated by double arrows and are also surrounded by electron dense deposits, resembling minerals, such as iron sulfides. (C) These electron dense deposits also lined intracellular bacteriocytes, packed with rod-shaped bacteria, such as the one shown here on the interior of the carapace on the exoskeleton. (D) High densities of mitochondria were often found in gill tissues, which were also lined with mineral deposits, resembling sulfides. These mitochondria were frequently filled with electron dense granules that may also be sulfides. Single arrows indicate mineral deposits lining the exterior membrane of bacteriocytes or membranes, double arrows illustrate inner and outer membranes of bacteria, mitochondria are indicated by (mt), asterices (*) show electron dense granules in mitochondria, and nuclei are indicated by (nu). Scale bars B= 20nm; D= 100nm; A =0.5µm; C = 1µm.

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Appendix 2.
The following references listed in Appendix 2, along with 2, 8-10 are cited only in Figure 4:

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CHAPTER 3

Chemosynthetic ectosymbiosis in Speleonectes tulumensis (Remipedia)

Abstract

Mutualisms between chemosynthetic microbes and invertebrates form the basis of foodwebs in dark, extreme habitats (i.e. hydrothermal vents) and have likely facilitated the invasion of underwater caves, as well. Dark, anchialine caves often include distinct water layers of varying concentrations of dissolved oxygen and sulfide and provide an ideal system for the discovery of chemosynthetically based systems and novel symbioses. These caves may be harsh environments for eukaryotes, but they contain gradients favorable for chemosynthetic symbiotic microbes. Here, we present chemosynthetic ectosymbiosis in the class Remipedia (Crustacea; Arthropoda) through electron microscopy and stable isotope analysis. Remipedes are considered to be predators through anecdotal observations of feeding in the lab and field and description of venomous apparati, but may supplement their diet with microbes. This finding sheds light on opportunistic feeding behaviors that may have evolved to combat resource -limited environments, such as underwater caves.

Introduction

Anchialine caves, dominated by crustacean fauna, are ripe for the discovery of chemosynthetic communities and novel types of mutualisms between microbes and their hosts. In these dark systems, the marine habitat is landlocked. As a consequence of severely restricted water access in these tidally influenced systems, they often exhibit stable physico-chemical gradients, such as brackish water layers overlying the marine habitat (Seymour et al., 2007). Distinct water layers also often include anoxic and highly sulfidic marine waters and redox zones that may support chemosynthetic bacteria (e.g., Canganella et al., 2007; Socki et al., 2002, Gonzalez et al., 2011, Chapter 1) (Figure 1). A diversity of eukaryotes benefit from chemosynthetic microbes that thrive and harness energy from the chemical gradients in these systems. These hosts generally live at steep chemoclines (sharp chemical gradients) between high-energy metabolites necessary for chemosynthetic metabolism, such as the methane or sulfides present in anoxic environments, and the oxygen used for oxidation of these compounds. Chemoclines are typical of the junction of anchialine water interfaces where anoxic marine water sulfates are biogenically reduced in anoxic sediments or at the marine- and oxygen-rich brackish water interface. Similar junctures occur at vent and seep fluids, reducing sediments, biogenic substrates (whale-wood fall material) and relatively oxygen-rich marine water. Many animals, including *Riftia* tube worms (Annelida) and *Rimicaris* spp. shrimp (Crustacea), that have endo or episymbiotic bacteria associate with these chemical gradients in order to supply

their symbiont community with the optimal compounds for chemosynthesis (Gebruck et al., 2000; Polz et al., 2000; Dubilier et al., 2008). Recently chemosynthetic symbionts have been found on a cave amphipod (Crustacea) from a sulfur fed groundwater system, which contains similar gradients (Dattagupta, 2011) as well as endosymbiotically in the anchialine endemic atyid shrimp, *Typhlatya pearsei* (see Chapter 2).

The unique anchialine cave habitat has led to the evolution of a crustacean fauna specific to its various water layers and generally found at low density (Neiber et al., 2011). Remipedia (Yager, 1981), a class of Crustacea are restricted to the marine layer of subtropical anchialine systems (Neiber et al., 2011) (Figure 1A). These hermaphroditic crustaceans likely predate or scavenge on atyid shrimp and blind cave fish (Carpenter, 1999; Koenemann et al., 2007) (Figure 1B). Some remipede setae appear to be enervated and glandular suggesting a function aiding with immobilizing and detoxifying prey (van der Hamm and Felgenhauer, 2008). Recently, several clades of these enigmatic crustaceans have been found to be venomous (von Reumont et al., 2013). Yet, remipedes have also been reported to carry balls of microbe-laden sediment in their mouthparts, presumably filtering out nutrients (Yager, 1981) and have been investigated for epibiotic microbes of uncertain taxonomy and metabolism (Yager, 1991).

Here we report chemosynthetic ectosymbiosis in the pancrustacean *Speleonectes tulumensis* (Remipedia) confirming that remipedes not only prey on other cave crustacean, but also on symbiotically associated chemosynthetic microbes. Electron microscopic examination of *S. tulumensis* remipedes showed evidence of chemosynthetic microbial symbioses. In addition, carbon stable isotope values of these crustaceans were similar to those of invertebrates known to harbor chemosynthetic symbionts. We also report the results of stable isotope enrichment experiments demonstrating that bacteria found on *S. tulumensis* carapaces are likely transferring dissolved inorganic carbon from the water column to the host-symbiont system. These results indicate that venomous remipedes are omnivores rather than predators.

Methods

Collection

Speleonectes tulumensis remipedes were collected from Cenote Crustacea, in Quintana Roo, Mexico, both for microscopical investigation of symbionts location or freezing for stable isotope analysis. Cenote Crustacea is known for its relatively high density of remipede crustaceans. All specimens were collected on SCUBA and kept alive until dissection or freezing.

Scanning and Transmission electron microscopy

Most subsamples for both TEM and SEM were immediately fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate (pH7.2). Some subsamples for SEM were fixed in 5% formalin in methanol. All samples were then stored at 4°C until post fixation at UC Berkeley Electron Microscopy Laboratory. Maxillipeds and mandibles were removed from *S. tulumensis* prior to further dissection into

subsamples of 6 or less segments to improve fixation. After 1-6 months in initial fixative, the tissues were then rinsed in 0.1 M sodium phosphate buffer and postfixed for 1 h in 1% osmium tetroxide in a 0.1 M sodium phosphate buffer.

After fixation, samples were rinsed several times with in 0.1 M sodium phosphate buffer then distilled water. Tissues were then incubated in the dark at 4°C in 0.5% aqueous Uranyl Acetate for one hour (as in Van der Hamm 2007). Following rinsing with distilled water, samples were dehydrated in an ethanol gradient (as in Van der Hamm 2007). Samples for SEM were critical point dried before viewing on a Hitachi TM-1000 Scanning Electron Microscope. Ultra thin (70nm) and semi-thin (100µm) sections of TEM samples embedded in Epon resin (Eponate 12, Ted Pella, Inc.) were prepared on a Reichert Jung-Ultracut E microtome with a Diatome ultracut 45° knife. After staining with uranyl acetate and lead citrate, sections were viewed using JEOL JEM-1200ex and FEI Tecnai 12 Transmission electron microscopes.

Stable Isotope Enrichment

Ten individuals each of *S. tulumensis* were collected from Cenote Crustacea to determine whether inorganic carbon uptake occurs in remipede-microbe pairs. These individuals were separated into closed 50ml BD falcon tubes filled with 25ml of marine layer cave water pre-sterilized by vacuum pump filtration using Millipore GS filters (0.22 µm pore size). Six mg of NaH^{13}CO3, an enriched inorganic carbon source, was added to the water of five individuals prior to incubation for 24 hours in the dark at ambient temperature. After incubation, specimens were stored frozen for transport to UC Berkeley and prepared for stable isotope analysis. Samples were then analyzed for carbon stable isotope analysis at UC Berkeley.

Results

Location of symbionts

Scanning electron microscopy (SEM) showed filamentous bacteria and polysaccharide excretions of these biofilms covered the exterior of *S. tulumensis* carapace and appendages, such as the antennae (Figure A-C). In some cases, ciliates also colonized the external surfaces of *S. tulumensis* (Figure 2D).

Stable isotope analysis

Mean natural stable isotope values for *Speleonectes tulumensis* were ∂C -34.4 (±SD 0.8), ∂N 6.59 (±SD 0.6), and ∂S -1.8 (±SD 1.1) (N = 17 with individuals ranging from 14-41mm).

∂13C values of *S. tulumensis* and microbe pairs (-29.41±0.54) kept in ∂13C enriched marine cave water after 24-hour dark incubations were more enriched in ∂13C than values of those pairs kept in control conditions (-37.41 ±2.02) (Figure 3). This significant difference in means ($p<0.001$) suggests inorganic carbon uptake by remipede and microbe pairs.

Discussion

Chemosynthetic ectosymbioses in Remipedia endemic to novel anchialine environments provide further evidence that mutualisms facilitate the colonization of extreme habitats. Dark, photosynthetically inactive, oxygen-poor habitats, such as underwater caves and the deep sea, were previously thought to be devoid of life due to low nutrient influx (Engel, 2013). The biogenically and geochemically produced sulfides often associated with these systems (i.e. deep-sea vents and seeps) have further lead these habitats to be labeled as "extreme." However, the presence of these compounds, which are toxic to eukaryotic aerobic respiration, also provides ecological opportunities for diversification of eukaryotes with chemosynthetic mutualisms.

Adaptations to sulfidic, anoxic systems: detoxification and symbiont metabolism

On a cellular level, anchialine crustacean lacuna morphology, mitochondrial arrangements, and mineral aggregations are similar to those of other organisms living in anoxic environments and hosting sulfide-oxidizing symbionts. Remipedes and cave shrimp contain electron dense granules surrounding and within lacuna, mitochondria and symbionts. These electron dense granules are likely sulfides, which may be used by sulfide-oxidizing symbionts, but are toxic to eukaryotic metabolism. The density of these minerals suggests that they are being locally concentrated after being pumped with incurrent water flow. Analogously, some lophotrochozoans (i.e. bivalves and annelids) concentrate sulfides for symbiont metabolism with sulfur-binding proteins in their body fluids (e.g., modified hemoglobins in *Riftia pachyptila*: Hourdez and Lallier, 2007). In other taxa these sulfide-binding proteins may serve dual purposes, detoxifying hemolymph or hemocoel of sulfides as well as transporting sulfides to chemosymbiont rich tissues (Zal et al., 1998). Compere et al., (2002) suggested that detoxification of sulfide-laden body fluid may also occur by packing organelles with harmful minerals for later export and describe *Riftia* tubeworm mitochondria that resembles those *Typhlatya* mitochondria presented here. Furthermore, both the polychaete annelid *Arenicola marina* and the bivalve mollusk *Geukensia demissa*, which lack sulfide binding proteins, likely use mitochondria to detoxify sulfides, oxidizing them while producing ATP (Hourdez and Lallier 2007 and ref therein). The analogous presence of mitochondria concentrated around electron dense granules (likely sulfide accumulations) within mitochondria of *Speleonectes tulumensis* may indicate sulfide oxidization in these taxa, as well.

Stable isotopes provide further evidence that remipedes host chemosynthetic and particularly sulfide oxidizing symbionts. Anchialine crustaceans have ∂C values that are depleted in 13C relative to photosynthetically fixed carbon (-18 to -28; Cavanaugh et al., 2006) and more similar to vent and seep bivalve mollusks with sulfide oxidizing endosymbionts (-27 to -35) than to vent crustaceans with ectobionts and annelid worms with endosymbionts (both -9 to -16) (Figure 5A). Carbon stable isotope values vary due to discrimination of carbon by fixation enzymes in addition to available sources of carbon (i.e.

dissolved CO2). It is therefore likely that Speleonectes symbionts are using Form II Rubisco or fixing methanogens, as their carbon stable isotope values are similar to symbiotic bivalves known to fix carbon using Form II Rubisco. In contrast, vent and seep tube worms and Rimicarid shrimp have bacterial symbionts which use From I Rubisco, resulting in a more enriched $\partial 13C$ value. Further evidence that these cave organisms may be fixing carbon chemoautotrophically (i.e. using chemosynthetic pathways and an inorganic carbon source such as dissolved carbonates) stems from enrichment experiments in which enriched carbon was supplied to host and symbiont in an inorganic form.

Why do remipedes host ectosymbiotic microbes? Based on isotope analysis (Pohlman, 1997) and behavioral observations (Carpenter, 1999; Koenemann et al., 2007), remipedes were previously thought to be top predators in their environment, scavenging and predating on *Typhlatya* spp. and other crustaceans. These data point to a combined diet of predation and symbiont grazing for remipedes. Similarly, hydrothermal vent alvinocarid shrimp gain most of their nutrition from their chemosymbioses as adults (Polz 2000), but shed their epibionts at each molt stage, lack the adaptation of endosymbioses. The evolution of behaviors to increase re-introduction of epibiotic bacteria are favored in ecydysozoan groups. For example, *R. exoculata* graze in aggregations of up to 3000 individuals m^{-2} on the bacterial mats that cover sulfide black smoker chimneys (Gebruk 2000). This high population density supported by localized bacterial mat growth likely results in localized molting. It is probable, therefore, that the re-inoculation of mutualistic ectobionts occurs by feeding on shed exoskeletons and that this behavior along with nutritional supplementation via grazing directly on mats has maintained the ectobiont state in alvinocarids at vents. Likewise, remipedes are less dependent on mutualistic nutrition, feeding on shrimp and other smaller crustaceans (Koenemann, 2007), and may therefore have less selective pressure towards evolution of endosymbiosis. This decreased dependence on mutualism may be further seen in a less dense microbial population on *S. tulumensis* than that seen on other chemosynthetic epibiotic taxa (i.e. Cave amphipods; Dattagupta 2009).

The finding of the chemosynthetic epibiosis in the class Remipedia provides new opportunities for comparative coevolutionary studies of these taxa with those from other, more productive chemosynthetic habitats as sister taxa likely also harbor chemosynthetic symbionts. Diffuse coevolution between symbionts and their hosts may be evidenced by patterns of cospeciation and patterns of divergence that have been driven by reciprocal evolutionary pressures between these groups (Janzen, 1980). In addition, future investigations into these symbioses may provide insight into whether trophic dependence and morphological adaptation drive coevolution in chemosynthetic mutualisms. It is likely that as we compare convergent adaptations described here to those found in deepwater and mollusk or annelid taxa, we will answer many questions about the evolution of chemosynthetic communities.

Acknowledgements

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Figure Captions

Figure 1. Known remipede Distribution and Diet. (A) Remipedes, endemic to anchialine systems have a subtropical global distribution. Adapted from Moritsch, Pakes, and Lindberg (accepted). (B) Image from video of the remipede S. tulumensis preying on a *Typhlatya* shrimp in Cenote Crustacea. Video taken by T. Thomsen. Image capture from video by D. Glenn.

Figure 2. Microfauna found in association with remipede Speleonectes tulumensis from Cenote Crustacea, an anchialine cave in the Yucatan peninsula. (A) S. tulumensis with maxillipeds (m), setae covered swimming legs (s) and antennae (a) labeled. Scanning Electron Micrographs (SEM) reveal rod shaped bacteria with what appears to be polysacharide excretions and a hardened matrix typical of biofilms on exterior of *S. tulumensis*. (B) Filamentous, cocci and rod shaped bacteria grow on exterior of remipedes, as well, such as on the antennae. (C) A close up of Interior of this bacterial "community" on antennae. (D) Strings of ciliates at base of antenae setae also colonize these remipedes.
Scale bars A= 10mm; B=300μm; D=20μm; C=10μm.

Figure 3. Enrichment of ∂13C of host-symbiont pairs from Cenote Crustacea, Quintana Roo, MX. Uptake of ∂13C labeled inorganic carbon in filtered cave water by symbiont and crustacean pairs after 24-hour dark incubations, indicating the presence of chemosynthetic activity. Left: *Typhlatya pearsi* host symbiont pair. Right: *Speleonectes tulumensis* host-symbiont pair.
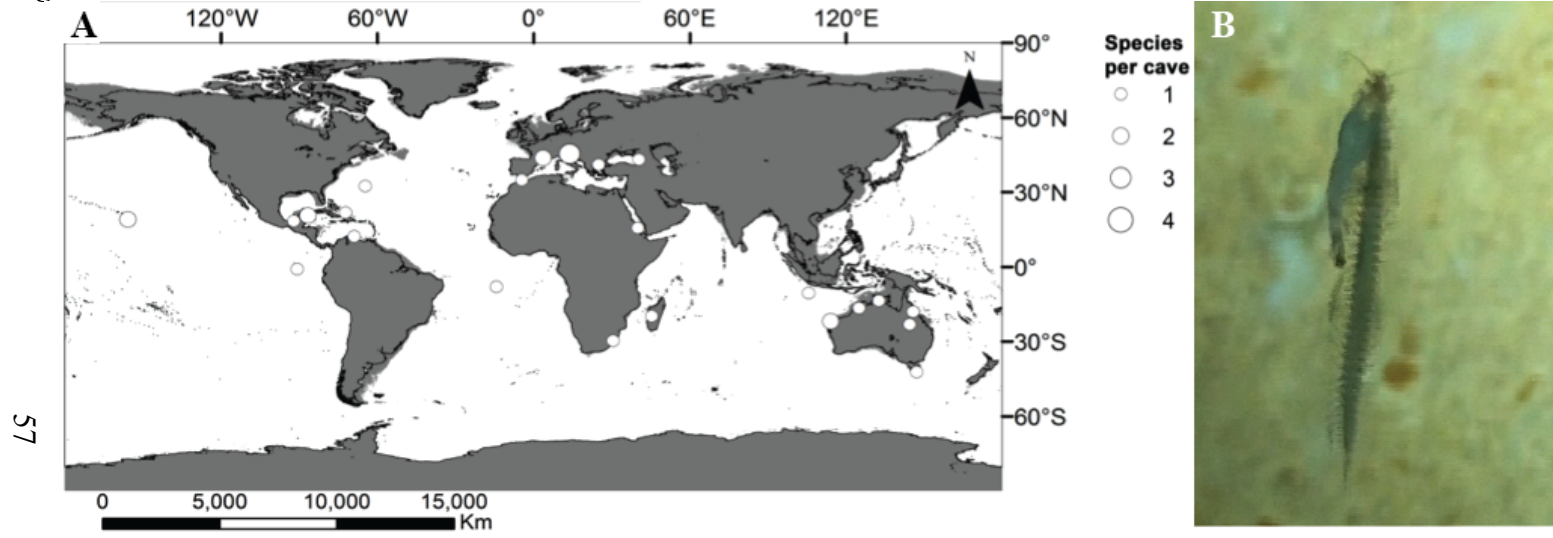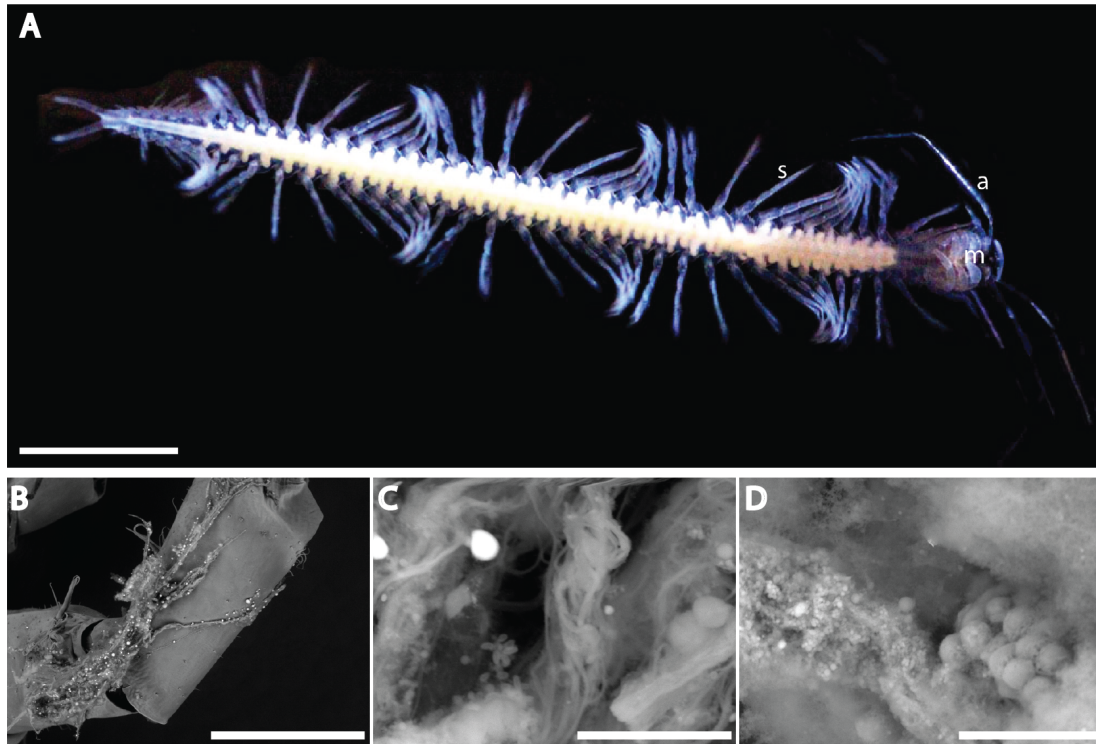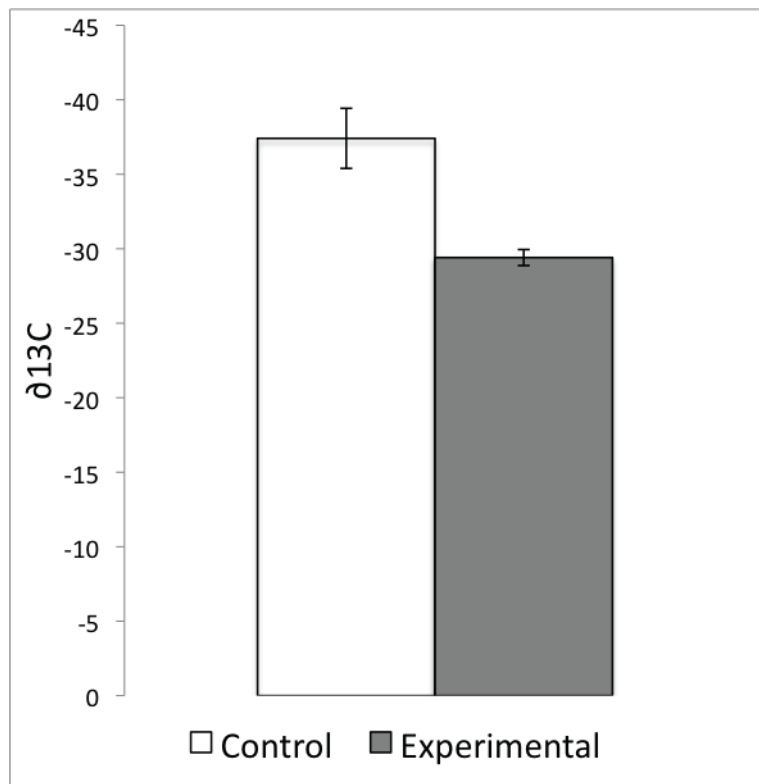
Figures

Figure 1.

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CHAPTER 4

Concurrent triple-isotope approach reveals chemosynthetic base to anchialine food web and intraspecific diet variation

Abstract

Dark cave environments are considered extreme- lacking in continuous resource supplies, and generally low in oxygen. Due to a presumably discontinuous supply of nutrients from the surface and the difficulty of sampling these subterranean sites, cave ecosystems food web dynamics are largely unknown. Anchialine environments house low-density macrofaunal communities endemic to stratified brackish and marine water layers. Advances in SCUBA and stable isotope methods were leveraged in the current study to reveal a chemosynthetic base of this crustacean dominated food web. I used novel concurrent Carbon, Nitrogen, and Sulfur stable isotope analysis to assess the diets of remipede, isopod and atyid shrimp crustaceans endemic to anchialine systems. Depleted carbon stable isotope values suggest a chemosynthetic base to this food web. Depleted sulfur stable isotope values further indicate that sulfide oxidizing and sulfate reducing bacteria are responsible for the influx of nutrients necessary to sustain this dark ecosystem. Concurrent analysis allowed for significant sampling of small biomass members of the community- a technique that allowed for greater capture of intraspecific diet variation. Results of Stable Isotope Analysis in R, a Bayesian model that incorporates uncertainty in trophic shifts to reveal finescale differences in foodwebs, indicate that sulfur, along with nitrogen and carbon, is necessary to solve diet solutions. *Speleonectes tulumensis*, a member of the Crustacean class Remipedia, was found to prey on both crustaceans and microbes. These anchialine cave endemics were previously thought to be predatory, but may alter the carnivorous proportion of their diet based on prey availability. *Metacirolana mayana* (Isopoda) was found to feed on a variety of crustaceans in the system including conspecifics. Since chemosynthetic endosymbiotic bacterial stable isotope values were unknown and therefore unavailable as a model input, *Typhlatya pearsei* shrimp diet was more difficult to determine via mixing models. Together these findings support a chemosynthetic food web in anchialine systems and intraspecific differences in diet within macrofauna sampled.

Introduction

Until recently, dark caves have been considered extreme, resource-limited systems unable to produce energy endogenously due to a lack of internal photosynthetic activity (Barton and Northup, 2007; Engel, 2013). Following this theory of nutrient-limitation, we assumed that anchialine caves, in which landlocked marine layers underlay meteoric brackish water (Seymour et al., 2007), host fauna fueled by surface produced energy. For example, marine-derived organics might travel through subterranean channels into inland caves with tidal influence. Nutrients may also enter caves as particulates through sinkholes or leech into the meteoric lens through the karst matrix itself. Alternatively, chemosynthetic bacteria may instead fuel anchialine food webs (Pohlman, 1997; Culver and Sket, 2000; Dov Por 2007). Although the presence or

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relative use of a this diet source has not been confirmed in caves that lack geothermal activity, depleted carbon isotope values of some cave crustaceans suggest a chemosynthetic source to this food web (Pohlman, 1997).

Here, I investigate whether chemosynthesis supports anchialine communities using the first concurrent triple-isotope analysis of foodwebs. Enzymes in different carbon fixation pathways vary in their use of the heavier carbon isotope, ^{13}C. Thus, the stable carbon isotope ratio of ^{13}C to ^{12}C, or $\delta^{13}C$, has traditionally been used to distinguish between chemoautotrophically fixed carbon and photosynthetically fixed carbon (Cavanaugh et al., 2006). For instance, in hydrothermal vent systems, carbon fixed through chemosynthesis is either enriched or depleted in ^{13}C compared to that of marine phytoplankton (Cavanaugh et al., 2006). Because isotopic composition of food is generally retained in the consumer, isotope differences are used to determine diet. Similarly, ^{15}N to ^{14}N ratios mixing models) vary between chemoautotrophic and photosynthetic pathways and change significantly and predictably among trophic levels (Cavanaugh et al., 2006). Therefore, $\delta^{15}N$ values may be used to determine the metabolic pathways leading to nitrogen uptake in a consumer as well as the consumer's trophic level (Cavanaugh et al., 2006). In addition, sulfur isotope ratios (^{34}S to ^{32}S ratios) have a wider distribution range than those of carbon and nitrogen across producers, so when combined with carbon and nitrogen isotope analyses, $\partial^{34}S$ can aid in the discrimination between primary producer inputs into aquatic foodwebs (Connolly et al., 2004). Sulfur, in particular has been found to be particularly helpful for the study of marine generalist predators in discriminating between on shore and off shore, as well as benthic and pelagic inputs into foodwebs (Newsome et al., 2007). Furthermore, due to fractionation during microbial sulfate reduction to sulfide, ^{32}S-containing sulfate is preferentially used, resulting in a depletion of ^{34}S in sulfide relative to that of sulfate (Socki et al., 2001). It is the combination of multiple isotopes which discriminate differentially between diet sources that increases the power of mixing model analyses (Newsome et al., 2007).

Anchialine caves are ideal systems in which to investigate how and why food web interactions change for several reasons. 1) Environmental characteristics of these submerged caves likely yield variation in potential nutrient sources. Anchialine systems are landlocked marine habitats, which often underlie water layers of discriminated salinity, allowing for potential terrestrial and marine nutrient input. Furthermore, the prevalence of anoxic-oxic interfaces at haloclines where marine and brackish water meet as well as at the sediment-marine water barrier may support sulfate and sulfide microbial metabolisms (Chapter 2). This stratification of resources may provide greater use for sulfur stable isotope discrimination in analyses of organisms that may feed on microbes with sulfur metabolisms. This sulfur discrimination is likely as carbon stable isotope values from these environmental microbes as well as from symbiotic bacteria (See Chapters 2, 3) are greatly depleted resembling values of sulfide-oxidizing bacteria (Cavanaugh et al., 2006). 2) The relative lack of diversity in anchialine caves, consisting of less than 10 macrofauna, makes these systems a good choice for studies employing mixing models to estimate diet (Newsome et al., 2007). 3) Like other restricted habitats, anchialine caves remain largely understudied due to their generally depauperate macrofauna. The only published analysis of anchialine faunal carbon and nitrogen isotope ratios reported depleted δ13C values consistent with a chemosynthetic dietary source, but did not examine δ34S values or analyze these data using a mixing

model (Pohlman et al.*,* 1997). As in many extreme or remote systems, few animals are available for observation and collection which makes studies of feeding behavior or gut content analysis impossible. This low faunal density increases the benefits of concurrent stable isotope design, bypassing the need for increased collection for later subsampling (e.g., for ^{13}C and ^{15}N versus ^{34}S).

The present study combines concurrent analyses of mixing models, δ^{15}N, δ^{13}C, and ∂^{34}S on individual samples with Bayesian mixing models to study foodwebs and resource switching across small spatial scales in anchialine environments. These findings illustrate that 1) anchialine fauna are dependant on chemosynthesis, akin to foodwebs at hydrothermal vents in the deep sea, and that 2) top anchialine predators (Remipedia) are omnivores. 3) That Atyid shrimp *T. pearsei* differ in diet between passages of varying geochemistry. This differentiation may drive major community structure changes in the environment. These results are informative to similar studies of intraspecific variation in diet. Without the addition of Sulfur to our analyses separation of various components of predators' diets would not be possible. These analyses also illuminate the possibilities of using concurrent stable isotope analysis to investigate diet and how resource use changes over time and space.

Methods

Sampling Dates and Site

On the following dates, divers collected animal, sediment, water, and vegetation samples for stable isotope analysis from in and around Cenote Crustacea, a cave system in Quintana Roo, Mexico: July 10-12, 2008, August 7-18, 2009 and June 23-July 3, 2010. Two passages, exhibiting different habitats, were sampled in this study (Figure 1). Since passages in the eastern section were discovered in 2009, western areas of the cave were sampled during all three years of this study and eastern sections were sampled only in the latter two years of this study. Sections differ in the following ways: 1) The western passage ranges from depths of 13m to 20m with a halocline at approximately 14m. In contrast, most of the sampled eastern passageways are 18-23m in depth and are, therefore, fully marine. 2) The western passage includes greater amounts of sediment, some areas of which contained dense patches of blackened microbe-rich sediment (Chapter 1), as compared with the eastern passages (Figure 1). In addition, much of eastern passage habitat is highly decorated, containing stalagmites, stalactites and boulders not found in the western portions of the cave (Figure 1). This suggests that the eastern passages have experienced greater rates of flow that carved decorations and carried away overlaying sediment over geologic time (Figure 1). 3) The western passage also appears to have higher crustacean densities than the eastern passages (Figure 1).

Collection

In order to reconstruct the anchialine food web, the following animals were collected by hand on SCUBA, identified using a microscope if necessary, and frozen for transport back to UC Berkeley: remipedes (*Speleonectes tulumensis*), amphipods (*Tuluweckia cernua* and *Mayalweckia cenoticola*), isopods (*Metacirolana mayana*), ostracods (*Danielopolina mexicana*), shrimp (*Typhlatya pearsi*), thermosbanaceans (*Tulumella unidens*).

In order to determine which primary producers were responsible for the most nutrient input into this anchialine system the following sediment, microbial, and vegetation samples were also collected and processed: Black microbial mat containing sediment from inside the cave passage as well as top soil from outside the cave. In 2009 and 2010, water samples were collected from the marine layer, the halocline and the brackish water layer in section A. Water samples were also collected from analogous layers in a room between Cenote Crustacea and section B, as well as the marine layer in section. All water samples were filtered with a vacuum pump on glass fiber APFF filters. Leaf samples (*Ficus* spp. and *Acacia* spp.) were collected in 2009 and 2010 in triplicate, as well. All samples were frozen for transport to UC Berkeley, where they remained frozen until they were prepared for isotope analysis. Subsets of sediment samples were acid washed to remove carbonates (protocol modified from Harris et al., 2001). Animal and plants were not acid washed as this method has been found to alter sulfur stable isotope ratios (Connolly and Schlacher, 2013)

Stable Isotope and Statistical Analyses

Animals, sediment and algae were freezedried for 2 days, whereas filter and leaf samples were oven dried at 60°C for 24 hours. All samples were analyzes with an IsoPrime100 gas source stable isotope ratio mass spectrometer to collect carbon, nitrogen, and sulfur stable isotope ratios and percent composition. Nb_2O_5, an oxidant previously thought to aid in the combustion of sulfur compounds, was added to animal and sediment samples (Ripley et al., 2011). Our study found no significant effect of this compound on animal C, N, or S percent composition but did see a negative effect with percentage composition in plants and sediments (see Appendix 1). As such, Nb_2O_5 was not added to sediment or plant samples during this study. Animals which were too large to run whole, were ground and a subset was run for concurrent $\delta^{15}N$, $\delta^{13}C$, and $\partial^{34}S$ analyses. Animals for which not enough material was available to run as an individual, (e.g., some juvenile amphipods and shrimp or thermosbanaceans) were pooled by species and collection location for analysis. Due to their high concentration of sulfur and low nitrogen, carbon content, pseudoreplicates of sediment were run once for $\partial^{34}S$ and once for combined $\delta^{15}N$ and $\delta^{13}C$ analyses. To remove inorganic carbon and determine organic carbon stable isotope values, sediments were also analyzed after acidification (see Harris et al., 2002). Data was corrected using a Fry correction.

I analyzed the effects of location within the cenote (east or west passage) on the isotopic signatures of remipedes, isopods and shrimp using Generalized Linear Mixed Models in AED package in R (citation). The general form of the models tested weere: Isotope value ~Passage versus Isotope value ~1 as the null model. Significance was determined by comparing Aikake Information Criteria (AIC) values between the full and null models using ANOVAs (alpha = 0.05). Stable isotope values of remipedes, isopods, and shrimp were than analyzed in SIAR using prey stable isotope means and standard deviations (Version 4.1 Parnell and Jackson, 2011). Discrimination factors were incorporated into all analyses and were estimated from known aquatic and terrestrial values (McCutchan, et al., 2003). All SIAR models were run with 1 million iterations. 5005 iterations of these analyses were initially discarded (Version 4.1 Parnell and Jackson, 2011).

Results

Habitat Differences
Biotic and abiotic characteristics of the west and east passages differed. Abundance of *S. tulumensis* remipedes and *T. pearsei* shrimp in the east passage was greater than in the west passage (Figure 1). In addition, *D. mexicana* ostracods are not found in the east passage of Cenote Crustacea. Although sediment was not quantified in these habitats, the east passage exhibited more sediment at the substrate than the west passage (Figure 1). These passages also differed in water column composition. The west passage was entirely marine, whereas the east passage contained both marine and overlying brackish water layers.

Intraspecific Variability
There is great intraspecific variability in anchialine cave crustacean carbon, nitrogen, and particularly sulfur stable isotope values (Table 1, Figure 2 and Appendix 2). Carbon stable isotope values are especially depleted in remipedes, shrimp, and *T. unidens* thermosbanaceans (Table 1, Figure 2 and Appendix 2). Shrimp exhibited the most depleted values for $\partial^{34}S$ (Table 1, Figure 2). Remipedes and *M. mayana* isopods illustrated greater $\partial^{15}N$ values on average than shrimp, *M. cenoticola* amphipods, and thermosbanaceans.

Intraspecific variability and habitat in remipedes – There was no evidence of differences between isotopic signatures in remipedes or isopods collected in the eastern or western passages of Cenote Crustacea (Appendix 3). No correlation was found between locations of collections (eastern or western passages) and isotopic signatures in remipedes (Figure 3A, Table 2). Furthermore, a comparison of generalized linear mixed models (GLMMs) found no significant difference between null models and those incorporating passage as a factor to describe variations in $\partial^{13}C$, $\partial^{15}N$ or $\partial^{34}S$ data collected from isopod specimens (Figure 3B, Table 2).

ANOVAs comparing generalized linear mixed models found no significant difference between null models and those incorporating passage as a factor to describe variations in $\partial^{15}N$ or $\partial^{34}S$ data collected from shrimp specimens (3C, Table 2, Appendix 3). However, an ANOVA comparing the null generalized linear mixed model for $\partial^{13}C$ of shrimp to a model including passage as a factor found a significant difference (Table 2). Therefore, $\partial^{13}C$ values of shrimp showed a significant passage effect with the western passage exhibiting more depleted values (Figure 3C, Table 2, Appendix 3).

Diet
The seven potential food items for both remipedes and isopods were: *T. pearsei* shrimp, *M. mayana* isopods, and *M. cenoticola* amphipods, *T. unidens* thermosbanaceans, *D. mexicana* ostracods, black microbe-rich sediment, and surface sediment (Figure 3A-B). However, the amount of overlap of individual remipede and isopod data points with the above food items depended on which stable isotope pairs were considered among $\partial^{13}C$, $\partial^{15}N$ and $\partial^{34}S$ (3A-B). Although all primary producers observed in and outside of the

cave were analyzed, a clear food source was not obvious for shrimp based on $\partial^{13}C$, $\partial^{15}N$ and $\partial^{34}S$ values (Figure 3C).

Remipede Diet– SIAR Bayesian mixing models incorporating $\partial^{13}C$, $\partial^{15}N$ and $\partial^{34}S$ showed that although the consumption of shrimp was predominant in a dataset exploring both passageways (95% credibility interval: 39–70%) (Figure 4A). *T. unidens* (95% credibility interval: 0–27%), *M. mayana* (95% credibility interval: 0–25%), and microbe rich black sediment (95% credibility interval: 0–10%) are also likely contributors to remipede diet (Figure 4A).

Isopod Diet– SIAR Bayesian mixing models incorporating $\partial^{13}C$, $\partial^{15}N$ and $\partial^{34}S$ also indicated that shrimp was the major prey item for isopods (95% credibility interval: 18–46%). Values for conspecifics (*M. mayana*) has the second highest predicted proportion of diet for the taxa (95% credibility interval: 8–52%) (Figure 4B). In addition, both T. unidens and microbe rich black sediment had 95% credibility intervals of 0–20% and may also contribute to isopod diet (Figure 4B).

Shrimp Diet– As *T. pearsei* shrimp were found to vary in carbon stable isotope values by passage, diets of shrimp from eastern and western passages were analyzed separately (Figure 4C). SIAR Bayesian mixing models incorporating $\partial^{13}C$, $\partial^{15}N$ and $\partial^{34}S$ suggested that microbe rich black sediment was the major input into *T. pearsei* diet in the eastern passage (95% credibility interval: 52–91%) which was a significantly greater contribution than in the western passage (95% credibility interval: 0–29%) (P=0) (Figure 4C). In contrast, although shrimp stable isotope datasets varied significantly between eastern and western passages did a primary proportional input to diet was not found (Figure 4C).

Discussion

Findings presented here advance our understanding of both anchialine ecosystem function and methods in stable isotope ecology. These data indicate 1) a chemosynthetic base to the anchialine food web, 2) prey solutions dependent on sulfur stable isotope analysis in tandem with $\partial^{13}C$ and $\partial^{15}N$ data, and 3) intraspecific variation in stable isotope values along fine spatial scales. Together these results illustrate the power of concurrent stable isotope analyses combined with Bayesian mixing models to delineate between varying diets of populations along environmental clines.

The Anchialine Food web

Carbon as well as sulfur stable isotope values measured in anchialine cave specimens are similar to those of invertebrates, such as seep bivalves associated with sulfide oxidizing symbionts, deriving nutrition from chemosynthetic sources (Dupperon et al., 2012). For example, the range of $\partial^{13}C$ values for anchialine crustaceans suggest a chemosynthetic base of the food web (mean $\partial^{13}C$ values of -26.7 for amphipods to -37.1 for shrimp). These values fall within those expected for chemoautotrophically-derived carbon (-25 to -40) (Fisher, 1995). *T. pearsei*, which hosts putative chemosynthetic endosymbionts (Chapter 2), exhibits the most depleted $\partial^{34}S$ values reported from this system. $\partial^{34}S$ may become depleted due to discrimination during microbially mediated

sulfur oxidation and sulfate reduction reactions in the cave environment (Stoessel et al., 1993).

Although *T. pearsei* stable isotope values indicate a chemosynthetic source, their primary diet was not found via mixing models presented here. There are several possible reasons for the inability of the model to identify a nutrient source for this organism. This endemic cave shrimp swims between the brackish and marine layers of Cenote Crustacea (Koenemann and Iliffe, 2013; pers. observ.) and its diet source may not have been collected during this study (Fig. 5). Atyid shrimp in caves and rivers in the Caribbean have been found to filter feed and scrape microfilms with their detritus with their fan like setae and chelipeds, respectively (Page et al., 2008). It is possible that *T. pearsei* in anchialine systems are behaving similarly and feeding off microbial biofilms on cave surfaces. However, recent evidence that these species harbor endosymbiotic bacteria suggests that *T. pearsei* are gaining this chemosynthetic nutrition symbiotically. Alternatively, these shrimp may be feeding on microbes living epibiotically as Rimicarid shrimp have been found to do (Gebruk et al., 2000). These feeding strategies are not mutually exclusive and may account for the variation found between passages in Cenote Crustacea.

It is likely that *S. tulumensis* and *M. mayana* are generalists, predating or scavenging on other crustaceans. Both remipedes and isopods exhibited mean $\partial^{15}N$ values (6.6 and 6.9, respectively) over 4‰ greater than the *T. pearsei* (2.0), *M. cenoticola* (1.7) and *T. unidens* (1.7) means. This differentiation in $\partial^{15}N$ suggests that the remipede and isopods were at least a trophic level above the shrimp, amphipod and thermosbanacean (Figure 5) (Cavanaugh et al., 2006). The siar models confirmed these hypotheses, but also introduced microbe-rich black sediment as a potential secondary food source for both isopods and remipedes. The obligate predation by remipedes on *T. pearsei* has recently been called into doubt (Koenemann and Iliffe, 2013). Although these crustaceans carry venomous toxins likely expelled by a injecting apparatus (van der Ham and Felgenhauer, 2007; von Reumont et al., 2013), they have survived months in captivity without feeding (Koenemann et al., 2007) and are found infrequently with prey in caves (Koenemann and Iliffe, 2013, pers. observ.). Lack of feeding combined with behavioral observations lead some to believe remipedes filter the water column for particulates (Koenemann et al., 2007). Yet, recent discovery of epibiotic microbiota (Chapter 3) combined with observations of grooming in aquaria (Koenemann et al., 2007) provides an alternative explanation: remipedes may be eating epibiotic bacteria as a complementary food source.

Generalist predators are thought to change their diet composition based on prey availability (e.g., Murdoch, 1969; Ostfield 1982), but there are no studies illustrating this process between symbiotic nutrients and prey. As both *S. tulumensis* and *M. mayana* diet models suggested a contribution of sediment microbes, it is possible that these crustaceans are feeding on microbes with similar isotope values from a surface other than the sediment, such as their carapaces. *M. mayana*, however, is most likely a scavenger and has been baited successfully by carrion (Pohlman et al., 1997).

Intraspecific Variation affects on Community Structure:

Intraspecific variation in diet, such as that reported for anchialine cave shrimp, may drive community structure changes, thereby affecting ecosystem function (e.g., Urton and Hobson, 2005). Stable isotope analysis (SIA) has long been used to estimate diet niche breadth in a range of organisms (e.g., rainforest ants: Bluthgen et al., 2003, near shore seabirds: Moreno et al., 2010) but has only recently been used along with mixing models to determine variations in diet between (e.g., migratory and sedentary; Voigt et al., 2013) and within (e.g., ontogenetically; de la Morinière et al., 2003, Davis et al., 2012) populations. Here, we see variation between environmentally distinct habitats. One passage contains a halocline (east) that may increase chemosynthetic metabolisms and thereby food availability for shrimp. Resource use shifts towards a chemosynthetic source in the east passage, but not in other sections of the cave lacking a redox zone at the marin-brackish water interface, may spurr the relative success of *T. pearsei* shrimp in this stratified environment. As shrimp are the primary food source for *S. tulumensis*, it makes logical sense that remipedes are also more abundant in the eastern passage where their food availability is high. These data point to a bottom up shift in resource availability in the east passage that may be driving community structure changes throughout higher trophic levels. Similar community structure changes are frequently seen as a consequence of atyid population density in Caribbean riverine environments (Page et al., 2008).

Our increased knowledge of intraspecific variation is due to two recent developments 1) the use of multiple stable isotopes in analyses (e.g., Newell et al., 1995; Hoekstra et al., 2002; Newsome et al., 2007, Moreno et al., 2010) combined with advances in sample processing that allow multiple isotopes to be measured concurrently, and 2) statistical analyses that can now reveal changes in proportional contribution of prey items within a population (e.g., Moreno et al., 2010). Limitations of SIA algorithms have largely been due to an inadequate number of stable isotopes used in analysis of complex foodwebs. Bayesian methods, such as stable isotope analysis in R (siar, version 4.1 Parnell and Jackson, 2009), incorporate uncertainty when calculating diet and may also benefit from larger samples sizes that are increased by concurrent stable isotope analysis. These novel methods have the greatest potential in low-density, extreme environments that are difficult to access and sample, such as deep sea hydrothermal vent (Gebruk et al., 2000) and Antarctic (Hoekstra et al., 2002) ecosystems, as well as the subterranean communities investigated here.

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Table Caption

Table 1. Anchialine Crustaceans stable isotope values with standard deviation.

Table 2. Analysis of GLLM models comparing stable isotope values of cave specimens as a factor of passage with the null model that passage does not drive variation. AICs from models as well as Degrees of freedom and F values from ANOVAs comparing models are reported. Significant Values are highlighted in bold.

Table

Table 1.

Animal	N	∂13C ±SD	∂15N ±SD	∂34S ±SD
S. tulumensis	16	-34.41 ± 3.17	6.59 ± 2.48	-1.76±4.59
M. mayana	36	-30.66±5.04	6.87 ±2.59	2.64±5.01
T. pearsei	56	-37.25± 3.76	2.04 ± 4.12	-9.59±6.44
M. cenoticola	14	-26.68±1.99	-1.73±3.19	-4.23±5.1
T. unidens	12	-37.08±5.11	1.76±4.32	1.11±9.81

Table 2.

Animal	*S. tulumensis*	*M. mayana*	*T. pearsei*
∂13C Null Model AIC	76.895	221.58	335.02
∂13C Passage Factor AIC	78.881	223.57	327.33
Degrees of Freedom	1	1	1
F Value	0.9121	0.9033	**0.001428**
∂15N Null Model AIC	68.333	173.64	334.65
∂15N Passage Factor AIC	69.808	175.48	336.64
Degrees of Freedom	1	1	1
F Value	0.3039	0.6974	0.9285
∂34S Null Model AIC	80.64	221.14	401.7
∂34S Passage factor AIC	82.582	220.45	400.63
Degrees of Freedom	1	1	1
F Value	0.8228	0.1044	0.08087

Figure Captions

Figure 1. Habitat differences within Cenote Crustacea. (A) Map of Cenote Crustacea illustrating Western and Eastern Passageways with access to outside cave collapse (cenotes) marked. (B) Environmental differences shown in two sets of passageways. Eastern sites have greater sedimentation and more biomass than western sites. White flecks in right photograph are *Typhlatya* shrimp. (C) Abundance of remipedes (dark bars) and shrimp (light bars) with standard error in western and eastern passages. Photographs by T. Thomsen.

Figure 2. Variation in Carbon, Nitrogen, and Sulfur stable isotope values of organisms in Cenote Crustacea. Filled icons indicate West passages and open icons indicate east passages.

Figure 3. Stable isotope values of anchialine crustaceans and their potential diet sources with standard deviation. (A) Remipede siar stableisotope biplots (B) Isopod siar stable isotope biplots (C) Shrimp siar stable isotope biplots. At left: $\partial15N$ vs $\partial34S$, at center: $\partial13C$ vs $\partial34S$, at right $\partial15N$ vs $\partial13C$. East passage samples (open circles) and west passage samples (open triangles).

Figure 4. SIAR modeling output of proportions of diet contributed by potential prey items in Cenote Crustacea to (A) Remipedes, which primarily prey on *T. pearsei* shrimp, *M. mayana* isopods, *T. unidens* thermosbanaceans and microbe-rich sediment. (B) Isopods, which also derive nutrition from *T. pearsei* shrimp, conspecifics, *T. unidens* thermosbanaceans and microbe-rich sediment, and to (C) Shrimp in the East Passage (at left) and in the West Passage (at right) Shrimp diets vary significantly by passage.

Figure 5. Anchialine system food web inputs. (A) A collapsed portion of the cave, or cenote, supplies access to photosynthetic inputs, like ficus and acacia. (B) Marine layer sediment contains black bacterial mats (at arrow). Note remipede at top left. (C) Shrimp are abundant in brackish water at cave ceiling. (D) Thermosbanacean at left and Amphipod at right. (E) *Typhlatya* shrimp. (F) Isopod. (G) Remipede.
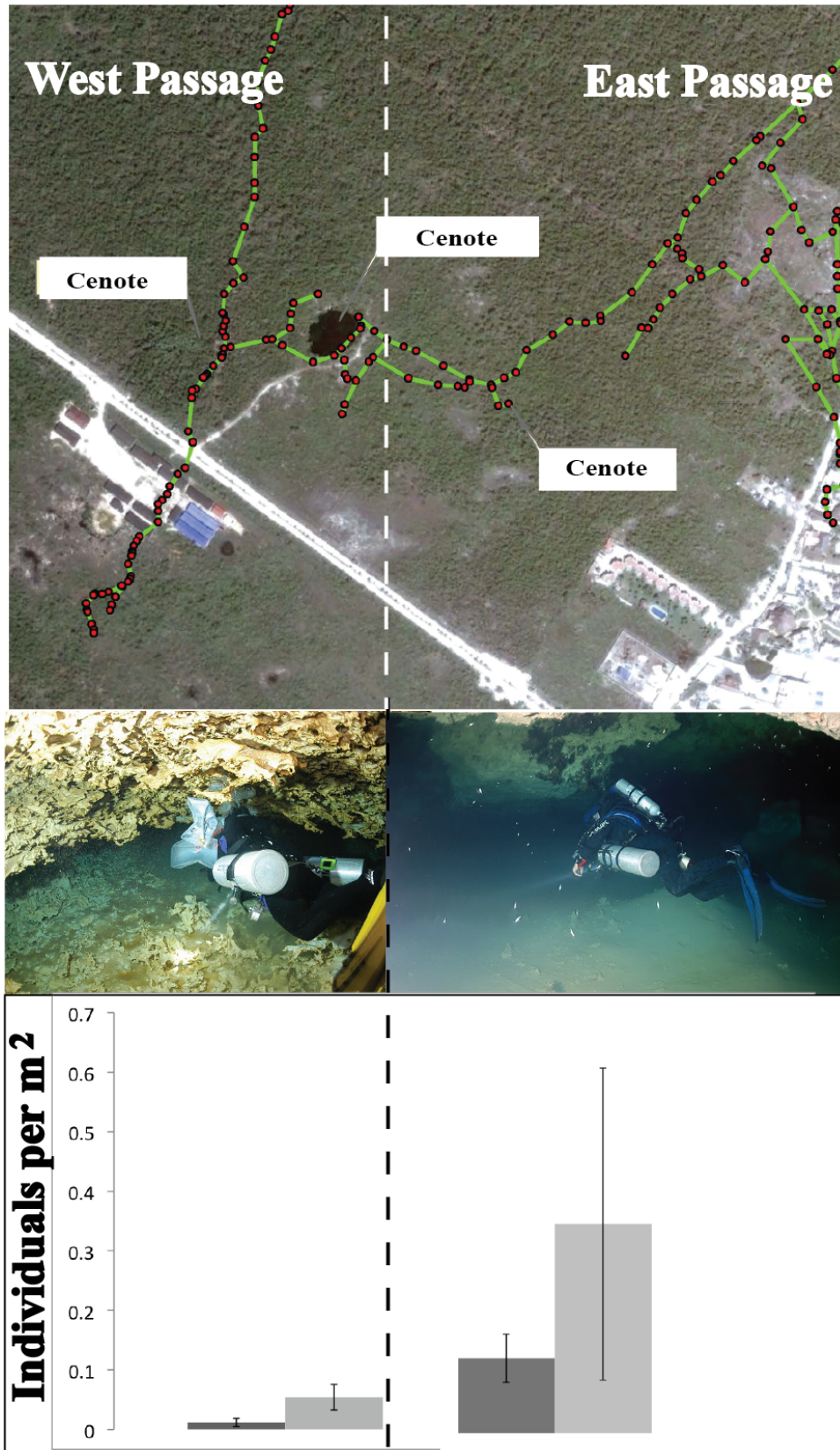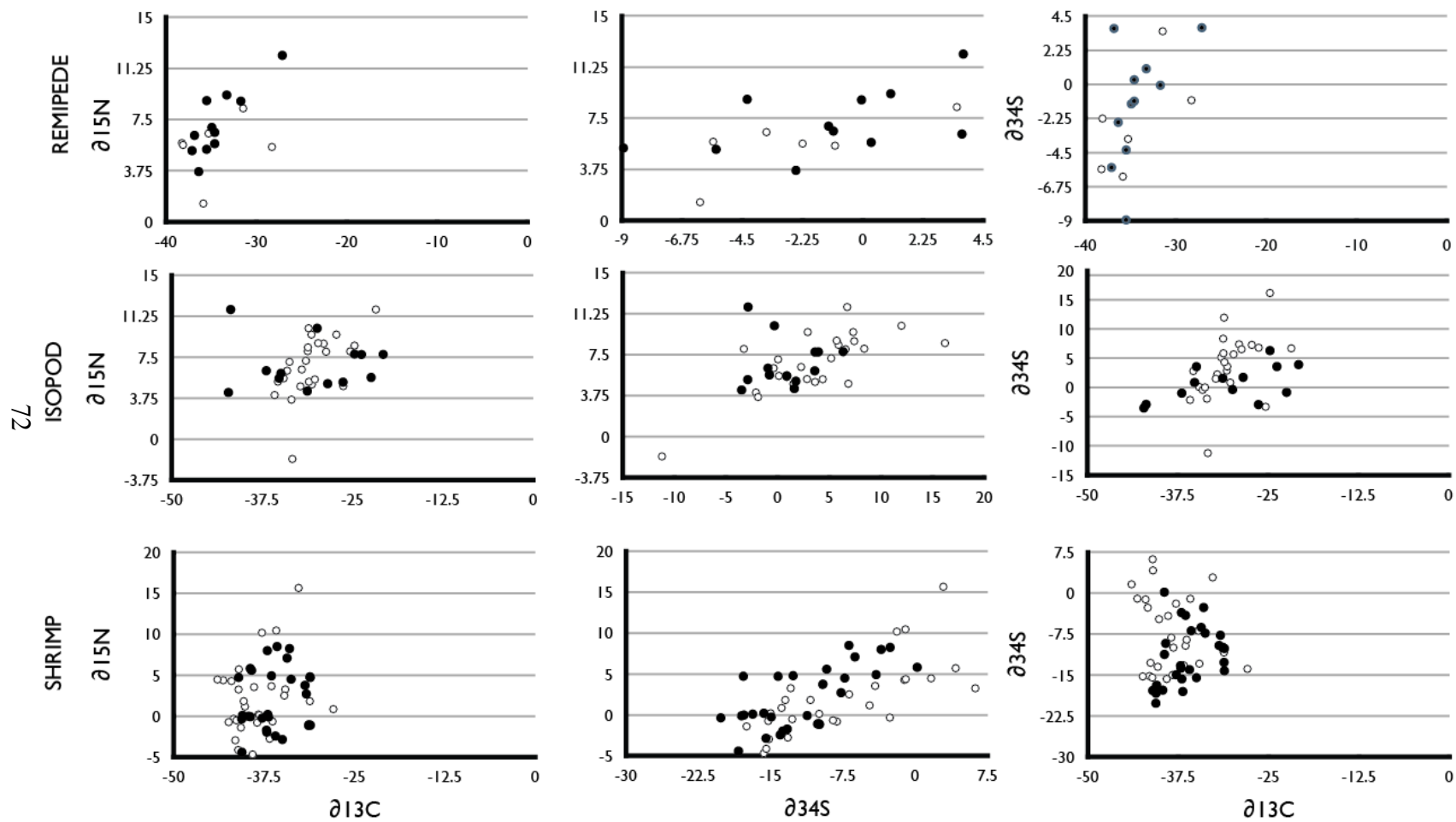
Figure 1.

Figure 2.

72

Figure 4.

A Remipede Diet

B Isopod Diet

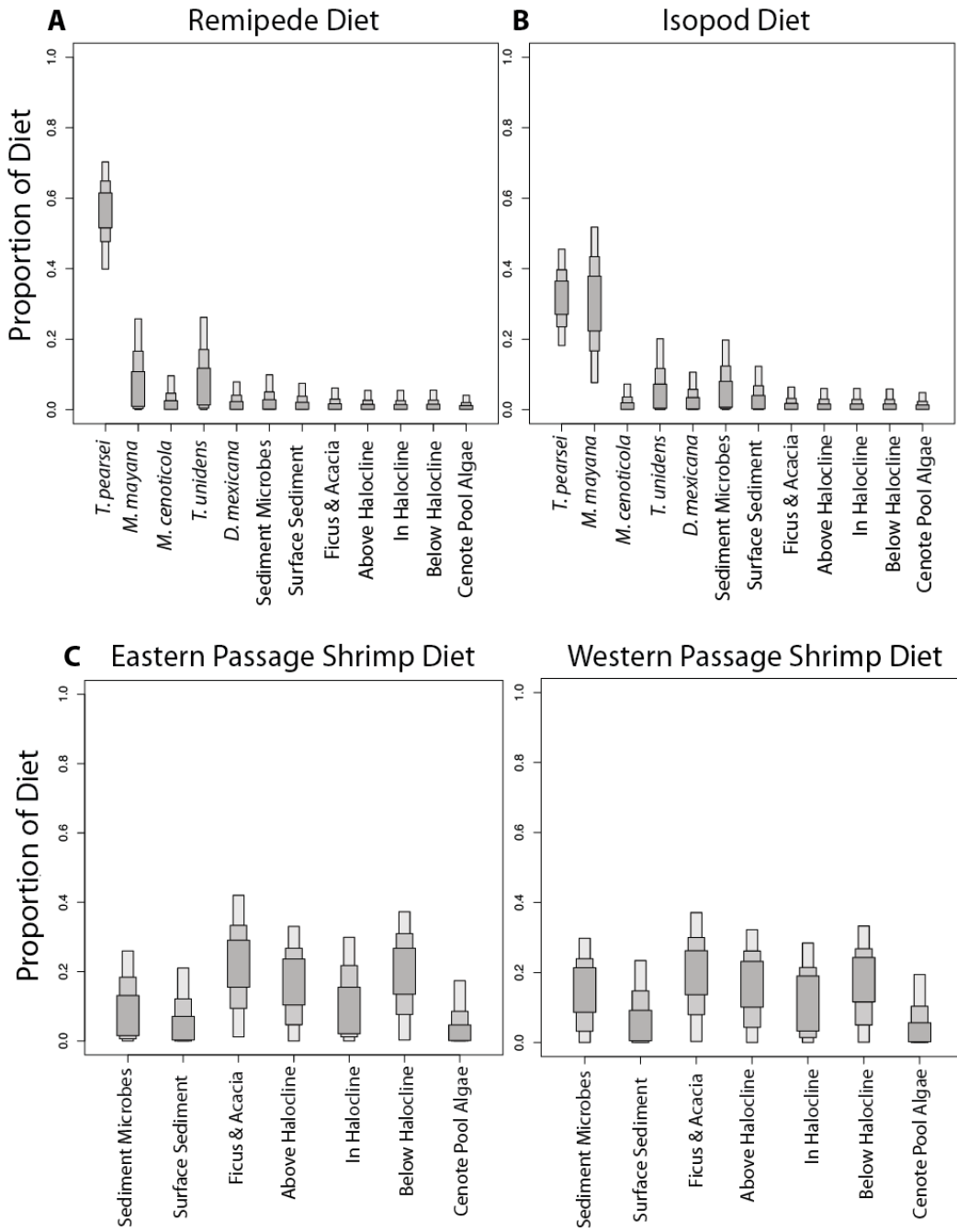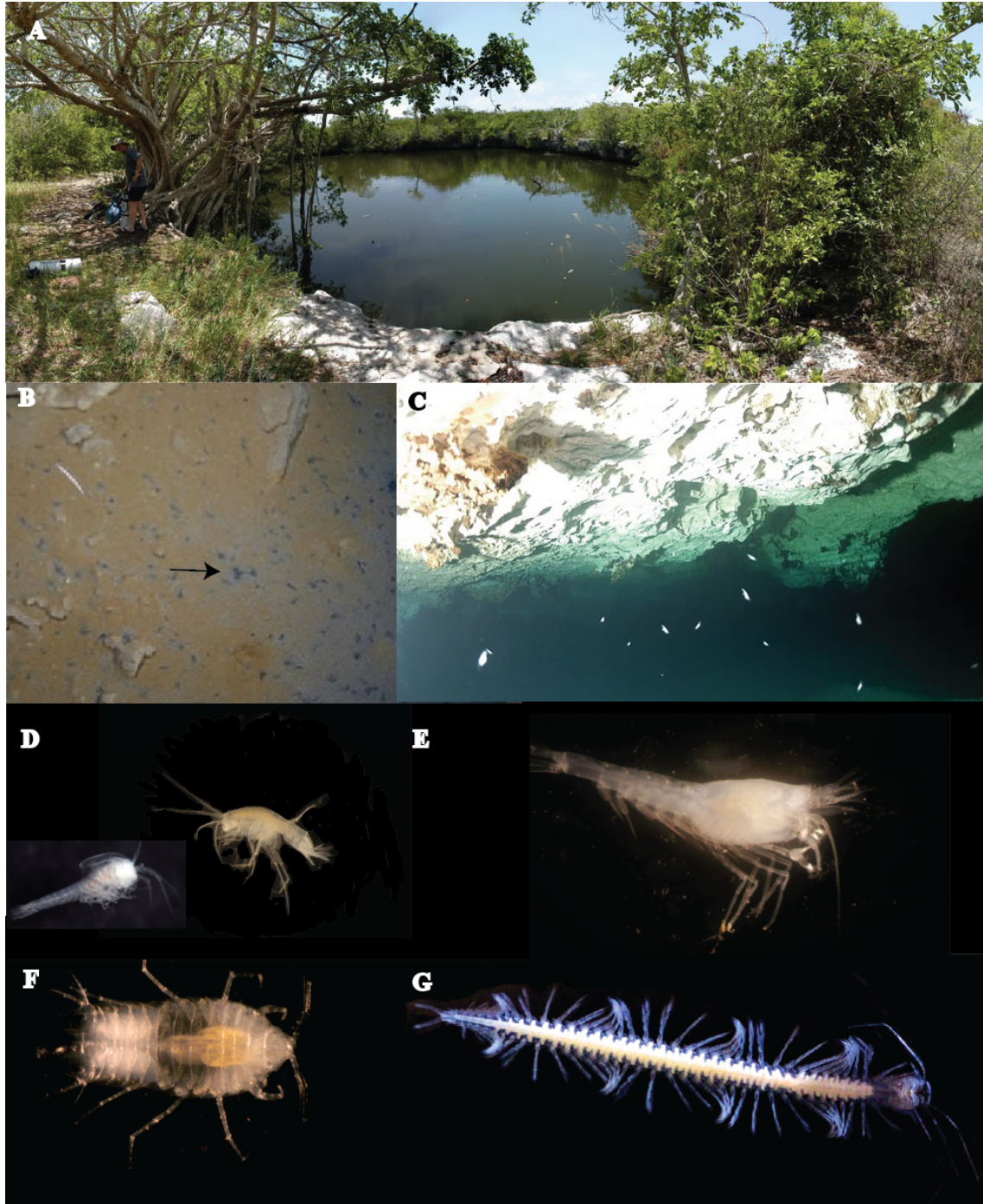C Eastern Passage Shrimp Diet

Western Passage Shrimp Diet

Figure 5.

Appendices

Appendix 1. Table reporting p-values for T-tests comparing percentage and values of Carbon, Nitrogen, and Sulfur stable isotope values in Soil and Peach Leaves with and without the additin of Niobium Pentoxide oxidant.

Sample Type (n; range in mg)	%N	∂N	%C	∂C	%S	∂S
Yolo Soil:						
N=11 per treatment (3x 9-11mg, 3x 28-32mg, 3x 57-63mg in each treatment set)						
Control average	0.10	4.31	0.85	-25.57	0.02	-2.31
Control standard error	0.00	0.14	0.01	0.02	0.00	0.13
paired t-test	0.035	0.059	0.017	0.243	0.824	0.480
Peach Leaves:						
N=9 per treatment (5x 1.5-2mg, 3x 4.5-5.5mg, 3x 8-9mg in each treatment set)						
Control average	2.96	2.06	48.41	-25.86	0.20	8.61
Control standard error	0.01	0.03	0.61	0.03	0.01	0.06
paired t-test	0.002	0.011	0.010	0.501	0.380	0.656

Appendix 2.
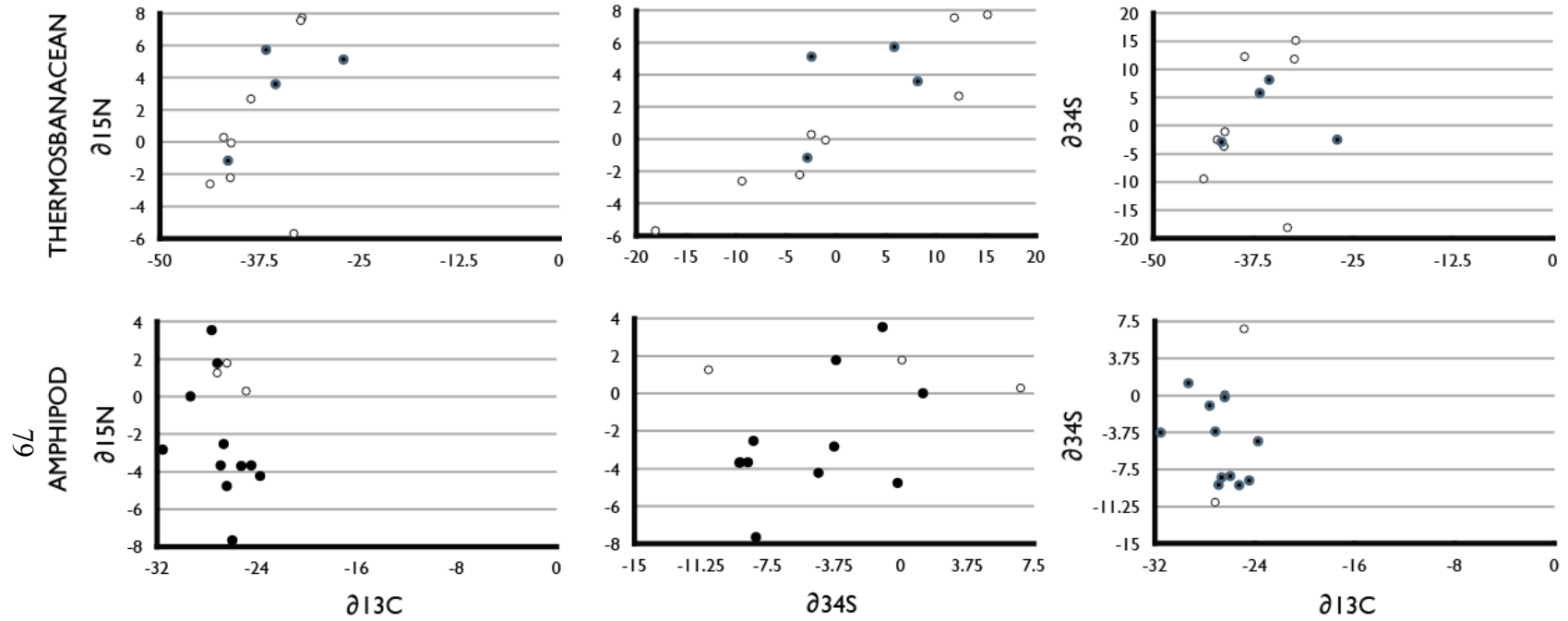
Appendix 2. Scatter plot illustrating variation in Carbon, Nitrogen, and Sulfur stable isotope values of thermosbanaceans (*T. unidens*) and amphipods (*M. cenoticola*) in Cenote Crustacea. Filled icons indicate West passages and open icons indicate east passages.
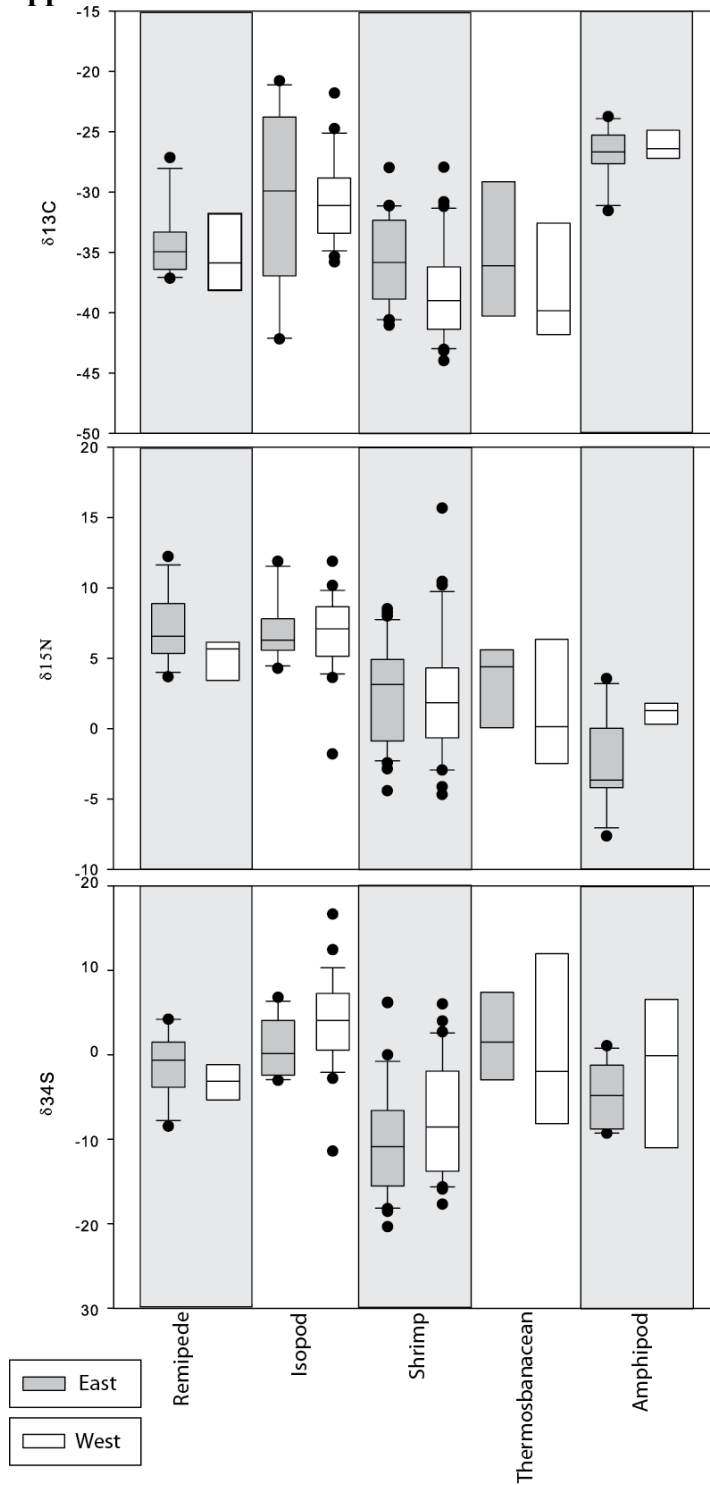
Appendix 3. Boxplots illustrating variation in stable isotope values of Carbon, Nitrogen, and Sulfur by organism (Remipede *S. tulumensis*, Isopod *M. mayana*, Shrimp *T. pearsei*, Thermosbanacean *T.unidens*, amphipod *M. cenoticola*. Boxplots show Mean, 75th and 25th percentile by passageway for crustaceans in Cenote Crustacea.

CHAPTER 5

Behavior of the remipede, *Speleonectes tulumensis*:
swimming in the dark

Abstract

How animals navigate through patchy aquatic and terrestrial environments to locate food and mates is poorly understood. Crustaceans often orient towards chemical cues while foraging in these fluid environments. Dark ecosystems, such as caves, provide an opportunity to isolate chemo-orientation from use of visual signals. Our study of the blind crustacean class Remipedia, endemic to anchialine caves, presents the first evidence of crustacean orientation toward chemical gradients. Anchialine caves are karst systems containing a distinct meteoric lens that overlies a marine layer, which are often characterized by dark, sulfidic and anoxic microhabitats. Here, we investigate chemical orientation by employing 300 hours of *in situ* video in underwater caves. Our observations of locomotion and navigation in the blind remipede, *Speleonectes tulumensis*, revealed its mode of turning and interaction with chemosynthetic microbial mats at anchialine sediments. *S. tulumensis* remained at mat edges for extended periods of time indicating the tracking of odorants effused by mats. We suggest that remipedes may be following gradients at mat edges in order to supply their microbial symbionts with sulfides necessary for chemosynthetic metabolisms.

Introduction

Connecting locomotory behavior with chemosensory function to determine how organisms navigate the biological-patches and chemico-physical gradients common to fluid environments remains a challenge. *In situ* study is likely necessary to understand behaviors in response to combined chemical cues or within complex natural environments. Investigations of this topic, a recent focus of marine and terrestrial studies (Woodson et al., 2007; Hay, 2009), have yielded the discovery of a diversity of arthropod sensors for sampling water and air-borne odor plumes (Page et al., 2011; Reidenback and Koehl, 2011). Yet, most of these studies have focused on behavioral responses to a single cue, usually generated from macerated prey items, in laboratory flumes in the absence of natural turbulence regimes or environmental heterogeneity. Even if the factor most strongly affecting behavioral response is isolated, responses may still be greatest with natural blends of peptides or other compounds (Hay, 2009). As orientation efficiency likely augments crustacean foraging ability, these chemosensory behaviors may be linked to fitness.

Currently, reactions to chemical cues are modeled as directional, but organisms may instead be attracted to specific concentrations along chemoclines radiating from a high concentration source (Polz et al., 2000) or the meeting points of two directionally opposed radiating sources. Such gradients occur at the junction of oxygenated marine water and mineral-rich reducing plumes (i.e., hydrothermal vent or methane seep plumes) (Polz et al., 2000) or may be created as microbes degrade organic material.

Flow regimes determine the stability of such gradients, with low turbulence creating more stable chemoclines. Anchialine caves provide ideal habitats in which to examine gradient following, as restricted water exchange in the marine layer results in low-flow regimes and consequent stable physico-chemical gradients (Cangenella 2007; Seymour et al., 2007). Underwater caves are often crustacean dominated (Dattagupta et al., 2009, Neiber et al., 2011) and we now know chemosynthetic microbes support the macrofauna in some underwater cave systems (Dattagupta et al., 2009; see Chapters 2 and 3). Yet, Troglobitic behavioral responses to these microbes are unknown. Endemic cave crustaceans have evolved increased densities of sensory organs (chemosensory hairs, pores, and sensilla) relative to their sister taxa (Yager, 1981; van der Ham and Felgenhauer 2008). Such chemosensory structures have been hypothesized to aid in mate searching or prey location (Fanenbruck et al., 2004), and may be used to sense sulfides and proteinaceous odors diffusing from microbial microhabitats.

Do anchialine crustaceans track chemical gradients and if so, why? Gradient following has been anecdotally observed and hypothesized to occur in a variety of invertebrates with chemosymbiotic associations (Gebruk et al., 2000; Polz et al., 2000), but has never been systematically studied. A strong candidate for this behavior and its study is the crustacean class Remipedia (Yager, 1981), which is endemic to anchialine systems and sister to the Cephalocarida (Giribet and Edgecombe, 2012). The Mexican remipede *Speleonectes tulumensis* (Yager, 1987) harbors chemosynthetic symbionts (Chapter 3). This study, focusing on turning and responses to environmental heterogeneity in the form of microbial mats, will help elucidate how these blind organisms find their way and locate prey in the mazes of their subterranean environment. Here, we describe gradient tracking, as a novel mode of navigation employing these blind cave crustaceans that navigate heterogeneous, dark environments with chemogradient-based cues as an example. Spatial analysis from 300 hours of *in situ* video confirmed that *S. tulumensis* is tracking bands surrounding dense chemosynthetic microbial mats (M. J. Pakes 2010, unpublished data). We hypothesize that epibiont-bearing remipedes will follow a gradient around microbial mats, in order to supply their symbionts with both sulfides and oxygen for microbial chemosynthesis. Other chemosymbiont-bearing fauna may also be attracted to gradients around microbial aggregations or metabolic substrates, such as sulfides, used in chemosynthesis.

Methods and Materials

Study site, filming, and taxon sampling

The hermaphroditic crustaceans Remipedia (Yager, 1981) are restricted to the marine layer of subtropical anchialine systems (Neiber et al., 2011). They likely predate or scavenge on atyid shrimp and blind cave fish (Carpenter, 1999; Koenemann et al., 2007). Remipedes have been seen carrying balls of microbe-laden sediment in their mouthparts, presumably filtering out nutrients (Yager, 1981). Yager (pers. comm.) investigated *S. tulumensis*, finding epibiotic microbes, which have recently been confirmed as chemosymbionts (See Chapter 3). Some remipede setae appear to be enervated and glandular suggesting a function aiding with immobilizing and detoxifying prey, including microbes (van der Hamm and Felgenhauer, 2008). Descriptions of remipede swimming and the metachronal movements of their trunk appendages have

largely been made from aquarium observations (Kohlhage and Yager, 1994; Carpenter, 1999; Koenemann et al., 2007).

Specimens were filmed and collected from Cenote Crustacea, an anchialine cave in Quintana Roo, Mexico, known for its high density of remipede crustaceans. A Sony SR-12 HD camera, fitted into a Light and Motion Stingray housing, was used for *in situ* observation at 60 to 200m of penetration from the cave entrance in the marine layer (14 to 19m in depth). Re-breather SCUBA was used during filming to eliminate bubbles and minimize sound disturbance. Focal remipedes were filmed at a distance of approximately half a meter and were followed until they were out of sight or had been followed for five minutes. Two sartek HID lamps provided illumination in August 2009 and January 2010 footage. Remipedes are easier to see when emitting fluorescence (Glenn et al., 2013). In July 2010, lamps were fitted with UV transmitting filter in order to view the fluorescence of remipedes. These blind animals were not likely affected by the change in lamps. MTS data files obtained from over 300 hours of filming and were converted to 422 format using Final Cut Pro 6.0.6. Focal animal sequences were extracted from video data taken in August, 2009, January, 2010, and July, 2010. Sequences visibly affected by diver-induced current or in which animals were not in focus were excluded from the data pool.

Maxillipeds and mandibles were removed from *S. tulumensis* prior to their dissection into subsamples of 6 or fewer segments. Fixation for SEM followed standard protocols (i.e. van der Hamm and Felgenhauer, 2008). Samples were critical point dried before viewing on a Hitachi TM-1000 Scanning Electron Microscope to identify chemosensory organs that might facilitate navigation.

In situ Turning study

Remipedes filmed swimming in the water column and at the sediment-water interface were scored during all exhibited turns. At the time of each turn, both turning angle (0-30^0, 31-60^0, 61-90^0, $>90^0$) and the number of metachronal waves on the inside and outside of the remipede were scored. Waves are quantified as groups of limbs moving metachronally in synchrony. If the number of waves was not visible, turns were not used in this analysis. Individuals that appeared to turn in response to an obstacle were also discarded. Turns per minute were calculated for individuals and averaged in each habitat type: SM sediment with greater than or equal to 12.5% surface cover by black or orange mats, SMO sediment with greater than or equal to 75% surface cover by black or orange mat, SN sediment containing less than 12.5% of mat cover, and WC water column. A Welch's ANOVA was used to determine whether mean differences in wave number on sides toward and away from direction of turn at varying turn angles are significantly different. Turn data were also used to determine whether habitat correlates with turning frequency and angle (see Appendix 1).

In situ Mat encounter study

Presence of vertical undulation and turn angle of focal remipedes at time of bacterial mat encounter were scored as 0^0 (straight swim), 1-30^0, 31-60^0, 61-90^0, 91-120^0, 121-150^0, or 151-180^0. Turns were scored relative to trajectory at time of mat encounter using a protractor and referencing a point on the video that was static (See Appendix 2). We also scored turns and undulations at a comparison time point of 10 seconds after mat

encounter. If the individual encountered another mat 10 seconds after the encounter point, a time point 20 seconds after the initial mat encounter was used for comparison. In the three instances in which the focal animal was no longer in view 10 seconds after the mat encounter, turn data were discarded. Wilcoxon Matched Pairs Sign Rank Tests were used to determine whether there were significant difference between behaviors exhibited at the time of mat encounters and at comparison time points using both turn and undulation data.

In order to determine how remipedes interact with mats, the trajectory of remipede interactions with mats was assessed for the interval beginning one second prior to mat encounter and ending one second after mat departure. In addition, mat areas were measured and estimated to calculate how much time the individual remipedes spent at different portions of the mat. Video was examined frame by frame at 30 frames per second using Fiji software (Rasband, 2011).

Mat Calculations and Percentage Mat Band Residence Time

Inner black mat and total mat area were measured in pixels (Figure 1 and Appendix 2). We calculated white mat area by subtracting black mat area from total mat area. Estimated Mat Areas (EMA) were measured as follows. Black (b) and total (t) mat areas were estimated by assuming roundness and averaging diameters (D) at 6 time points (Db and Dt, respectively):

$$\text{EMAb} = \pi(Db/2)^2 \ \& \ \text{EMAt} = \pi(Dt/2)^2$$

Since there were no statistical difference between EMAs and actual mat areas for either black or total mats, we used estimated mat areas in further analyses.

EMAb and EMAts were then multiplied by .5, .75, 1.25, etc. in order to calculate 50%, 75%, 125%, etc. Mat Bands, respectively (Figure 1). Because of the low flow of the caves, we assumed diffusion. Therefore, these bands were assumed to contain decreasing gradients of chemical concentration from the mat centroid. The radii of these EMAs (EMAradii) were then calculated:

ie. For estimated 50% black band radius: $\sqrt{(0.5\text{EMAb}/\pi)}$ = the 50%EMAbradius

and

ie. For estimated 75% total band mat radius: $\sqrt{(0.75\text{EMAt}/\pi)}$ = the 75%EMAtradius

In addition, black and white mat centroid x and y coordinates and the location of the anterior end of the remipede head at each frame were recorded. Using a Cartesian coordinate system, these centroids and %EAbradii %EAtradii, we determined where the focal individual's head was located during each frame with respect to mat area bands. Calculations of percentage mat band residence time were calculated. For standardization, the time at which remipede reached minimum distance to the mat centroid was logged as zero, making movement thereafter in positive time and movement prior in negative time.

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Results

Study site, filming, and characterization of sensory organs
In over eight hours of *in situ* observation taken during 14 rebreather SCUBA dives in August, 2009, January, 2010, and July, 2010, we filmed over 300 *S. tulumensis* focal individuals. Observations were obtained between 9am and 7pm CST and length varied between 3 seconds and 306 seconds. Remipedes were filmed in Cenote Crustacea's marine layer, whose geochemical characteristics range with depth from the halocline (14.6 m at the time of measurement) to the sediment (18.9m): 34.0-34.6 ppt, 25.3-25.6^{o}C, 0.6-0.5 mg/L O_2, pH 6.9-7.3.

A high density of pores, setae and sensilla occurred in all body areas examined, anterior-posterially from antennae to caudal filaments (Figure 2A-H). Setae, secondary setae, and pores were located at the junctures of mouthpart and walking appendages (Figure 2E).

In situ Turning study
Turning and undulatory behavior were seen both near the sediment and in the water column. Undulations are more frequent near the sediment, where individuals make vertical body waves ranging in amplitude from a quarter of a body length to greater than a body length. These undulations often occurred in succession or before or after a series of horizontal turns.

The number of waves on the inside and outside of 346 remipede turns of varying angles filmed in August, 2009, January and June 2010 were assessed (Figure 3). Remipedes decrease the frequency of beats on the inside of their turns relative to the outside of their turn as their turn angle increases (Figure 3). The mean differences in wave number on sides to and away from direction of turn at varying turn angles are significantly different (Welch's ANOVA all p values < 0.0001).

Total turns per minute varied between habitat types with the greatest turning frequency occurring in sediments with the least mat cover (SN) (Figure 4). In all microhabitats characterized, small angle turns (0-30°), were most frequent (data not shown). Large angle turns (>90°), representing directional changes, were more frequent in the water column and near the sediment containing low mat cover (SN) than near sediments containing high mat cover (SM, SMO) (data not shown).

in situ Mat encounter study
Speleonectes tulumensis (n=15) filmed swimming along the sediment encountered bacterial mats 26 times during observations in August 2009 and January 2010 footage. These individuals most often turned at approach (Figure 5). Turn angle was significantly greater upon mat encounter than at comparison times (Wilcoxon Matched Pairs Sign Rank Test p <0.05), in which vertical undulations were conservatively scored as straight swims). Most mats encountered by remipedes had a black central portion (130.1 mm^2 ± 62.3 SE) and a surrounding white portion (319.0 mm^2 ± 61.2 SE) (Figure 6). Total mat size ranged from 27.9-1520.9 mm^2 (385.2 mm^2 ± 75.4 SE) (Figure 6).

In the 27 mat encounters scored, *S. tulumensis* travelled towards the mat until a distance threshold of 6.1 mm ± 1.0 SE from the black mat centroid (6.3 mm ± 0.9 SE

from the total mat centroid) (Figure 7). Individuals spent the most time in the 101-150%
black mat band, which was often well into the exterior white mat band (51-125%total
mat band) (Figures 1, 8). One scored encounter involved a speckled black and white
mat.

Discussion

How do organisms orient to patchy cues?
Understanding the links between agility and sensory capabilities during orientation may
elucidate differential foraging success in heterogeneous environments. Such
environments may either 1) contain patches of resources spaced across a generally stable
fluid environment or 2) contain odor plumes broken up into temporally and spatially
intermittent patches (or filaments) by turbulence (Reidenbach and Koehl, 2011). The
first type of heterogeneity occurs in anchialine systems, generally characterized by low
nutrient availability (Neiber et al., 2011). Here, low flow likely increases boundary layer
height causing odor plumes to diffuse from sparse chemical sources, such as a microbial
mats, and results in stable gradients (Figure 1).

In such systems where cues are patchy, one might expect abundant
chemosensory organs. Recent laboratory experiments have found that blue crabs
(*Callinectes sapidus*) are efficient at sampling and tracking odor plumes using
chemosensory organs along their entire body (ie. Page et al., 2011; Reidenbach and
Koehl, 2011). While antennules are generally used for long-range detection of odor
plumes, leg receptors, which have been shown in turbulent flow to sense odors at near
range (Reidenbach and Koehl, 2011), may operate more efficiently in low flow
environments such as caves where plumes remain uninterrupted at further distances from
sources. We also noted a variety and great density of chemosensory organs on *S.
tulumensis*, indicating that odor sampling likely occurs along the entire length of their
bodies. Continuous remipede appendage beating may also facilitate cue recognition
through repeated sampling at such distances.

Increasing efficiency through behavior
 Behaviors, such as efficient turning and gradient tracking, may also facilitate foraging
in patchy landscapes. Remipedes' oar-like swimming described as metachronal
(Kohlhage and Yager, 1994) gives them their name, (*remigium* = oar). This study
increases our understanding of how these crustaceans, exhibiting a homonomous body
plan, have leveraged their simplified appendages for increased mobility. Instead of
beating evenly on both body sides, remipedes slow the stroke of their inner legs in order
to turn, using a strategy much like walking sticks (Dü rr and Ebeling, 2005). Other
metamerically moving pancrustaceans, like *Drosophila melanogaster*, use an alternative
strategy of altering the step length of their inner legs when turning (StrauB and
Heisenberg, 1990). Furthermore, gradient tracking allows individuals to access an
optimal mix of desired physico-chemical parameters and has previously only been
documented in prokaryotes. For example, magnetotactic bacteria align intracellular
magnetic iron oxide or sulfide minerals with magnetic fields to swim parallel to
geomagnetic fields and ultimately positioning themselves favorably in vertical chemical
gradients (Posfai M and Dunin–Borkowski, 2006 and references therein). We provide a

framework for the combination of these turning behaviors during navigation around chemosynthetic microbial mats in anchialine sediments. Bacterial communities, capable of sulfate reduction likely emit sulfides into the boundary layer.

Gradient tracking

Video analysis of *S. tulumensis* supports the use of chemoreceptors for gradient tracking of chemical cues emitted from microbial mats (ie. Fraenkel and Gunn, 1960). Since remipedes hover above sediment, it is unlikely that they are directly grazing on bacteria. Instead, they may be indirectly benefitting from emitted compounds, such as sulfides that are toxic to crustacean aerobic respiration in high concentrations (Hourdez and Lallier, 2007), but beneficial to the sulfide oxidizers that may be colonizing *S. tulumensis*. Observations of klinokinesis (increasing rate or angle of turning as the cue intensity increases) suggests that *S. tulumensis* is searching for a chemical cue instigated by initial encounter with a favorable mat-derived chemosensory stimulus (Fraenkel and Gunn, 1960). In addition, klinotaxis was observed nearest to the chemical source in mat dense areas and finally in proximity to the mat (Fraenkel and Gunn, 1960). There, remipedes decrease turning rate, indicating decreased sampling. This behavior was followed by tracking of black mats edges. Ultimately, the searching pattern observed indicates attraction rather than avoidance to mat edges, where a gradient of compounds have likely diffused from the mat centroid.

Gradient following behaviors are likely widespread in symbiotic invertebrates. The hydrothermal vent shrimp *Rimicaris exoculata* is hypothesized to inhabit substrate at the sulfide-rich vent plume and oxygen rich marine interface in order to supply its epibiotic sulfide oxidizing bacteria with metabolic substrates (Gebruk et al, 2000). These mineral rich plumes are geothermal and sometimes exhibit extremely high temperature. *Rimicaris* is thought to seek areas of intermediate temperature and therefore chemical concentration via specialized eyes that can detect infrared radiation (Van dover et al., 1989). Vent shrimp gradient following in a patchy environment are likely analogous to remipede behavior. If *S. tulumensis* epibionts are sulfide oxidizers, this remipede is likely tracking gradients to provide both the sulfides and oxygen necessary for chemosymbionts metabolism, thus supplementing its own nutrition. Similar behaviors have been hypothesized in methane seep and hydrothermal vent systems (Gebruk et al., 2000, Polz et al., 2000). Further anchialine investigation may inform evolutionary studies into the behavior of deep ocean chemosymbiotic organisms that are difficult to access and keep in the laboratory. Connecting locomotory behavior with sensory function will improve chemonavigation theory in a variety of chemosymbiotic clades (i.e., crustaceans, annelids, and mollusks) that occupy patchy fluid environments. Further investigations are needed to determine whether the turning threshold and following behavior observed is due to increased diffusion of microbial matrix compounds, increased sulfide diffusion, or decreases in oxygen concentrations near mats.

Acknowledgements

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Figure Captions

Figure 1. Schematic of remipede turning around microbial mats of two different sizes. Figure illustrates inner black microbial mat and outer white mat as well as increasing total distance mat bands as used in study. Note that the smaller mat at left requires a remipede of the same size to turn at a smaller angle to follow a gradient of choice (ie. 125% mat distance band). (Angle differences described and calculated in Appendix 1). In addition, decreasing sulfide and dissolved microbial protein concentration diffusing from center of mat and caused by low-flow regime is shown in two-dimensions.

Figure 2. Scanning Electron Micrographs of the numerous and varied sensory organs found on *Speleonectes tulumensios* (Remipedia). (A) Setae and pores at end of antennae and (B) sensilla, which are shown here on carapace, but were also found on antennae and other appendages. Variations of setae at the juncture of **c** propodus and endopod, (D) segment, (E) maxilliped propodus and carpus juncture. Note long, slender secondary setae in (D) that was prevalent throughout. (F-H) illustrates density of pores, setae and sensilla on caudal filaments. (F) shows entire caudal filament, while (G) illustrates tip of filament with three types of sensory organs and at (H) center a close up of sensilla can be seen. Scale Bars in (F) 500µm; in (A, C) 100µm; in (E, G) 50 µm; in (D) 20µm; in (A) 10µm .

Figure 3. The waves on the inside of 346 remipede turns of varying angles ($0\text{-}30^o$, $31\text{-}60^o$, $61\text{-}90^o$, and $>90^o$) filmed in August, 2009, January and June 2010 were subtracted from those on the outside of turns. This Figure shows the mean difference of waves counted on the outside and inside of individuals as they turned at various angles, illustrating that the difference in number of waves increases with increasing turn angle.

Figure 4. Turns per minute by individuals filmed *in situ* in various microhabitats illustrating variation in total rate of turns by habitat, with rate greatest in sediment with <12.5% black mat cover (SN).

Figure 5. Remipedes encountered mats 26 times in the footage captured in August 2009 and January 2010 (no encounters were captured in July 2010). The numbers of times behaviors, such as straight swim (0^0), sideways turn ($1\text{-}90^o$) or vertical undulation, were performed upon mat approach are shown in the above Figure. In the vast majority of encounters, remipedes avoided mats by turning.

Figure 6. Average mat area (mm^2) for 26 mats used for *in situ* mat encounter study. Black and Total mat areas were calculated by taking pixel areas from imageJ and converting them into mm^2, using average remipede length of 31.23 mm ± 2.0 SE as compared with remipede length in video. White mat area was calculated by subtracting black from total mat area.

Figure 7. Trajectory of (A) 26 remipedes as they approach the black centres of mats and (B) 27 remipedes as they approached the exteriors of mats. (One mat had no discernable interior black mat). Distance from centroid of total mat noted in mms. To standardize

time, time 0 was defined as the time at which remipedes reached the distance at which they will turn away from the mat. (A) This created a lower threshold of -100 ms and upper threshold of 67 ms, spanning 167 ms in turns with respect to the black mat. (B) In contrast, this created greater lower (-67 ms) and upper thresholds (233 ms), spanning 300 ms in turns with respect to the total mat. Time points from -1033 to 1133 ms had at least 20 individual turns included, whereas timepoints greater than 1033 and less than -1133 ms had between 6 and 18 individual turns, accounting for greater variation in these analyses.

Figure 8. Average residence time in milliseconds (± standard error) per percentage mat band is shown for (A) black mats and (B) total mats. Remipedes spent significantly more time in the 101-125 and 126-150% black bands, which was inside of exterior white bands.
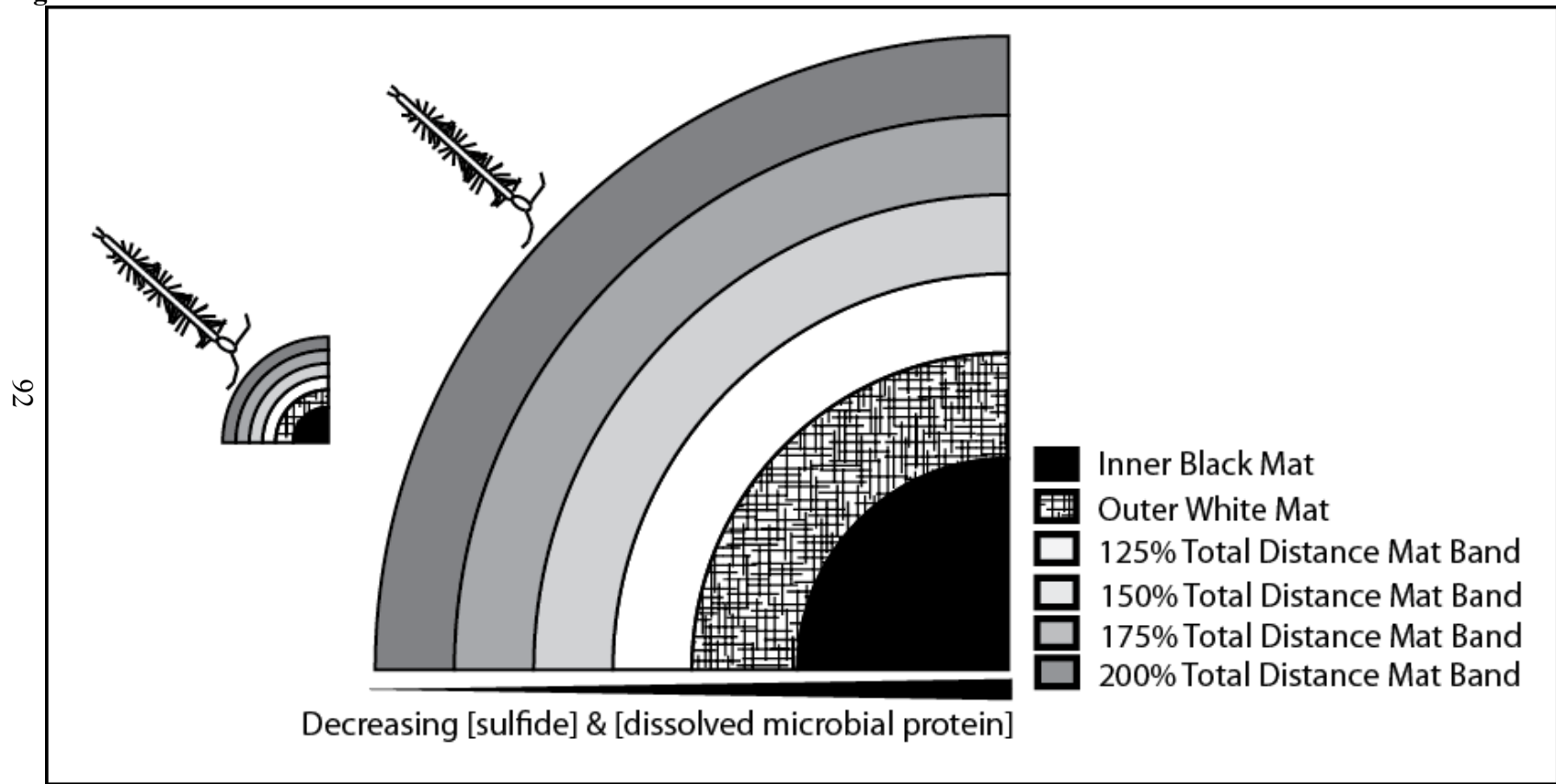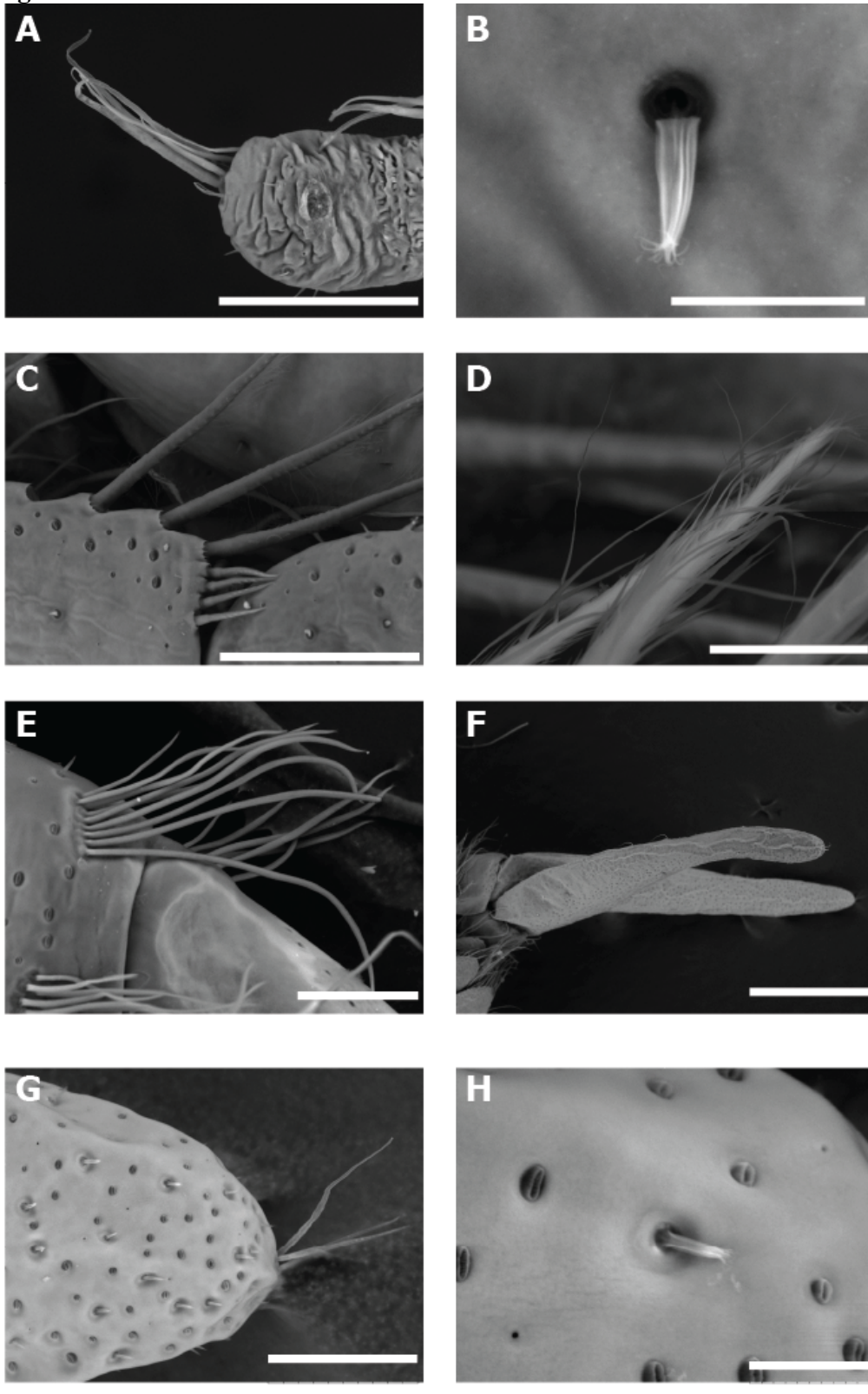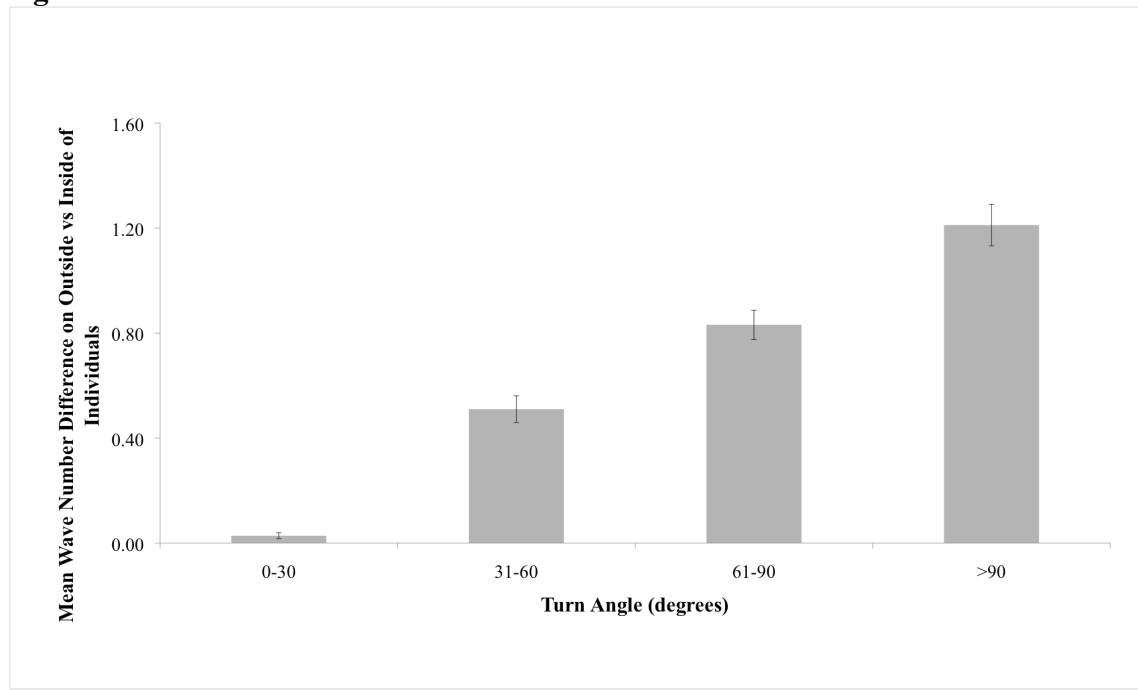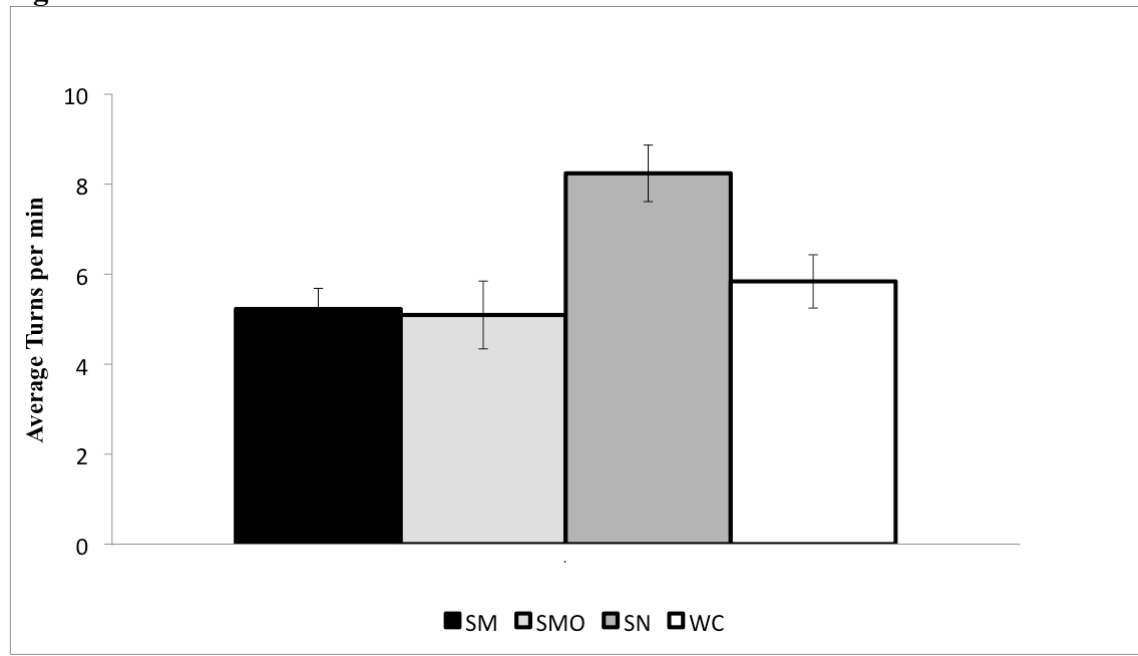
Figures

Figure 1.

Inner Black Mat
Outer White Mat
125% Total Distance Mat Band
150% Total Distance Mat Band
175% Total Distance Mat Band
200% Total Distance Mat Band

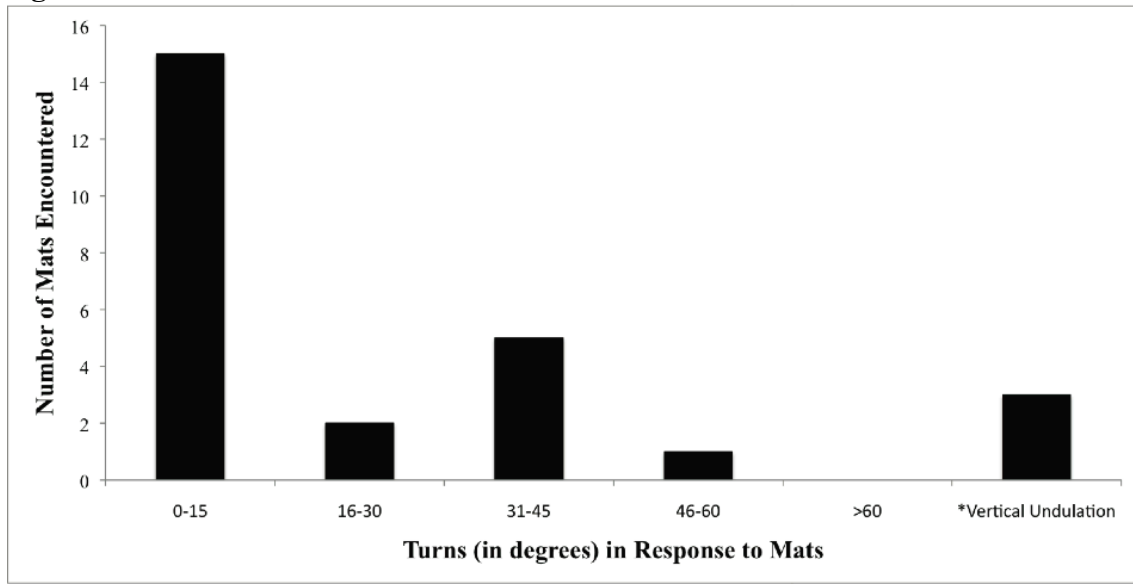Decreasing [sulfide] & [dissolved microbial protein]

Figure 2.
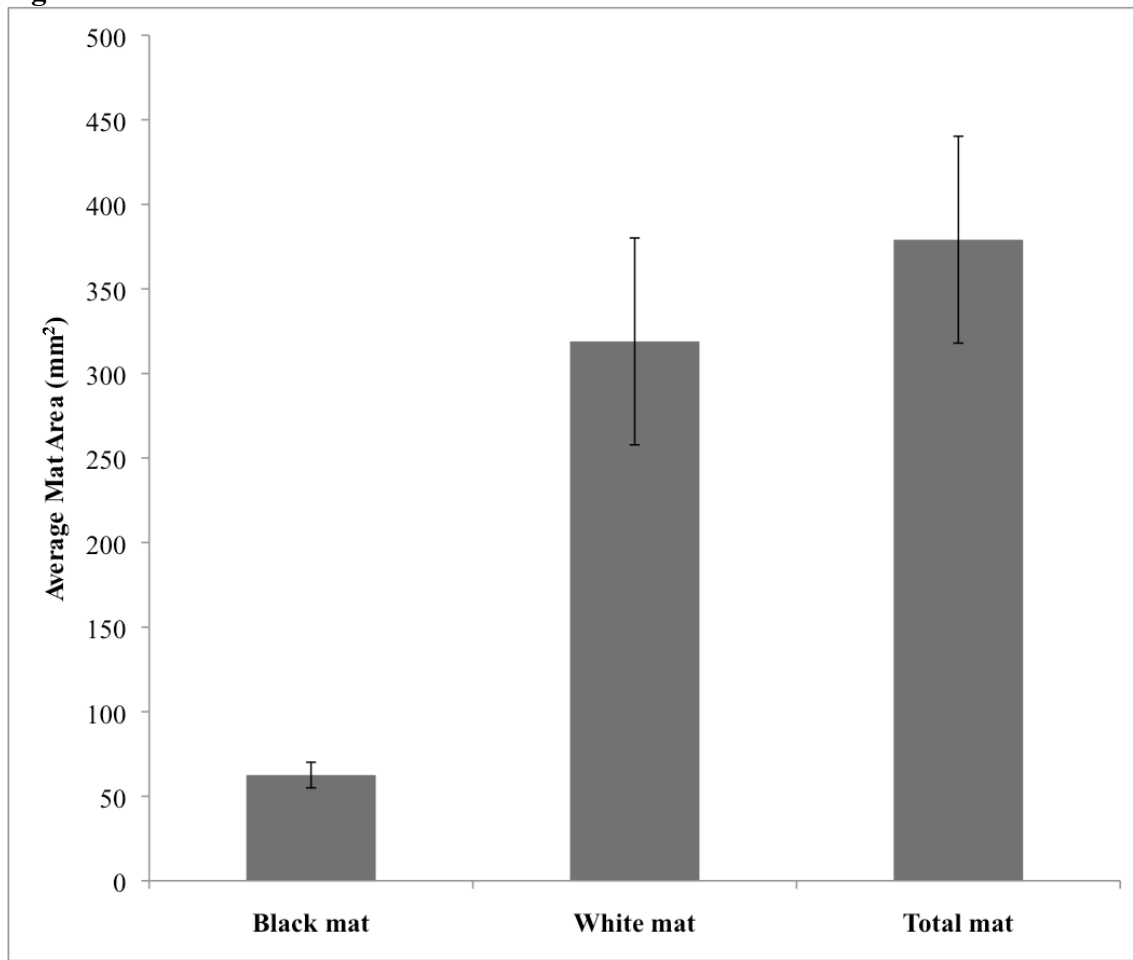
Figure 3.

Figure 4.

Figure 5.
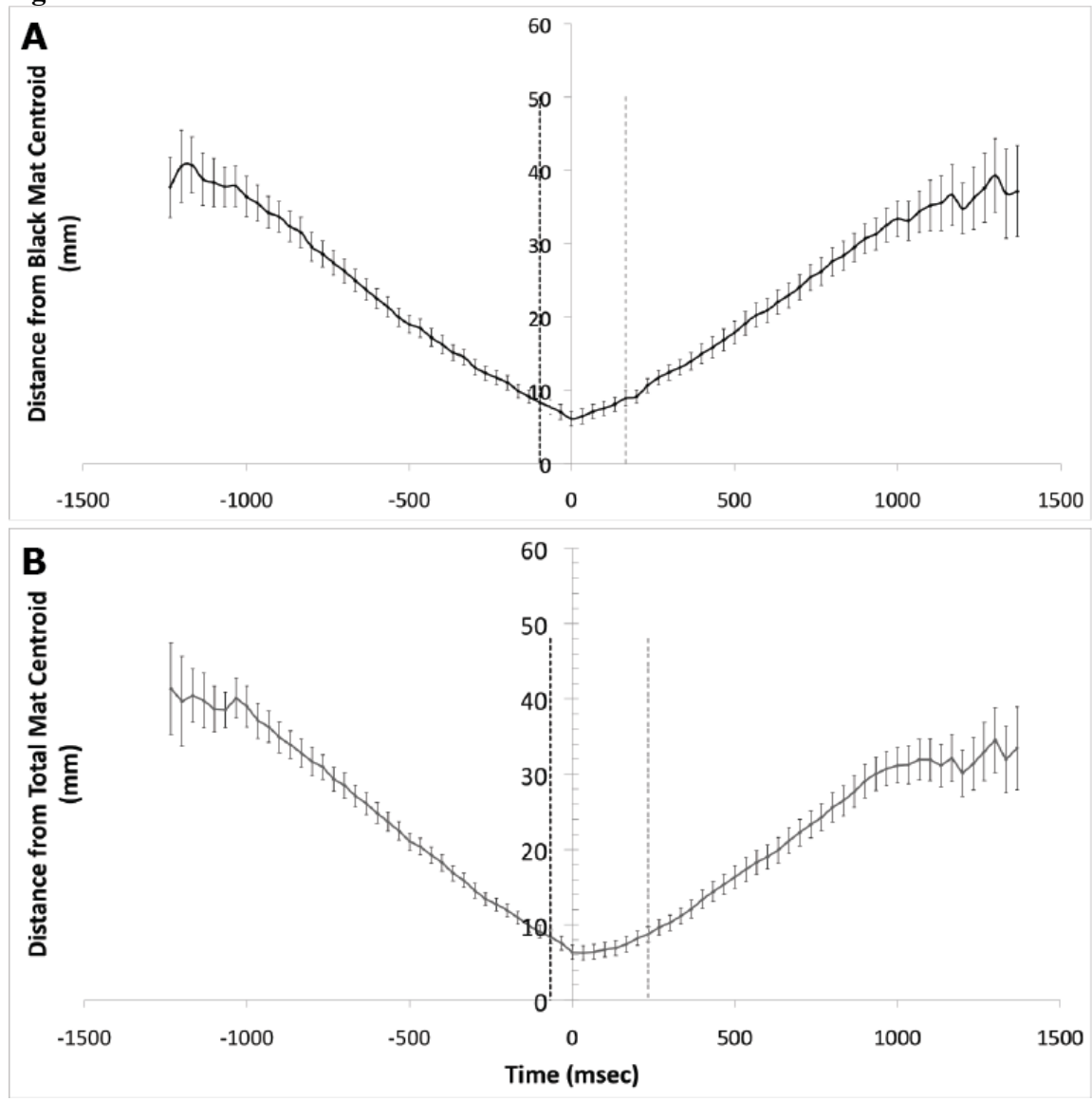
Figure 6.

Figure 7.

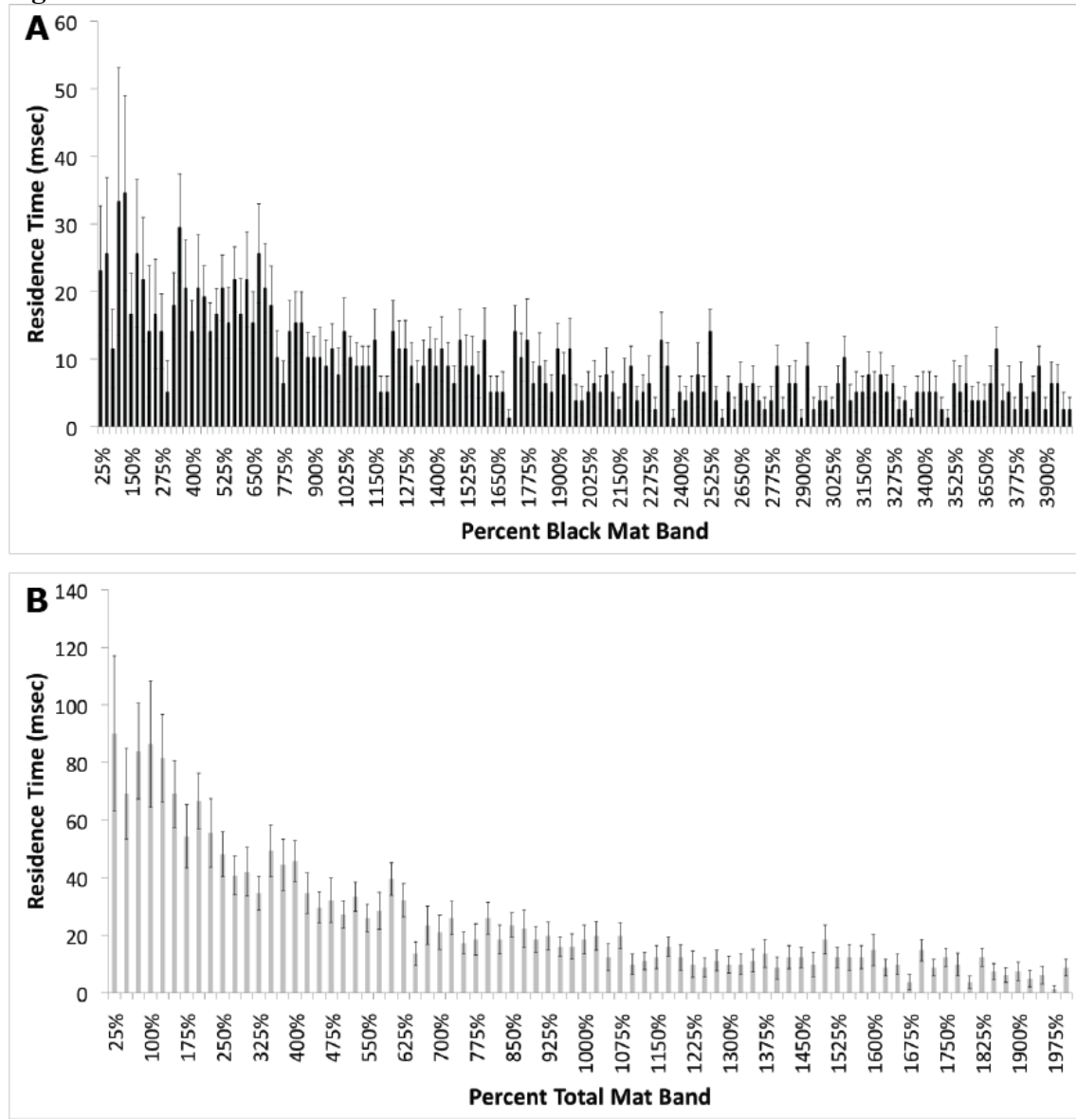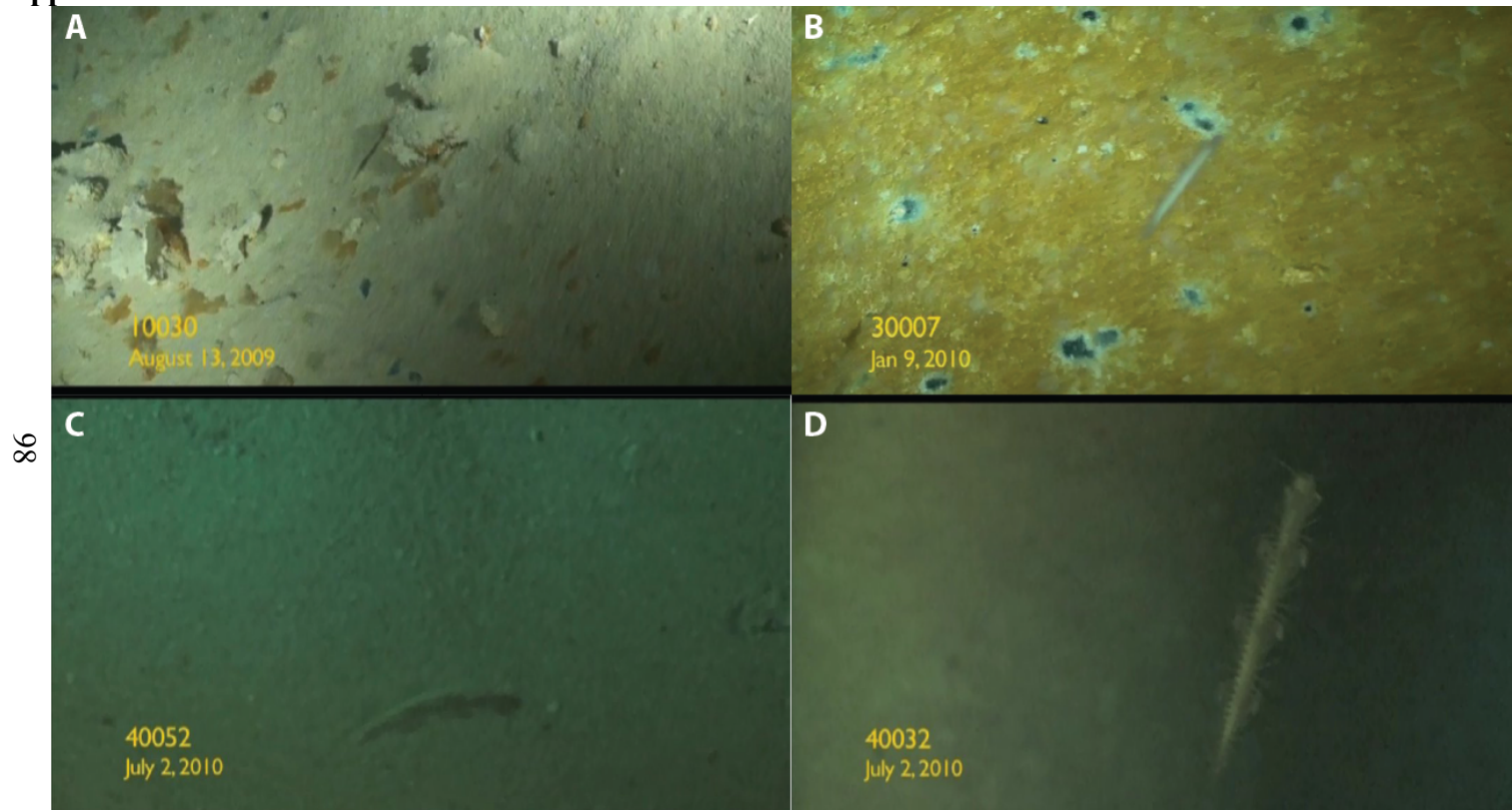**Figure 8.**
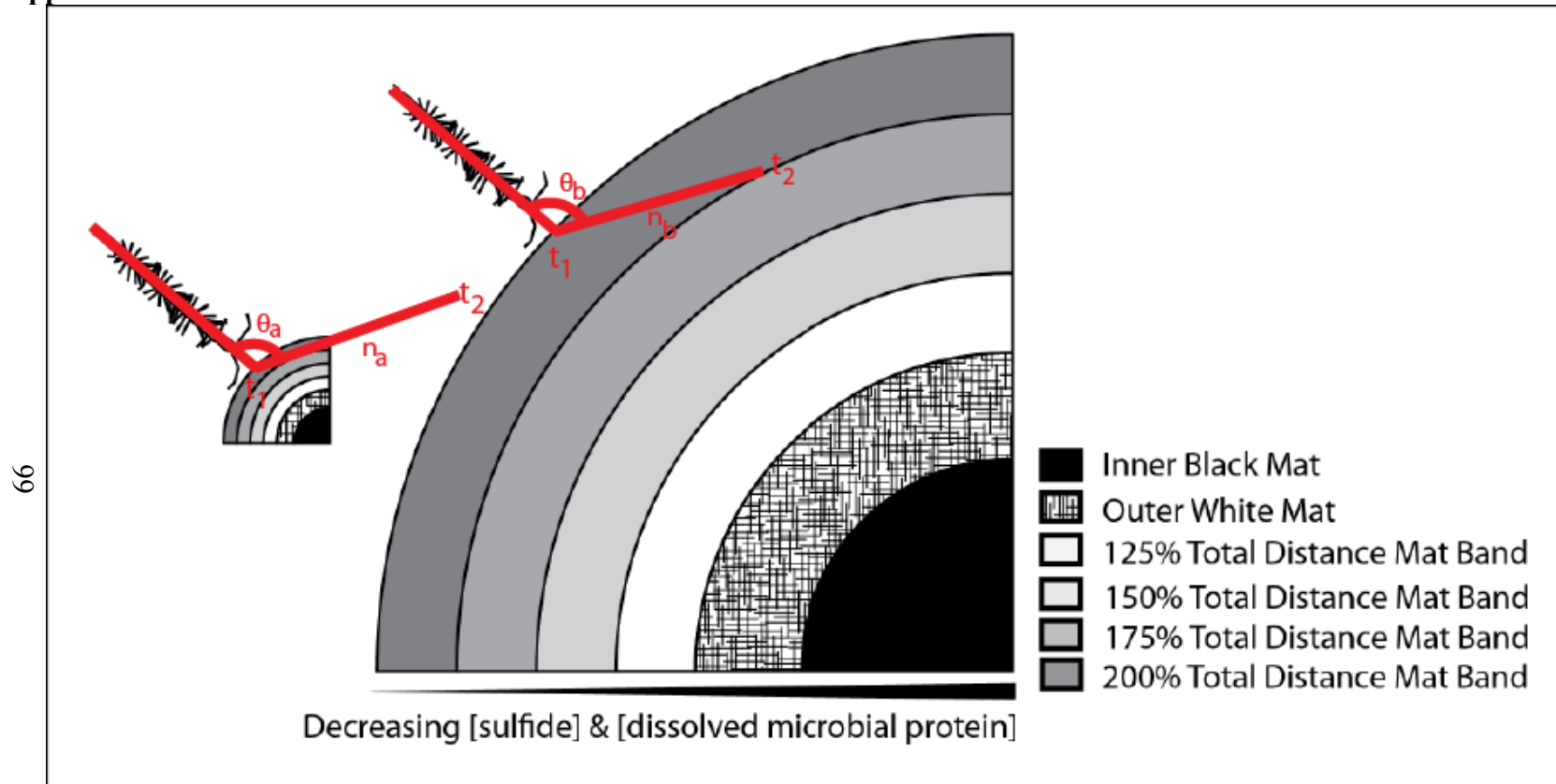
APPENDICES

Appendix 1.

Appendix 1. Microhabitats and Sediment types found in Cenote Crustacea. Clockwise from top left: (A) Sediment with greater than or equal to 12.5% surface cover by black mats (SM), (B) sediment with greater than or equal to 75% surface cover by black of orange mat (SMO), (C) Sediment containing less than 12.5% of mat cover (SN), and (D) water column (WC).

Appendix 2. Schematic of remipede turning around microbial mats of two different sizes with angle measurements. Figure illustrates inner black microbial mat and outer white mat as well as increasing total distance mat bands as used in study. Note that the smaller mat at left requires a remipede of the same size to turn at a smaller angle $\theta_a = 112°$ (versus $\theta_b = 115°$) to follow a gradient of choice (ie. 175% mat distance band), regardless of travelling the same distance ($n_a = n_b$). In addition, decreasing sulfide and dissolved microbial protein concentration diffusing from center of mat and caused by low-flow regime is shown in two-dimensions.

Conclusions

Changing caves:
Shifting focus to the dynamic anchialine environment

Review

Underwater cave research will increase our understanding of how life evolved in dark, extreme ecosystems. However, the investigation of underwater subterranean environments requires specialized field training and is often limited by accessibility and sampling time. As such, there are few systematic studies of ecosystem function, community structure, and evolutionary processes in these systems. Here, I review research in anchialine habitats– systems in which a landlocked marine layer flows under less saline layers. It is my aim to illustrate recent advances relating to the evolution and ecosystem function of crustacean communities in these extreme systems and to point to areas in which research is especially deficient.

Initial theories regarding cave diversity and biomass operated on several assumptions 1) that cave ecosystems were extreme due to their lack of constant nutrient supply from surface photosynthetic sources (Engel, 2007), 2) that caves are stable systems buffered from climatic changes (e.g., Dov Por, 2008, Neiber et al. 2011). While Engel (2007) and others have reviewed evidence of chemosynthetic energy production in subterranean systems, these studies are rare and often lack evidence of in situ metabolic activity. Furthermore, growing evidence suggests that caves are dynamic, changing over both neontological and paleontological time scales.

Should chemosynthetic energy fuel anchialine habitats, the stratified water layers that shaped microbial primary producers, would in turn likely affect macrofauna. Each microhabitat in these stratified systems has a distinct associated faunal community. In the Yucatan and Caribbean, this community is largely crustacean-dominated (Neiber et al., 2011) and includes many species of caridean shrimp and amphipods that are restricted to the meteoric waters above the halocline (Debrot et al. 2003; De Grave et al. 2008). Some atyid shrimp, however, inhabit both marine and overlying brackish components of anchialine systems (Sanz & Platvoet, 1995; Alvarez et al., 2005; Hunter et al. 2008, Pakes pers. observ.) In addition, the members of the anchialine endemic class Remipedia are known as marine-layer specialists and likely restricted to this underlying marine layer of anchialine systems (Mejía-Ortíz et al. 2007; Neiber et al. 2011).

Energy Production in Anchialine Habitats

Growing evidence that supports the existence of chemosynthetically fueled subterranean ecosystems has undermined the theory that cave nutrients are exclusively surface-derived. For example, freeliving and symbiotic chemolithoautotrophs have been discovered in geothermal submerged limestone (karst) caves (Hose et al., 2000; Dattagupta et al., 2007) and dry caves (Barton and Northup, 2007). In addition, phylogenetic affiliation of chemosynthetic communities has been reported from sunlight

anchialine blueholes (Cangenella et al. 2007, Gonzalez et al., 2011). Likewise dark anchialine systems have been sampled for both the morphological and phylogenetic affinities of their communities and these data have been used along with habitat geochemistry to suggest that chemoautotrophic metabolic activity occurs in these caves (e.g., Seymour et al. 2007; Humphreys et al. 2012).

Chapter One of the present study is the first to integrate culture-independent and culture dependent methods in a cave study. The resulting findings stemming from geochemical analyses, enrichment, and beta-diversity studies of microbial community structure begins to elucidate the significant role of chemosynthetic microorganisms in anchialine systems. Here, 16S rRNA gene sequencing of isolated bacteria and subsequent phylogenetic analyses revealed the presence of chemolithoautotrophic Gammaproteobacteria and Deltaproteobacteria in the sediment. This result complemented evidence that the proteobacterial clades were enriched in the sediment community relative to the water column.

The potential for these chemosynthetic bacteria in anchialine faunal nutrition and community structuring is illustrated by anchialine symbiosis studies (Chapters 2-3) and food web analyses (Pohlman, 1997; Chapter 4) that suggest a chemosynthetically derived nutrient base to the Yucatan anchialine food web (Chapter 4). As the cave investigated in the current study includes a passage known for its high density of anchialine taxa (Koenemann et al., 2007) and another passage with greater diversity, but lower biomass (Neiber et al. 2012) it provides a natural experiment with which to correlatively test affects of chemosynthetic input and community structure dynamics. Findings suggested a bottom up pressure to the anchialine food web, wherein *Typhaltya pearsei* and their chemosynthetic endosymbiotic bacteria (Chapter 2) may be more abundant in the stratified passage than the fully marine due to increased metabolic capabilities at the halocline.

As nutritional associations between chemosynthetic microbes and eukaryotes form the basis of life in many extreme environments, these findings are not surprising. It is the location of these symbionts and the potential for their study that makes this system unique. Chemosynthetic symbioses are found associated with seven eukaryotic phyla support (Cavanaugh et al., 2006, Dubilier et al., 2008). Such mutualisms support a diversity of communities, including those found in intertidal, hydrothermal vent, methane seep, whale and wood fall ecosystems (Cavanaugh et al., 2006, Dubilier et al., 2008), as well as cave ecosystems (e.g., Boston et al., 2001; Barton and Northup, 2007; Dattagupta et al., 2009). In anchialine caves, restricted tidal flow (Iliffe and Kornicker, 2009), results in stable gradients including hypoxic and sulfidic marine layers, underlying oxygenated brackish water layers (Iliffe and Kornicker, 2009). Interfaces between chemosynthetic substrates (CH_4, H_2S) and oxygen (Pohlman et al., 1997) in these caves make ideal habitats for mutualisms between microbes and macrofaunal hosts. This dissertation described putative chemosynthetic extracellular symbioses in the cave shrimp, *Typhlatya pearsei,* remarkable in that it marks the first occurrence of chemosynthetic intracellular symbiosis in an arthropod (*T. pearsei*) (Chapter 2). Furthermore, here I report a member of the endemic cave crustacean class Remipedia, *Speleonectes tulumensis*, likely hosts ectobiotic bacteria (Chapter 3) and may alter it's behavior to increase colonization or the concentrations of chemosynthetic subtrate available for its symbiotic microbes (Chapter 5).

Changing environments and changing communities

Ecological Time scales– Because caves are sheltered from sunlight and direct surface disturbance, they have often been described as static systems. However, several recent findings have refuted this claim on ecological and evolutionary timescales. For example, my dissertation research provides some of the only 16S rRNA and 16S rDNA of microbial communities in extreme habitats and shows variation in persistent and active bacterial communities (Chapter 1). This discrepancy suggests that turnover is high in certain microhabitats of anchaline caves, such as sediment microbial mats. These results while novel, are not surprising when considering temporal changes in halocline and sulfide layers (e.g., Seymour et al. 2007). Evidence points to tidal fluctuations influencing the depth of physicochemical layers. Such daily fluctuation or disturbance creates a dynamic habitat for successional communities and further mix microbial populations in situ.

Other temporal changes to water chemistry may also be seasonal or sporadic (Iliffe and Schram, 2013). Seymour et al., indicated that some anchialine systems may experience fluctuations in water level and therefore halocline of up to 10% of oceanic oscillations (2007). While haloclines may only change on the order of centimeters over ecological time scales, these oscillations likely affect the microbiota within the system that operate on the small spatial scale of microns. In addition, I have noticed an increase in particulate matter in the water column and a decrease in taxa following winter storms. Whether these storms increase organic material flowing into the cave and create later blooms of life must be examined with more care in the future. However, it should be noted that in these winter months (December and January) shrimp exoskeletons have been observed to be more abundant in the water column and may be a source of chemosynthetic substrates and carbon in the redox gradient and sediment microbial communities (pers. observ.)

Evolutionary Time Scales– Anchialine habitats are physically and biologically complex. In these systems the marine layer which flows under less saline layers, each with its associated fauna. These organisms may depend on input from both overlying terrestrial (meteoric water) and tidally influenced, land-locked marine environments. The effect of salinity tolerance on species connectivity and through it distribution in these complex systems are likely great, but as yet untested. How then do sea levels past and present determine diversity of anchialine fauna?

The influence of sea level on connectivity and consequently on anchialine diversity may vary as a function of a cave's topography (Iliffe 2000). Niches may be kept in tact as sea level tracks horizontally and vertically through continuous cave passages. This continuity of habitat provides connections between adjacent vertical levels of the cave complex, increasing a taxon's survivability through time. Falling sea levels dry out formerly available aquatic habitats, resulting in migration or extinction (Jablonski 1985; Finnegan et al. 2012). Depending on karst island topography, sea level fall will interrupt subterranean underwater passages, by breaking their continuity with emerging land patches. This splintering of suitable habitat into underwater islands with fragmented populations of species will drive evolutionary divergence and speciation (Jaume et al.

2008; Zaksek et al. 2009; Fiser et al. 2012; Moritsch et al., 2014). Conversely, sea level will connect previously fragmented passages via flooding, allowing organisms to disperse, in some cases reconstituting splintered species ranges (Smith 2001; Cromer et al. 2005; Zaksek et al. 2007; Botello and Alvarez 2010).

Other factors, including proximity to the sea and tidal current flows may affect dispersal distances (Christman and Culver 2001). Climate change likely for example results in different meteoric water inputs (via changes temperature and precipitation) which alter the suitability of cave regions or networks. Urbanization and industrialization also contribute to pollution and water diversion within these extreme ecosystems. This stratified system may be particularly susceptible to increasing global sea temperatures, which may result in admixture between cave water layers, decreasing habitat complexity and suitable habitat for many microhabitat specialists (Mortisch et al., accepted).

If sea level rises as much as the predicted 1.4 m in the next century (Rahmstorf 2007), the marine-meteoric interface will likely move inland, away from the coast. How will this affect cave locality and regional cave diversity? As species richness and phylogenetic diversity have declined with distance from the coast for both remipedes and atyids during past sea level rises (Moritsch et al., accepted). The decline in diversity of atyids relative to remipedes in this scenariuo was likely a result of their different evolutionary histories. While remipedes are only known from their pleisomorphic marine habitat, extant atyid shrimp lineages have repeatedly become established in meteoric and other low salinity habitats (Page et al., 2008). As such, remipedes and atyids, as well as other anchialine organisms, would likely respond differently to sea level and temperature changes. Similarly, collapses of the meteoric lense have been observed in the geologic record in Bermudian anchialine systems (Van Hengstum and Scott, 2012). Although micropaleontological studies incorporating radio carbon dates are few, they have great potential in revealing the dynamic nature of subterranean habitats (Van Hengstum and Scott, 2012). More studies integrating sea level changes over evolutionary time scales will inform our understanding of how previous perturbations may have shaped current distributions in these extreme ecosystems.

This dissertation has revealed several ways (predation, chemosynthetic symbiosis, scavenging, and grazing) in which anchialine organisms make a living in these extreme systems. The anchialine environment is complex and ecosystem function depends on intra-, inter, and abiotic interactions (Estes et al., 2013). Therefore, in order to determine how anchialine ecosystem will be altered with changing climate and water use, it is imperative that future studies gather habitat (geologic, geochemical) parameters as well as trophic dynamics data on cave endemics. These data will allow for distribution modeling in addition to aiding in our discovery of new anchialine communities and understanding of their ecosystem interactions.

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