

ECOPHYSIOLOGY AND FOOD WEB DYNAMICS OF SPRING ECOTONE
COMMUNITIES IN THE EDWARDS AQUIFER, USA

by

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DEDICATION

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	v
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
LIST OF ABBREVIATIONS.....	xi
ABSTRACT.....	xiii
CHAPTER	
1. INTRODUCTION.....	1
REFERENCES CITED.....	5
2. HABITAT ASSOCIATIONS PREDICTS RESPONSES OF SEVERAL SPECIES OF RIFFLE BEETLES TO ENVIRONMENTAL STRESSORS.....	8
INTRODUCTION.....	8
MATERIALS AND METHODS.....	12
Animal collection, housing, and initial acclimation.....	12
Exposure to acute stressors and assessing critical thresholds.....	13
Exposure to chronic stressors experiments.....	15
Data analysis.....	18
RESULTS.....	19
Exposure to acute stressors and assessing critical thresholds.....	19
Exposure to chronic stressors experiments.....	20
DISCUSSION.....	24
ACKNOWLEDGEMENTS.....	28
REFERENCES CITED.....	35
3. INTERACTIONS AT SURFACE - SUBTERRANEAN ECOTONES: THE STRUCTURE AND FUNCTION OF FOOD WEBS IN SPRING OPENINGS..	41
INTRODUCTION.....	41
MATERIALS AND METHODS.....	44
Study sites and field sampling.....	44

Compound specific isotope analysis of essential amino acids	48
Data analysis	51
RESULTS.....	54
Bulk isotope analysis, dietary mixing models, and niche modeling.....	54
$\delta^{13}\text{C}_{\text{AA}}$ analyses and Comal Springs elmids	58
DISCUSSION	60
ACKNOWLEDGEMENTS	66
REFERENCES CITED.....	75
4. METABOLIC RESPONSES TO LONG-TERM FOOD DEPRIVATION IN SUBTERRANEAN AND SURFACE AMPHIPODS.....	87
INTRODUCTION	87
MATERIAL AND METHODS.....	90
Site description and organism collection.....	90
Estimation of basal metabolic rates.....	92
Food deprivation and metabolic responses.....	92
Metabolite analysis.....	94
Data analysis	94
RESULTS.....	95
O ₂ consumption rates.....	95
Effects of food deprivation on metabolite content.....	95
DISCUSSION	97
ACKNOWLEDGEMENTS	100
REFERENCES CITED.....	103
5. CONCLUSIONS	110
REFERENCES CITED	113

LIST OF TABLES

Table	Page
3.1: Bayesian mixing model outputs for Comal and Finegan springs, with the percent contribution of basal resources to each consumer species	71
3.2: Results of the probabilities of niche overlap among riffle beetles and dryopid beetle at Comal Springs and Finegan Springs	73
3.3: Results of the probabilities of niche overlap among amphipods at Comal Springs and Finegan Springs	74

LIST OF FIGURES

Figure	Page
2.1: Map with sampling locations for the four riffle beetle species.....	29
2.2: Threshold T for LOR (A), and the threshold DO concentration for LOR (B) in <i>H. comalensis</i> and <i>H. glabra</i> (mean \pm 1 SE).....	30
2.3: Time-series of proportional survival of <i>H. comalensis</i> (A), <i>H. glabra</i> (B), <i>H. vulnerata</i> (C) and <i>M. pusillus</i> (D) exposed to different T treatments for 60 days.....	31
2.4: Estimated Median Lethal T (LT ₅₀) values \pm standard error (SE) (A), Estimated Median Lethal Time (Lt ₅₀) values \pm 95% confidence intervals for each tested T (B) for the four elmids species.....	32
2.5: Mass-specific O ₂ consumption rates of the four elmids species across the four experimental T s (A) and the calculated Q_{10} values for each species across three T ranges (B) (mean \pm 1 SE).....	33
2.6: Estimated Median Lethal Concentration (LC ₅₀) values \pm standard Error (SE) (A), Estimated Median Lethal Time (Lt ₅₀) values \pm 95% confidence intervals for each tested DO concentrations (B) for <i>H. comalensis</i> and <i>H. glabra</i>	34
3.1: Stable isotope ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) biplot for Spring Run 3 (A), Spring Island (B), and Finegan Springs (C).....	67

3.2: Normalized essential amino acid $\delta^{13}\text{C}$ values for basal sources from the literature (A), basal food resources from Comal (B), and <i>H. comalensis</i> and <i>M. pusillus</i> (C)	68
3.3: Linear discriminant analysis figure (LDA) graph portraying normalized $\delta^{13}\text{CAA}$ values for literature basal resources (fungi, bacteria, algae and plants), Comal Springs basal resources (periphyton, leaves, and wood), and <i>H. comalensis</i> and <i>M. pusillus</i> from the Comal system.....	69
3.4: FRUITS model output for <i>H. comalensis</i> (A, C) and <i>M. pusillus</i> (B, D) using <i>in situ</i> organic matter sources (CPOM and periphyton) (A, B) and outputs for runs utilizing literature data for producer groups (fungi, bacteria, algae, and plants) (C, D).....	70
4.1: Oxygen consumption in darkness for <i>Stygobromus pecki</i> and <i>Synurella</i> sp. at ambient temperature of 23°C.....	101
4.2: Changes in the levels of body metabolites in <i>Stygobromus pecki</i> and <i>Synurella</i> sp: Carbohydrates (A), Proteins (B), Lipids (C) during long-term food deprivation.....	102

LIST OF ABBREVIATIONS

Abbreviation	Description
T	Environmental temperature
T_b	Body temperature
T_o	Thermal optimum
DO	Dissolved oxygen
CT_{Max}	Critical thermal maximum
CDO_{Min}	Critical DO minimum
LOR	Loss of response
DM	Dry mass
Q_{10}	Temperature coefficient
GLM	Generalized linear model
GEE	Generalized estimating equation
Lt_{50}	Median lethal time
LT_{50}	Median lethal temperature
LC_{50}	Median lethal concentration
OM	Organic matter
CSIA	Compound-specific stable isotopes
EAA	Essential amino acids
HDPE	High density polyethylene

CPOM	Coarse particulate organic matter
FPOM	Fine particulate organic matter
SR3	Spring run 3
SI	Spring Island
AA	Amino acid
TEFs	Trophic enrichment factors
HCL	Hydrochloric acid
JAGS	Just Another Gibbs Sampler
MCMC	Markov chain Monte-Carlo
simmr	Stable isotope mixing model in R
LDA	Linear discrimination analysis
FRUITS	Food reconstruction using isotopic transferred signals
Thr	Threonine
Leu	Leucine
Val	Valine
Ile	Isoleucine
Phe	Phenylalanine
Lys	Lysine
ANOVA	Analysis of variance
ww	Wet weight

ABSTRACT

Spring orifices serve as ecotones between groundwater and surface water habitats. It is thought that organisms living in physicochemically stable spring ecotones should exhibit small tolerance ranges; however, previous experiments examining this prediction are equivocal. I examined this hypothesis by investigating effects of elevated temperature and decreased dissolved oxygen on several riffle beetle species (Coleoptera: Elmidae), including two ecotone specialists. Results indicate that ecotone-associated species exhibit stenothermal tolerance profiles when compared to surface species. I also examined resource use in invertebrate communities at two spring ecotones using stable isotopes of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) and amino acid-specific stable isotopes ($\delta^{13}\text{CAA}$). Results indicate that spring ecotones contain trophically complex communities with substantial niche partitioning among species. I finally examined the hypothesis that subterranean organisms in systems with ample energy resources (such as guano caves or spring ecotones) may not exhibit reduced metabolic rates. I assessed metabolic and biochemical responses of a subterranean amphipod (*Stygobromus pecki*) that inhabits spring ecotones and compared these responses to their epigeal relative. Results indicate that *S. pecki*, despite its occupation of relatively resource rich spring opening ecotones, still exhibit lower metabolic rates relative to their epigeal relative. Cumulatively, this body of research provides new and critical information on the ecology and evolution of spring ecotone communities, which are among the least studied and poorly understood aquatic ecosystems.

1. INTRODUCTION

Groundwater is the largest hydrological pool on the planet after the oceans (Marmonier et al. 1993, Gibert and Deharveng 2002). Groundwater ecosystems develop in sediments or rocks and are generally protected against surface environmental changes and may exhibit a high degree of stability for millions of years. Subterranean biologists have long contended that low energy and organic (OM) inputs, lower habitat availability and complexity, and simple webs make groundwater ecosystems markedly different from surface water ecosystems (Poulson & Lavoie 2000, Gibert and Deharveng 2002). The paradigm that typical subterranean conditions (i.e., complete darkness, low energy, and consistent physicochemical conditions) in groundwater ecosystems imposes selection pressures on organisms to exhibit a suite of specific traits, often characterized by loss of eyes and pigments, long and/or setaceous appendages, and low metabolic and reproductive rates, is now being revised as many studies show that some of these ecosystems harbor diverse biological assemblages composed of a diversity of characteristics (Gibert and Deharveng 2002, Fernandez et al. 2016, Hutchins et al. 2016).

Springs are defined as a physical location where groundwater emerges from the subsurface and flows into epigeal environments. Spring openings serve as ecotones that link multiple distinct ecosystem types: terrestrial and aquatic habitats, and groundwater (hypogean) and epigeal habitats (Cantonati et al. 2012). Biological assemblages inhabiting spring opening ecotones are often composed of a organisms representing a variety of habitat preferences and adaptations, including taxa solely restricted to the proximity of spring openings (i.e., crenobionts), more widespread epigeal taxa that are also found in spring influenced environments (i.e., crenophilic species), and subterranean

adapted taxa that are associated with shallow hypogean environments. Thus, spring ecotones serve as points of high biodiversity in many surface water networks, by containing assemblages composed of organisms representing a variety of morphological and physiological adaptations, life history strategies, and trophic ecologies. Although springs are classified as groundwater-dependent ecosystems, they are usually approached from an epigeal perspective, and spring ecotones remains relatively understudied. As the demand for groundwater increases, integrating information on spring ecotonal communities and making it readily available to resource managers has become imperative for the protection of aquifers and their biota, as well as the downstream catchment that receives groundwater flows.

The Edwards Aquifer in central Texas, USA, is a karst limestone aquifer that is one of the most biodiverse groundwater ecosystems in the world in terms of stygobionts (i.e., obligate subterranean aquatic organisms) (Longley 1981, Hutchins 2017). Groundwater from the Edwards Aquifer discharges from a diversity of springs and spring complexes throughout the region. Studies presented in this dissertation examined the ecophysiology and trophic ecology of invertebrate fauna that occur within and in proximity of spring orifice ecotones of the Edwards Aquifer. The three themes of the studies presented here, corresponding to the three chapters of this dissertation are: (1) identify the temperature and dissolved oxygen (DO) tolerances of crenic macroinvertebrates and comparing these responses to related surface taxa, (2) examine resource uses and trophic ecology of spring ecotone macroinvertebrate communities in two large spring complex systems to understand the role of competition and resource partitioning in these ecotone environments, and (3) assess metabolic responses to food deprivation in a spring ecotone

specialist subterranean invertebrate and compare these responses to a related surface species. The rationale for each is summarized below.

Groundwater dominated systems (i.e., springs) are generally more protected from climatic variability due to consistent inputs of groundwater that exhibits limited temporal physicochemical variability (Vanderkamp 1995). Thus, it is widely thought that spring-associated species should be adapted to stenothermal conditions (Hubbs 1995) and spring-obligate taxa will be extremely sensitive to increased thermal variability. Reduced spring discharge during a prolonged drought or due to excessive groundwater pumping can lead to changes or increased variability in temperature and DO concentrations that could be problematic for spring-obligate species. In contrast, it is predicted that more cosmopolitan surface water associated species should be adapted to deal with more variable conditions and that changes in spring ecotone water quality may select against spring obligate taxa. In Chapter 1 of this dissertation, I examined the hypothesis that crenic species should exhibit more limited ranges of environmental tolerances in a suite of riffle beetles (family: Elmidae) species. This study is the first of its kind to examine responses of multiple elmids (a group that is often used as a water quality indicator species) that are spring- and surface water associated.

It is hypothesized that spring ecotones are more resource limited (allochthonous and autochthonous inputs) than surface water dominated systems, but to date there is little data on resource availability and food web dynamics within spring ecotones (Odum 1957, Tilly 1968). If spring ecotones are in fact more resource limited, then there may be intense competition for food resources and habitat space among taxa found within spring ecotones. Co-occurrence of diverse groups of organisms from different environmental

settings (e.g., epigeal, crenic, and hypogean species) in spatially-confined spring opening ecotones may be due to their ability to avoid competitive exclusion through partitioning food resources (Hardin 1960, Schoener 1974). Mechanisms facilitating coexistence of diverse invertebrate communities in spring systems is not understood but resource partitioning due to specialized feeding habits may have a role in influencing this pattern (Bowles et al. 2003, Hutchins et al. 2014). In Chapter 2 of this dissertation, I examined patterns in resource and habitat use in macroinvertebrates in two spring systems using bulk stable isotopes of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) and compound-specific stable isotopes in amino acids. I additionally used bulk isotope data to estimate niche overlap between phylogenetically related species found within spring ecotones.

Traditionally, subterranean ecosystems were thought to be severely resource and energy limited due to spatiotemporal patchiness of resources, due to the intermittent input of allochthonous (surface-generated OM) inputs, and a lack of *in situ* autotrophic production (Poulson 1964, Hüppop 1985). It is also widely thought that subterranean-adapted organisms living in low energy habitats should be able to withstand long periods of food shortages due to a lower metabolic rate relative to epigeal counterparts. In addition, it has been observed that subterranean taxa exhibit markedly different patterns of internal energy reserves (proteins, carbohydrates, and lipids) during prolonged periods of food deprivation (starvation) when compared to phylogenetically related epigeal species (Hervant et al. 1999). However, previous studies have speculated that subterranean organisms which occupy systems with ample energy resources (e.g., bat guano caves or spring ecotones) may not exhibit reduced metabolic rate and differential use of energy reserves (Culver and Poulson, 1971). The equivocal nature of previous

findings suggests that there is still a need to examine starvation responses of subterranean-adapted species that are known to occupy environments with greater resource availability to assess if metabolic adaptation to low energy systems is relaxed in more resource rich environments. In Chapter 3 of this dissertation, I conducted an experiment that assessed metabolic and biochemical responses of two amphipod species to prolonged food deprivation. I also compared basal metabolism of both species by measuring O₂ consumption rates. The two amphipod species compared in this study were a subterranean amphipod, *Stygobromus pecki* (Crustacea: Crangonyctidae) that inhabits spring opening ecotones and a related epigeal amphipod, *Synurella* in the same family (Crangonyctidae).

Cumulatively, the series of studies in this dissertation adds a substantial amount of new information on the ecophysiology and evolutionary biology of the species that occupy spring ecotones. In addition, the studies in this dissertation address several long-standing hypotheses in ecology and subterranean biology. This dissertation highlights the importance of flow permanence and connection to adjacent riparian vegetation for persistence and stability of invertebrate communities occurring in spring ecotones. Information in this dissertation will aid in conservation and captive breeding of spring ecotone species, many of which are imperiled, and the management of their unique habitats.

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2. HABITAT ASSOCIATIONS PREDICTS RESPONSES OF SEVERAL SPECIES OF RIFFLE BEETLES TO ENVIRONMENTAL STRESSORS

INTRODUCTION

Environmental temperature (T) regulates body temperature (T_b) in poikilotherms, which governs critical processes such as behavior, locomotion, metabolic rate, and cardiorespiratory function (Huey and Stevenson 1979, Kiefer et al. 1998, Farrell 2002). During acute exposure to a broad range of T , an asymmetric function describes the relationship between T_b and performance (i.e., growth) where performance is maximized at an intermediate T : the thermal optimum (T_o) (Angilleta et al. 2002). Given the ability to make a choice, many organisms select a T that often corresponds with thermal optima for physiological processes (Beitinger and Fitzpatrick 1979). Deviation from the optimum leads to declines in performance (Pörtner and Knust 2007), and as a result, many organisms utilize thermoregulatory strategies such as sheltering and use of thermal refugia to maintain body T at or near optima (Thorpe 1994).

Temperature is also a critical factor affecting respiration in aquatic invertebrates (Verberk and Bilton 2013). Environmental T affects the concentration of dissolved gasses in an aqueous environment, creating interdependence between the effects of T and dissolved oxygen (DO) on respiratory function. The effects of elevated environmental T are exacerbated in aquatic systems by the mismatch between DO supply and its biological demand (Verberk and Bilton 2013). Elevated T increases metabolism (and thus O_2 demand) in ectotherms, but increasing T also decreases gas solubility in water, resulting in lower ambient DO concentrations (Brown 1987, Resh et al. 2008). A number of previous studies have examined the effects of T and DO on respiratory function and

survival in aquatic insects (i.e., Harpster 1944, Verberk and Bilton 2013). However, there has been relatively little examination of the effects of T and/or DO on the function and survival of invertebrates found in stenothermal environments (i.e., spring ecosystems) (Colson-Proch et al. 2009, Mermillod-Blondin et al. 2013).

Environmental tolerance measures have been used for >50 years to determine capacities of organisms to tolerate thermal and DO changes in complex holocoenotic environments (Lutterschmidt and Hutchison, 1997). Behavioral and physiological responses of organisms to elevated T and hypoxia are determined by using a dynamic method, the critical thermal maximum (CT_{Max}) and critical DO minimum (CDO_{Min}), where a linear change in in test T (increase) or DO (decline) persists until a sub-lethal/critical endpoint is reached (Brett 1944, Fry et al. 1947, Fry 1957). The critical endpoint is the T or DO concentration at which an observable response, such as lack of movement is reached in aquatic poikilotherms. Although these types of studies provide critical endpoints for T and DO, there is typically a sequence of behavioral stress responses before the onset of the critical endpoint (Dallas and Ketly 2011). Despite their widespread use and long history, critical endpoint methodology has some limitation because experiments often examine relatively short exposure times (hours to days) to acute changes in environmental conditions, during which species may mediate effects of environmental variation through behavioral adjustments. Thus, researchers often also use static exposure methods to assess chronic effects of longer-term consistent exposure to environmental stressors on organisms (Terblanche et al. 2007). Therefore, a powerful approach to examine the effects of changes in environmental conditions (i.e., T and/or DO) can be obtained by using a more unified approach, which examines response of

organisms with regard to the intensity (acute or dynamic method) and duration (chronic or static method) of environmental stressors (Cooper et al. 2008, Rezende et al. 2014).

It has been hypothesized that organisms found exclusively in thermally stable environments should exhibit a narrow range of T tolerances (i.e., organisms are considered stenothermal) (Mermillod-Blondin et al. 2013, Farless and Brewer 2017). Conversely, in thermally fluctuating or variable environments, organisms should evolve a eurythermal profile, with their thermal optima extended throughout a broad range of T (Huey and Kingsolver 1989, Issartel et al. 2005, Mermillod-Blondin et al. 2013).

However, temperature is not the only physicochemical condition that has high temporal consistency in spring-influenced aquatic ecosystems. Because flows at spring systems are composed of discharging groundwater, there is often high consistency in conditions such as DO, ionic concentrations, and pH (Odum 1957, Sear et al. 1999). It is been hypothesized that spring associated (obligate crenic) species are largely limited to habitats immediately adjacent to spring outflows because they are more likely to exhibit stenoecious tolerance profiles (e.g., Cooke et al. 2015).

This study examined thermal and dissolved oxygen tolerances and optima of four aquatic beetle species, *Heterelmis comalensis*, *Heterelmis glabra*, *Heterelmis vulnerata*, and *Microcyloepus pusillus* (Coleoptera: Elmidae) from Edwards Plateau region of central Texas, USA. *H. comalensis* and *H. glabra* share morphological and ecological similarities, are associated with spring outflows, and are hypothesized to be adapted to more stenoecious environmental conditions (Gonzales 2008, Cooke et al. 2015). *H. comalensis* is a federally endangered species with a very limited geographic distribution limited, but there is virtually no information on its environmental tolerances. In contrast,

H. vulnerata is a geographically widespread species that is found in a diversity of flowing water habitats (Brown 1972, Phillips 1995). *M. pusillus* is another widespread elm mid species that is found in a diversity of well-oxygenated surface streams (Brown 1987). It occurs in the same habitat as *H. comalensis* in the Comal system, but it is not strongly associated with the presence of spring openings. In the present study, I conducted several experiments examining the effects of acute and chronic exposure to higher T and lower DO on the three species. The first set of experiments examined differences in environmental tolerances in the two species which are associated with spring conditions and presumably the most limited range in environmental tolerances, *H. comalensis* and *H. glabra*, by determining their CT_{Max} and CDO_{Min} in acute exposure experiments. The second set of experiments examined responses of spring-associated and non-spring (surface water) associated species to persistent environmental conditions. In these experiments, I assessed if beetle survival was affected by continuous and persistent (chronic exposure experiments) to different T conditions over an extended time period and compared mortality patterns in the spring-associated riffle beetles (*H. comalensis* and *H. glabra*) and the two more cosmopolitan species (*H. vulnerata* and *M. pusillus*). I additionally estimated basal metabolic rate responses (as O_2 consumption rate) of these beetle species at various temperatures as they were held in chronic exposure experiments. In combination, these experiments assessed the hypothesis that spring associated species exhibit a narrower range of environmental tolerances than more widespread species. To date, no studies have examined responses of multiple spring- and surface water associated species to increased T and lower DO on elmids. The information obtained through this study will aid in conservation of spring-associated species which are more

imperiled than species in other groundwater habitats (Hutchins 2017). In particular, this study will aid in conservation of *H. comalensis* and the management of its designated critical habitat area.

MATERIALS AND METHODS

Animal collection, housing, and initial acclimation

The karstic Edwards, Trinity, and Edwards-Trinity aquifers span approximately 87,283km² in west and central Texas, USA (Barker and Ardis 1996). Groundwater from the Edwards and Trinity aquifers discharges from large springs throughout the region. *Heterelmis comalensis* only occurs at two of the larger spring complexes discharging from the Edwards Aquifer: Comal Springs and San Marcos Springs (Gonzales 2008); but this study only used *H. comalensis* from Comal Springs (29°43'5.32"N, 98° 7'52.94"W) (Fig 2.1). Along with *H. comalensis*, *M. pusillus* was also collected from Comal Springs. *H. glabra* is morphologically similar to *H. comalensis*, but it is associated with a number of streams and rivers in the southwestern United States, Central America, and Mexico (Brown 1972). In the west Texas region, there are several elmids populations morphologically consistent with *H. glabra*, but they only occur in spring-associated habitats and show some genetic divergence from other *H. glabra* (Gonzalez 2008). The largest spring-associated population occurs at Finegan Springs and nearby Dolan Springs (29°53'58.45"N, 100°59'51.17"W), which flow into the upper Devils River in the southern portion of the Edwards Plateau (Fig 2.1). For the purposes of this study, these spring-associated populations are hereafter referred to as *H. glabra*. *H. glabra* used in experiments were collected from the Dolan Springs/Finegan Springs site. *H. comalensis*, *M. pusillus*, and *H. glabra* were collected by hand picking and use of poly-cotton lures

(Huston et al. 2015). *H. vulnerata* was collected by hand picking at the confluence of Plum Creek and the Guadalupe River near Luling, Texas (29°39'17.97"N 97°35'56.40"W) (Fig 2.1). For all species, adult individuals collected in the field were immediately placed in PVC containment tubes with mesh openings, and transported to the lab at Texas State University, San Marcos, Texas in coolers filled with water from the source location.

Adult beetles were housed at the Freeman Aquatic Biology Building Wet Lab facility for at least two weeks prior to experimentation in temperature controlled flow-through chambers with pre-cleaned limestone river cobbles and fed well-conditioned terrestrial detritus (i.e., leaves and twigs; their presumed food source; Nair et al. *in prep*). Animals were held in conditions approximating the conditions found at their collection sites (23°C, >4 mg DO/L). Water supplying the Wet Lab and the flow-through system was untreated groundwater directly from the Edwards Aquifer.

Exposure to acute stressors and assessing critical thresholds

As an initial experiment to characterize the potential temperature and DO thresholds for the two spring associated species, I conducted acute threshold experiments on *H. comalensis* and *H. glabra*. I measured responses of these two species to changing *T* or DO concentration from ambient conditions (i.e., 23°C and/or 4 mg DO/L) in a stepwise manner by increasing 1°C or lowering DO 1 mg/L every 24 hours. To assess CT_{Max} and CDO_{Min} , an experiment was performed on four individual adult beetles simultaneously on three occasions, yielding $n = 12$ independent observations of individual responses to increasing temperature and declining DO. Individual flow-through chambers received a continuous supply of groundwater from a 5-L reservoir and flow rates into chambers

from this reservoir were set to replace volume of the 60-ml holding chambers approximately every minute. The conditions in the larger reservoir were manipulated (increased temperature or declining DO) (see below for methods) and this water was fed into chambers via a closed system with a peristaltic or magnetic drive pump placed in the reservoir.

CT_{Max} of riffle beetles was assessed by subjecting beetles to elevating T while keeping normoxic conditions ($DO \geq 4$ mg/l). An adjustable digital heater unit (Innovative heat Concepts, LLC, QDPTY1-1) placed in the reservoir was manually adjusted to increase T at a rate of $1^{\circ}C$ every 24 hour period. Temperature was raised by $1^{\circ}C$ from the previous temperature and held at the new temperature for 24 hours before the next increase. DO was kept ≥ 4 mg/L by continuously monitoring DO in the main reservoir chamber using a Dissolved Oxygen Control System (Qubit Systems, Kingston, ON, Canada); the control system would bubble standard laboratory air into the reservoir if DO were to drop below 4 mg/L. Throughout experiments, adult beetles of both species were regularly monitored for a loss of response (LOR) to a stimulus (gentle agitation of the experimental chamber). For each individual of both species, the temperature of the LOR was recorded. In addition, beetles were observed for other indicative behavior, such as being immobile with legs curled up or sudden agitated and uncoordinated movements every 2-3 hours. Beetles exhibiting an LOR were immediately removed from an experimental chamber and placed in an individual container at initial acclimation conditions ($23^{\circ}C$) and observed for 24 hours to note whether it recovered or died.

CDO_{Min} of adult beetles was assessed by subjecting beetles to declining DO, while maintaining T at $23^{\circ}C$. Decreases in DO concentrations were accomplished by

bubbling N₂ gas through the reservoir to strip oxygen and decrease DO concentrations to desired levels (Martinez et al. 1998, Ostrand and Wilde 2001, Chiba et al. 2004, Denisse and Diaz 2011). DO changes and maintaining DO targets was done using a Dissolved Oxygen Control System (Qubit Systems, Kingston, ON, Canada), which measures DO and maintains a programmed DO automatically through the release of N₂ via a large airstone bubbler placed in the main water reservoir. DO concentration was decreased by 1 mg/l from initial acclimation DO of 4 mg/L and held constant for 24 hours before the next decline. Beetles were checked for LOR or other changes in behavior (agitated and uncoordinated movements) every 2-3 hours. Beetles exhibiting LOR were immediately removed from the chamber and placed in an individual container at initial acclimation conditions (23°C, >4 mg/L) and observed for 24 hours to note whether it recovered or died.

Exposure to chronic stressors experiments

In the second set of experiments, survival of riffle beetles to long-term conditions was assessed at four temperatures (23°C, 26°C, 28°C, and 31°C) and four DO concentrations (4 mg/L, 3mg/L, 2 mg/L, and 1 mg/L). This range of temperatures and DO concentrations were based upon the acute exposure experiments for *H. glabra* and *H. comalensis*. In chronic *T* treatments, groups of ($n = 5$ replicates with each replicate consisting of three individuals) *H. comalensis*, *H. glabra*, *H. vulnerata*, and *M. pusillus* were gradually acclimated to target treatment temperatures, and upon reaching these target temperatures, were held for a period of 60 days. For the initial acclimation process to reach the target treatment *T*, chamber temperature was raised from the initial *T* of 23°C at a rate of 1°C per day to facilitate acclimation of the organism. In the chronic DO

exposure treatments, adult *H. comalensis* and *H. glabra* were gradually acclimated to the target DO of their respective treatment and then exposed to the DO treatment for a period of 14 days. For the initial acclimation process to reach the target DO concentration treatment, DO was decreased from at the rate of 1 mg/L per day until the target treatment DO was reached.

Individual holding chambers received a continuous supply of water from a reservoir that maintained the target *T* or DO treatments, which flowed into individual chambers at the rate of 60 ml/min. Water temperatures and DO levels in the reservoir container was maintained with the Qubit system (see description above). Beetles were checked multiple times per day for LOR to an external stimulus (gentle agitation of the holding chamber). If a beetle was observed to exhibit an LOR, it was removed from its chamber and placed in new container at pre-experiment conditions (23°C, >4 mg/L) and observed every few hours for 24 hours to note recovery or death.

During chronic temperature exposure experiments, I estimated metabolic activity of the four riffle beetle species (i.e., *H. comalensis*, *H. glabra*, *H. vulnerata*, and *M. pusillus*) at the treatment temperatures of 23°C, 26°C, 28°C, and 31°C. Estimation of metabolic rates was conducted after beetles had reached treatment target temperatures and had been maintained at those temperatures for 21 days. Estimates of metabolic rates (measured as O₂ consumption rate) were individually performed on an experimental unit (i.e., a holding chamber containing *n* = 3 beetles). I measured O₂ consumption rate *n* = 3 times for each temperature treatment for each species. To measure O₂ consumption rate, all individuals in a randomly selected chamber/experimental replicate were gently removed using a pipettor and transferred to a water-jacketed respiration chamber

(cuvette) fitted with a Clark-type electrode (OX1LP-1mL Dissolved Oxygen Package, Qubit Systems, Kingston, ON, Canada). The respiration chamber was maintained at the treatment temperature with the water jacket surrounding the cuvette. Oxygen consumption rate of each group of beetles was calculated as the difference between the initial and final DO concentration in the chamber after a one-hour period. After the 1-h period, beetles were gently removed from the respiration chamber and placed back in their original housing chamber. Oxygen consumption rate of the group of beetles was divided by the mean dry mass (DM) of $n = 3$ adult beetles of each species to express the mass-specific rate of oxygen consumption (mg O₂/mg DM/h). Adult beetle dry mass was empirically determined in the lab with a Mettler Toledo microbalance; variation in dry mass among adult beetles of a species was minimal. Using mass-specific O₂ consumption rates, I calculated Q_{10} values for all four beetle species at the intervals between the temperatures used in the chronic exposure experiments (i.e., 23-26°C, 23-28°C, and 23-31°C) using the formula

$$Q_{10} = \left(\frac{k_2}{k_1} \right)^{\frac{10}{t_2 - t_1}}$$

where, k_2 and k_1 are mass specific O₂ consumption rates at temperatures t_2 and t_1 . Q_{10} describes how a metabolic rate changes with each 10°C change in temperature and values ~2 are common and indicate thermally dependent metabolic rates in many invertebrate taxa; values greatly different from 2 may be indicative of thermal stress (Chown and Gaston 1999; Hodkinson 2003).

Data analysis

For the first set of experiments, critical T and DO thresholds of *H. comalensis* and *H. glabra* were determined as the arithmetic mean of the observed LOR endpoints of each individual beetle for each species. I additionally noted temperatures at which *H. comalensis* and *H. glabra* exhibited stress-indicative behavior, rapid crawling movement (elmids are typically stationary or crawl slowly) and uncoordinated movement around the experimental chamber. Temperatures of the onset of LOR (or other behaviors) was compared between *H. comalensis* and *H. glabra*, using one-way ANOVA ($n = 12$ for each species). Data were examined for normality and heterogeneity of variances prior to analyses. Significance was inferred at $\alpha \leq 0.05$. All statistical analyses were performed in R (version 3.2.4, R Core Team, 2016).

For the second set of experiments, I examined the proportional survival of groups of adult beetles in containers (i.e., number of dead/alive beetles per experimental chamber) of each riffle beetle species as a function of T (*H. comalensis*, *H. glabra*, *H. vulnerata*, and *M. pusillus*) or DO concentration (*H. comalensis* and *H. glabra*) treatments and stress duration (in days) using a generalized linear model (GLM) with binomial error distribution and logit link function, because I utilized proportional data and data did not meet requirements and assumptions of ANOVA. The effects of each variable (i.e., temperature or DO concentration, species identity, and experimental duration) were analyzed through an Analysis of Deviance (“Anova” function in “car” package in the R platform, Fox & Weisberg, 2011). The predictive strength of logistic regression models was assessed by McFaddens pseudo- R^2 . If a significant main effect of T or DO was detected, pair-wise post-hoc comparisons were performed with Tukey’s

HSD using the `glht`, `multcomp` package. A Generalized Estimating Equation (GEE) model in a logistic regression setting was used to estimate the L_{t50} (median lethal time for 50% of the experimental population) for each species from temporal survival data in temperature and DO exposure experiments. LT_{50} and LC_{50} (median lethal DO concentration for the DO experiments involving *H. comalensis* and *H. glabra*) was estimated using a two-parameter log-logistic curve fit approach in R extension package “`drc`” (Ritz et al. 2015).

Comparison of mass specific O_2 consumption rates across the four species at each temperature treatment level was assessed with two-way ANOVA with species and T as independent variables and mass specific O_2 consumption rates as the dependent variable. If a significant effect of species or T was detected, pair-wise post-hoc comparisons were performed with Tukey’s HSD. Calculated Q_{10} values at each T interval (23-26°C, 23-28°C, and 23-31°C) were compared across species with separate one-way ANOVAs at each T interval. If a significant effect of species identity was detected, post-hoc comparisons were performed with Tukey’s HSD. For all ANOVAs, data were examined for assumptions of normality and homogeneity and significance was inferred at $\alpha \leq 0.05$.

RESULTS

Exposure to acute stressors and assessing critical thresholds

For the spring associated species (*H. comalensis* and *H. glabra*), I observed a similar sequence of responses to gradually increasing temperatures. As T increased, both species showed an overall increase in movement around the experimental chamber from “baseline” behavior (clinging to a small piece of screen or the bottom of the chamber). As

T further increased, some individuals of both species exhibited very rapid movement around the chamber (i.e., swimming-like behavior) that was uncoordinated. However, this behavioral sequence was not observed for all individuals before the onset of LOR. The LOR state of both beetles was easily recognized in that beetles would remain immobile at the bottom of the flow-through chamber with their legs curled up, even when gentle agitation was applied to the chamber. Both species showed a similar behavioral sequence in responses to increasing T and there were no substantial differences in CT_{Max} between species. The temperature at which LOR was observed ($\bar{x} \pm 1 \text{ SE}$) in *H. comalensis* ($34.2 \pm 0.8^\circ\text{C}$) was not different from the LOR onset T observed in *H. glabra* ($35.9 \pm 1.0^\circ\text{C}$) ($F_{1, 20} = 1.92, p = 0.18$) (Fig. 2.2 A).

In the Critical DO Threshold experiments, *H. comalensis* exhibited a significantly higher LOR ($1.6 \pm 0.4 \text{ mg DO/L}$) than *H. glabra* ($0.5 \pm 0.23 \text{ mg DO/L}$) ($F_{1, 19} = 59.48, p < 0.001$) (Fig. 2.2 B).

As previously described, riffle beetles that exhibited LOR in T and DO experiments were placed in ambient conditions and were observed for 24 hours. No individuals of *H. comalensis* recovered from T and DO exposure experiments (i.e., 100% mortality in both sets of experiments). In contrast, *H. glabra* exhibited 75% mortality when allowed to recover from increased T (9 out of 12 individuals did not recover) and there was 0% mortality to DO exposures (all individuals recovered).

Exposure to chronic stressors experiments

In the long-term chronic exposure temperature experiments, the proportional survival of all species (*H. comalensis*, *H. glabra*, *H. vulnerata*, and *M. pusillus*) declining over the course of the experiment (60 days) (Fig. 2.3 A – D) was assessed. For all species, there

was significant effect of T (Temperature: $x^2 > 87$, $df = 3$, $p < 0.0001$), which indicated that at higher temperature treatments, species exhibited lower survivorship. Survivorship in all species also declined throughout the experimental time period showing significant effect of time ($x^2 > 265$, $df = 1$, $p < 0.0001$). In addition, there was a significant Time x Temperature interaction ($x^2 > 12.39$, $df = 3$, $p < 0.01$), indicating that the effect of temperature was not independent from time.

In all the four species, relatively small differences in environmental T led to substantial decreases in survival (Fig. 2.3 A – D). However, *H. comalensis* ($x^2 > 30$, $df = 3$, $p < 0.0001$) exhibited significantly higher mortality than the other species at elevated temperature treatments of 26°C and 31°C (; but not at 28°C, $x^2 = 7.24$, $df = 3$, $p = 0.06$). These differences are reflected in predicted median lethal T values (Fig. 2.4 A). The median lethal temperature ($\bar{x} \pm 1$ SE) of $26.96 \pm 0.25^\circ\text{C}$, for *H. comalensis* was approximately 2.5°C lower than *H. glabra* ($29.477 \pm 0.32^\circ\text{C}$), ~4°C lower than *H. vulnerata* ($30.89 \pm 0.81^\circ\text{C}$), and ~15°C lower than *M. pusillus* ($41.9 \pm 4.7^\circ\text{C}$) (Fig. 2.4 A). However, difference in survival rates between *H. glabra*, *H. vulnerata*, and *M. pusillus* was non-significant (Tukey's HSD for glht; $p > 0.60$ for all temperature comparisons).

The lethal time to 50% mortality (Lt_{50}) for *H. comalensis* at 23°C was 55 ± 10.3 days (mean \pm 95% confidence interval) (Fig. 2.4 B). Estimated Lt_{50} values decreased substantially at all temperature treatments $> 23^\circ\text{C}$ ($x^2 = 250.9$, $df = 3$, $p < 0.001$), with the 26°C $Lt_{50} = 18.3 \pm 7.3$ days, 28°C $Lt_{50} = 35.6 \pm 21.7$ days and the 31°C $Lt_{50} = 13.5 \pm 3.86$ days (Tukey's HSD for glht: $p < 0.05$ for all comparisons) (Fig. 2.4 B). Similarly, *H. glabra* showed a substantial shortening of Lt_{50} values from 74.7 ± 21.5 days at 23°C to

35.6 ± 12.4 days at 26°C ($p < 0.001$), to 33.7 ± 6.6 days at 28°C to day 26.7 ± 5.7 at 31°C ($p < 0.05$ for comparisons); however, Lt_{50} at 26°C did not differ significantly from the Lt_{50} value at 28°C for *H. glabra* ($p = 0.920$). *H. vulnerata* also showed a significant decrease in Lt_{50} values from 55 ± 21.1 days at 23°C to 29 ± 12 days at 26°C and 39.4 ± 8.2 days at 28°C ($p < 0.05$ for comparisons), but the Lt_{50} value at 26°C did not differ from Lt_{50} at 31°C (30.5 ± 9.4 days) ($p = 0.980$). *M. pusillus* also showed a significant decrease in Lt_{50} from day 76 ± 19.3 at 23°C to 30.3 ± 13.5 days at 26°C ($p < 0.05$ for comparisons) and 49.1 ± 8.4 days at 31°C, but the Lt_{50} value at 23°C did not differ from Lt_{50} at 28°C (50.1 ± 4.4 days) ($p = 0.853$). Some of the variation in proportional survival of the beetles at specific temperature treatments (e.g., lack of difference between 26°C and 28°C treatments or higher survival at 28°C when compared to 26°C) for *H. comalensis*, *H. glabra*, *H. vulnerata*, and *M. pusillus* could be due to individual variation in factors such as age and fitness prior to the start of the experiments.

Mass specific O₂ consumption rates estimated for all species during chronic temperature exposure experiments varied among the different study temperatures (Temperature effect: $F_{1,40} = 42.6$, $p < 0.001$; Fig. 2.5 A). There was also a significant main effect of experimental temperature on mass-specific O₂ consumption rates across all species (Species effect: $F_{3,40} = 3.473$, $p = 0.025$). The interaction term ($T \times$ Species effect) was also significant ($F_{3,40} = 6.32$, $p = 0.001$), indicating the interdependence of the effects of species identity and temperature on mass-specific O₂ consumption rates (Fig. 2.5 A). Namely, the mass specific O₂ consumption rates did not substantially differ between *H. comalensis* and *H. glabra* at most of the temperatures, but both species exhibited a steep increase in mass specific O₂ consumption at 31°C, indicating a strong

non-linear increase of metabolic rates in these two species. A non-linear increase of this magnitude was not observed in *H. vulnerata* or *M. pusillus*.

Calculated Q_{10} values in the 23-26°C interval ranged from 0.01- 4.99 across species, but significantly differed among the four species examined in this study ($F_{3,8} = 6.4$, $p = 0.019$ (Fig. 2.5 B)). *H. comalensis* had a significantly higher Q_{10} value (3.02 ± 1.18 , mean and ± 1 SE) when compared to all other species ($p < 0.034$ for all comparisons), but *H. vulnerata* and *H. glabra* did not significantly differ from each other ($p = 0.900$). Q_{10} values calculated at the 23-28°C interval varied from 1.11- 10.79, but did not significantly differ among species ($F_{3,8} = 1.76$ $p = 0.233$); however, Q_{10} values significantly differed among species at the 23-31°C interval, ($F_{3,8} = 10.73$, $p = 0.004$). *H. comalensis* did not differ from *H. glabra* ($p = 0.532$), but had significantly higher Q_{10} values than *H. vulnerata* ($p = 0.004$) and *M. pusillus* ($p = 0.026$). *H. glabra* had significantly higher Q_{10} values at this interval than *H. vulnerata* ($p = 0.026$), but *H. vulnerata* and *M. pusillus* did not significantly differ from each other ($p = 0.696$).

The experiment examining chronic effects of lower DO exposure on adult *H. comalensis* and *H. glabra* found that in both species virtually all individuals survived to the end of the 15-d experimental period in the 4 mg DO/L treatment (100% and 93% survival of *H. glabra* and *H. comalensis*, respectively); however, there was a significant decline in survivorship in both species with DO concentrations below 4 mg/L ($\chi^2 = 19.76$, $df = 1$, $p < 0.0001$). Although the general pattern of declining survivorship was similar between species, *H. comalensis* exhibited lower survivorship at a given DO concentration when compared to *H. glabra*, particularly at DO concentrations of 3 mg/L and 1 mg/L (Tukey's HSD for glht: $p < 0.05$ for comparisons). The apparent lower

tolerance of *H. comalensis* to DO concentrations < 4 mg/L relative to *H. glabra* is also reflected in the difference in the median lethal concentrations (LC_{50}) between the two species: LC_{50} of *H. comalensis* was 1.26 ± 0.083 mg/L (mean \pm standard error) while that of *H. glabra* was 0.67 ± 0.113 mg/L (Fig. 2.6 A). Similarly, the lethal time to 50% mortality (Lt_{50}) differed between the two species in the chronic DO experiments. Lethal time to mortality became shorter in both species with declining DO and did not substantially differ between species at 3 and 2 mg DO/L. However, Lt_{50} for *H. comalensis* (4.02 ± 3.2) was significantly shorter (as indicated by non-overlapping 95% confidence intervals) than that of *H. glabra* at 1 mg DO/L (11.61 ± 1.96) (Fig. 2.6 B).

DISCUSSION

In this study, survival of multiple riffle beetle species in response to T and DO concentration manipulations was a function of both stress intensity (differing levels of heat and hypoxia) and exposure duration (acute and chronic exposures). The decline in survival in all of the species examined by this study with decreasing quality of their environment, is consistent with classical dose-response relationship (Enriquez and Colinet 2017, Rezende et al. 2014). This study examined responses of two spring-associated species (*H. comalensis* and *H. glabra*) to acute temperature and DO exposures and subsequently conducted a longer-term chronic temperature exposure study that compared responses of multiple species which differed in their main habitat type association (spring- or surface water-associated) and found that responses were a function of their main habitat type association. I additionally found that the two spring-associated species differed somewhat in their responses to chronic exposures to T and DO stress. Cumulatively, the results of this study indicate that a wide range of environmental

tolerances can be present in species in a single family of insects that co-occur in a biogeographic region, but that environmental tolerances are primarily related to the main habitat type the in which each species occurs.

The acute temperature critical threshold experiments found that the two spring-associated beetles, *H. comalensis* and *H. glabra*, exhibited thresholds (for LOR) at 34.2°C and 35.9°C, respectively. Although these two species are considered to be spring-associated, the observed critical *T* thresholds are within the same range of threshold temperatures for other plastron-utilizing aquatic beetles which have more cosmopolitan geographic distributions. For example, Harpster (1941) and Harpster (1944) found that *Stenelmis quadrimaculata* (Elmidae) and *Helichus striatus* (Dryopidae) exhibited elevated mortality rates when held at temperatures >30 to 33°C, assuming adequate DO supply. Multiple investigators have questioned the application of “endpoint” tolerance limits to organismal populations in the wild (Rezende et al. 2014) and instead point to the use of a “thermal tolerance landscape” (TTL), which portray the probability to survive thermal stress as a function of both intensity and the duration of the stressor (i.e., environmental *T*). Indeed, the use of a combination of static and dynamic methods to assess temperature stress and mortality provide a more comprehensive framework to examine thermal tolerance limits (Cooper et al. 2008).

Although short-term acute exposure endpoint studies may not be reflective of organismal *in situ* tolerances, I used the initial acute thermal tolerance experiments to provide data on the range of temperatures for the chronic exposure studies. In the acute *T* exposure study, I found that first indicator of stress response in *H. comalensis* and *H. glabra* was an increased rate of movement around the experimental chamber. In the acute

exposure experiments, the onset of movement in *H. comalensis* occurred $\sim 1.3^\circ\text{C}$ lower than in *H. glabra* (ANOVA comparing onset of movement temperatures: $F_{1,21} = 6.40$, $p = 0.020$). A similar pattern was observed in the longer-term chronic T exposure experiments, where *H. comalensis* showed lethal temperature (LT_{50}) at a T that was approximately 2.5°C lower than that of *H. glabra*. Thus, results from the acute exposure studies have value in that they allowed us to determine an initial range of temperatures to use in the chronic exposure study and also elucidated some patterns which were apparent in the chronic exposure study.

In the longer-term chronic T exposure study, the two spring-associated species (*H. comalensis* and *H. glabra*) had substantially different patterns in mortality and metabolic (as O_2 consumption) responses than the two cosmopolitan species (*H. vulnerata* and *M. pusillus*). Both *H. comalensis* and *H. glabra* had substantially higher mortality and Q_{10} values than *H. vulnerata* and *M. pusillus* at higher temperatures. In fact, estimated Q_{10} ($23\text{-}31^\circ\text{C}$) for *H. comalensis* and *H. glabra* were much higher than many values reported for invertebrates, sometimes by an order of magnitude (Hodkinson 2003). Values of this magnitude indicate substantial thermal stress and hyperactive behavior (Hodkinson 2003). Cumulatively, these results suggest that *H. comalensis* and *H. glabra* are spring specialists with a more stenothermal profile than *H. vulnerata* and *M. pusillus*. It is often stated that spring-associated species should exhibit narrower environmental tolerance ranges than non-spring associated species, and that the reason why spring-associated species are found in the proximity of springs is due to their inability to tolerate the more variable conditions experienced away from spring openings (e.g., Smith et al. 2003). Results from this study support this hypothesis, but it is important to note that both *H.*

vulnerata and *M. pusillus* also frequently occur in the proximity of springs and therefore have the potential to occupy the same habitat space as spring-associated elmids. Indeed, *M. pusillus* occurs in and around spring openings at both Comal Springs (the site of *H. comalensis*) and Finegan Springs (the site of *H. glabra*) (Nair et al. *in prep*).

In this study, I also observed that there are differences in responses of the two spring-associated species to chronic exposure to temperature and DO. In general, *H. comalensis* was less tolerant to variation in *T* and DO than *H. glabra*. Although both species are considered spring specialists, the between-species differences in *T* and DO tolerances are likely related to adaptation to the specific environmental characteristic at their respective spring sites. *H. comalensis* and *H. glabra* occur in spring complexes located in different geographical regions with different volumetric flow rates. Finegan Springs is located along the southwestern edge of the Edwards Plateau and is a more arid area than Comal Springs. In addition, the discharge at Finegan Springs is substantially lower (~660 L/s) (B Schwartz, *unpublished data*) than Comal Springs (~8269 L/s) (data from waterdata.usgs.gov). Due to differences in flow rates and regional climate, the population of *H. glabra* may be exposed to greater fluctuations in *T* and DO than *H. comalensis*.

H. comalensis is federally listed as endangered and has an extremely limited geographic distribution. Flows at its main population site (Comal Springs) are protected by conservation measures related to the Edwards Aquifer (the water source for the springs), but the predicted increase in groundwater pumping and the potential decreased spring flows could lead to a decline in water quality (e.g., higher water temperatures and lower DO concentrations) (Chen et al. 2000). In addition, the Edwards Plateau region is

prone to periodic droughts, which could lead to decreased spring flows and water quality. Long-term temperature data from several spring runs in the Comal Springs complex showed that, as expected, the temperature of the water emanating from springs was highly consistent and with a long-term mean of $\sim 23.68 \pm 0.06$ °C (January 2005 – December 2016; data from the Edwards Aquifer Authority, <http://eaahcp.org/documents-publications/>). However, maximum water temperatures during low flow periods in spring runs can range from 25.40 to 26.10°C (Data from the Edwards Aquifer Authority, <http://eaahcp.org/documents-publications/>). The present study estimated the median lethal temperature (LT₅₀) for *H. comalensis* as 26.96°C and the Lt₅₀ for mortality at 26°C as 18 days. Thus, a prolonged drought and/or increased pumping has high potential to affect *H. comalensis* populations in the wild and I strongly suggest that future studies should perform a rigorous risk assessment analysis for *H. comalensis* for various drought and pumping scenarios (Newman and Unger 2003).

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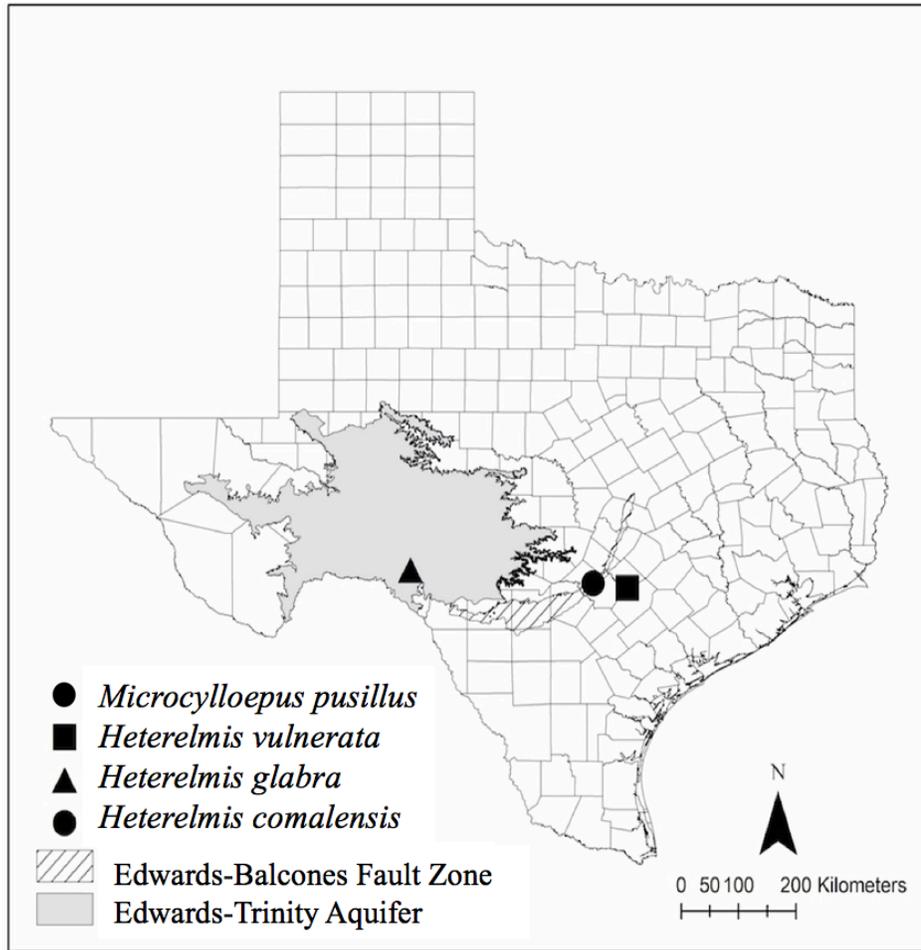


Figure 2.1: Map with sampling locations for all the four riffle beetle species used in this study

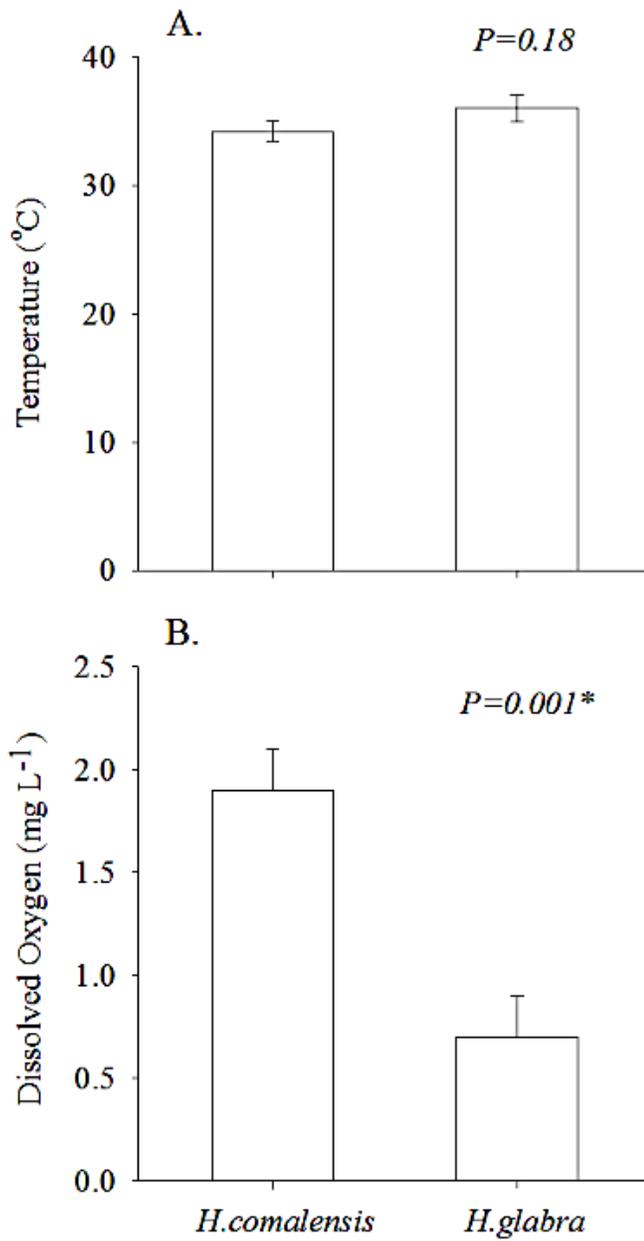


Figure 2.2: Threshold T for LOR (A), and the threshold DO concentration for LOR (B) in *H. comalensis* and *H. glabra* (mean \pm 1 SE). * $p<0.05$

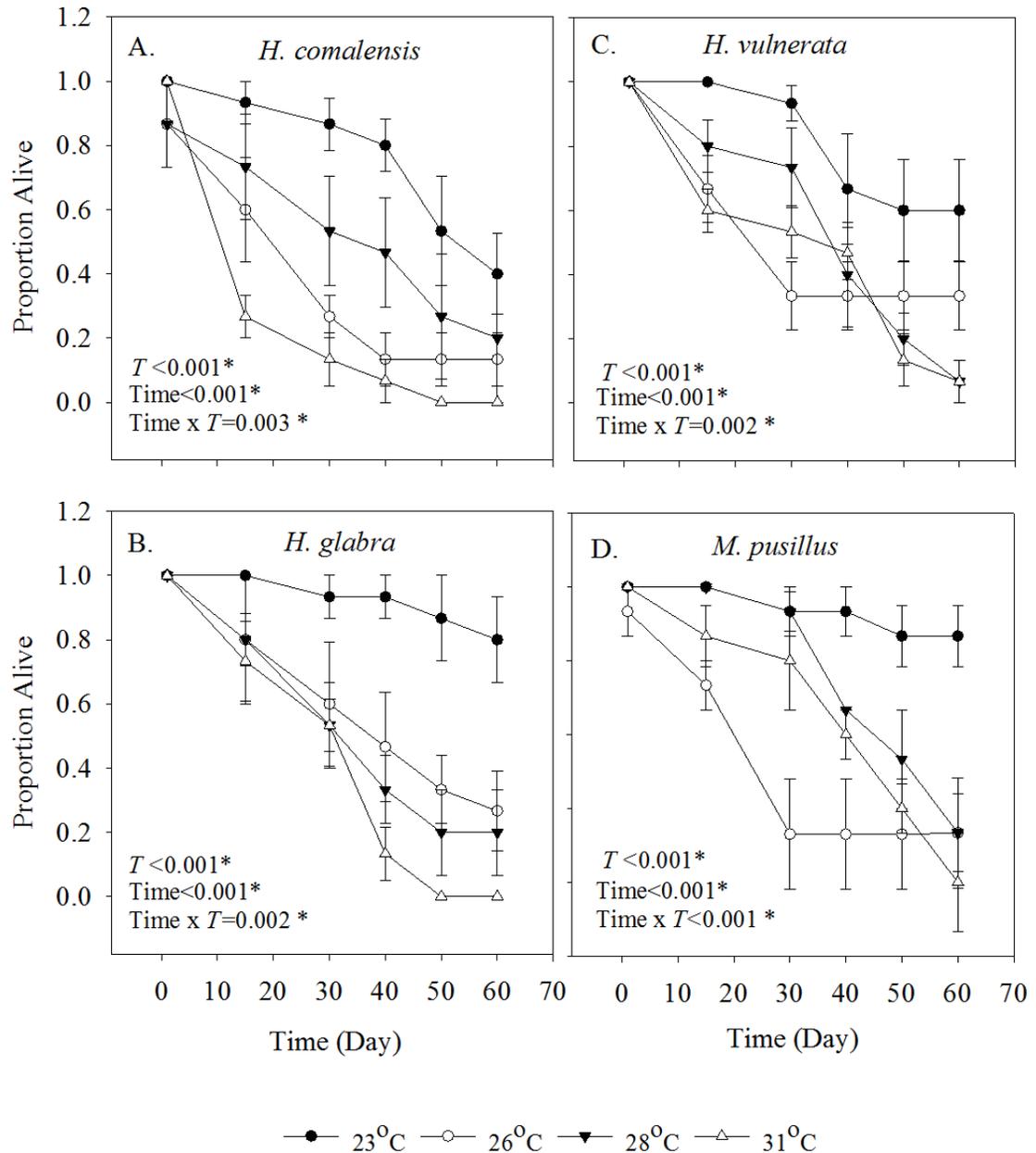


Figure 2.3: Time-series of proportional survival of *H. comalensis* (A), *H. glabra* (B), *H. vulnerata* (C) and *M. pusillus* (D) exposed to different temperature treatments for 60 days. p-values for the main effects of Time, Temperature (T), and the Time x Temperature (T) interaction are presented. * indicates significance at $\alpha < 0.05$. Error bars are ± 1 SE

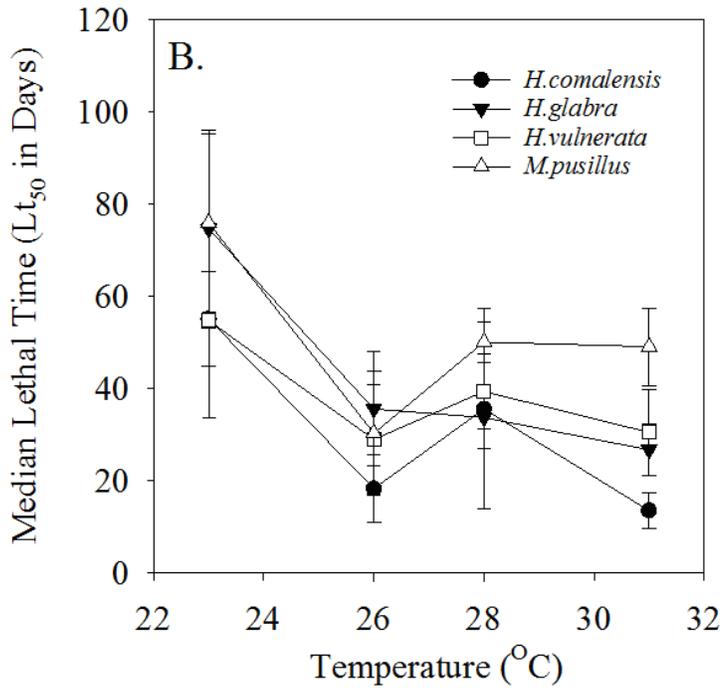
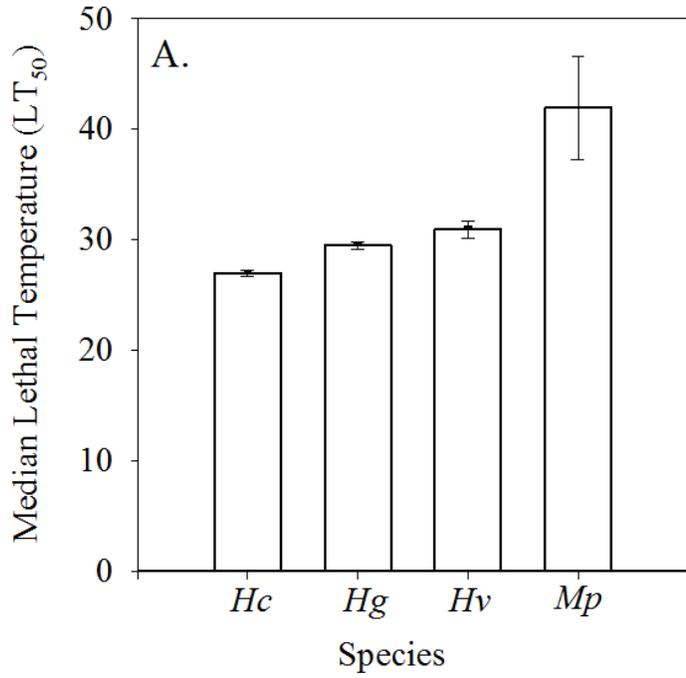


Figure 2.4: Estimated Median Lethal T (LT_{50}) values \pm standard error (SE) (A), Estimated Median Lethal Time (Lt_{50}) or time at which 50% population is dead) values \pm 95% confidence intervals for each tested T (B) for the four elmid species

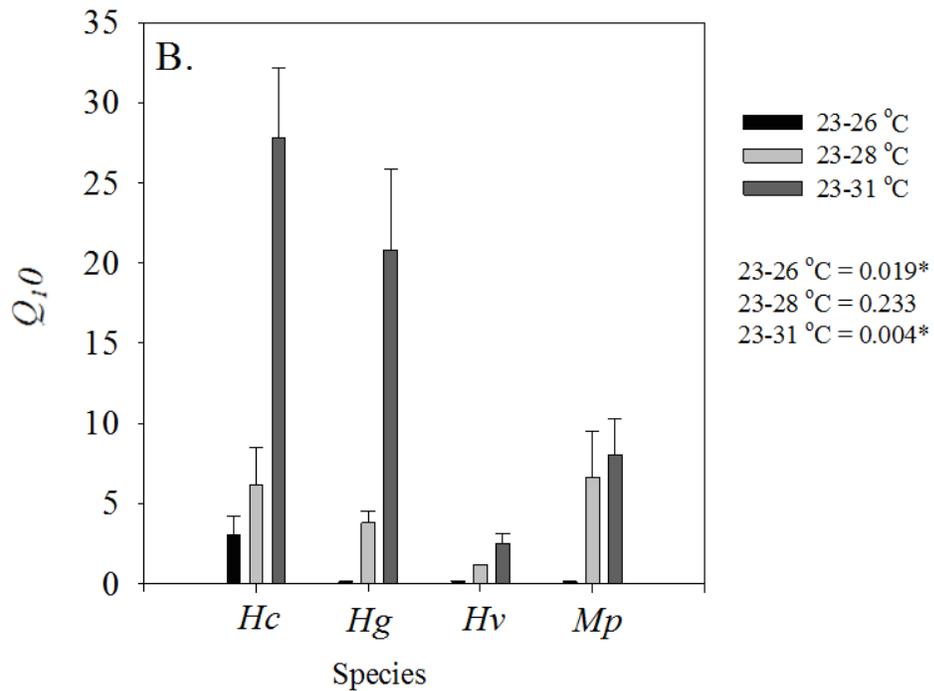
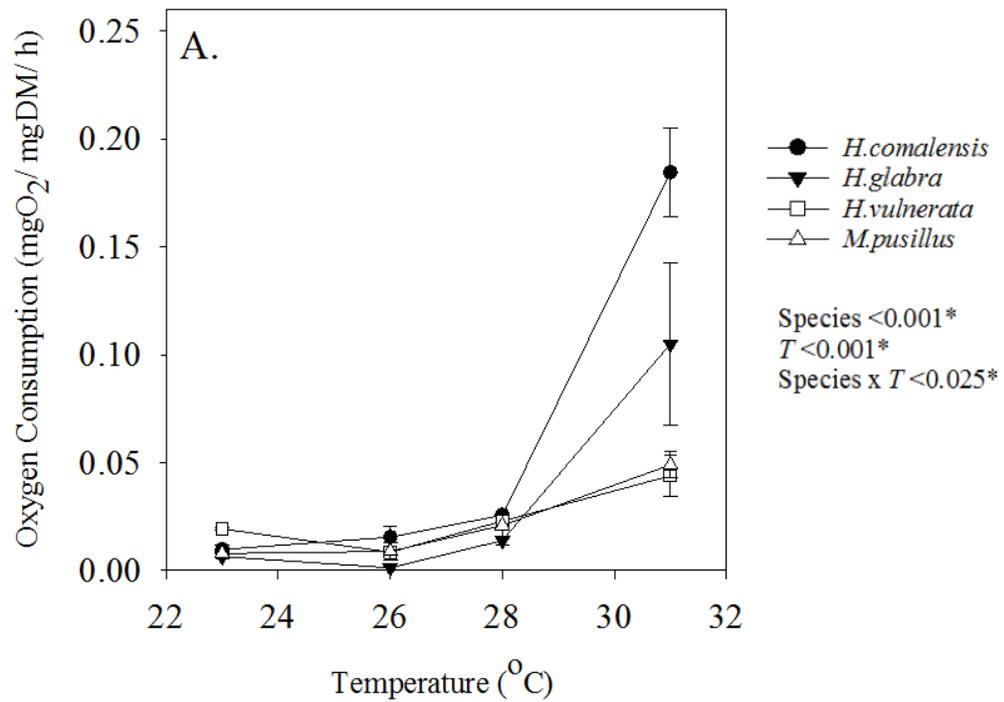


Figure 2.5: Mass-specific O₂ consumption rates of the four elmid species across the four experimental *T*s (A) and the calculated Q_{10} values for each species across three *T* ranges (B) (mean ± 1 SE). * $p < 0.05$

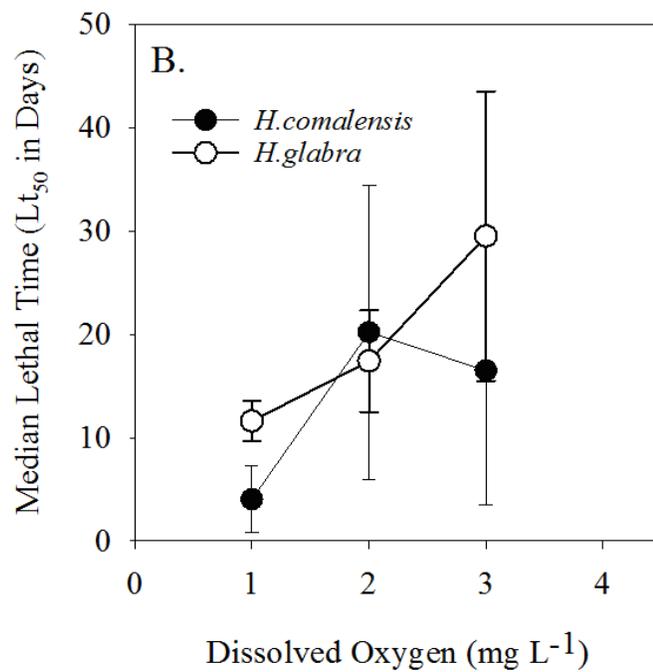
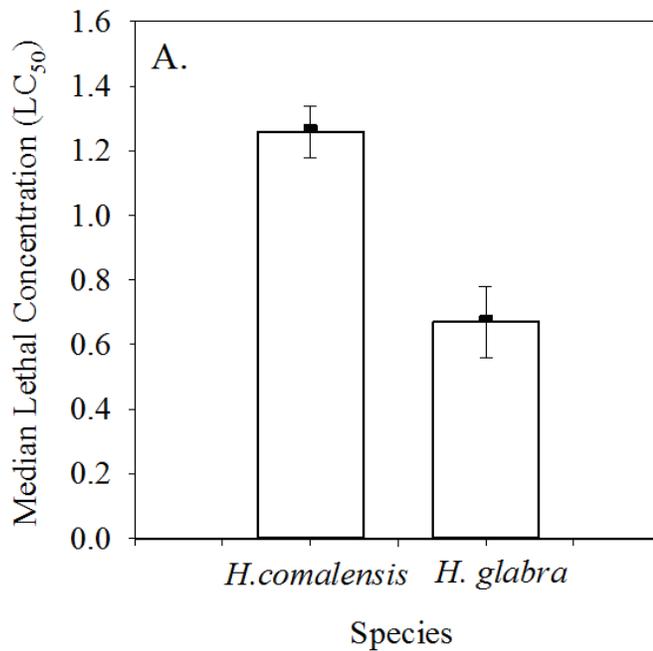


Figure 2.6: Estimated Median Lethal Concentration (LC₅₀) values ± standard Error (SE) (A), Estimated Median Lethal Time (Lt₅₀) values ± 95% confidence intervals for each tested DO concentrations (Lt₅₀ not calculated at 4 mg/L due to almost 100% survivorship) (B) for *H. comalensis* and *H. glabra*

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3. INTERACTIONS AT SURFACE - SUBTERRANEAN ECOTONES: THE STRUCTURE AND FUNCTION OF FOOD WEBS IN SPRING OPENINGS

INTRODUCTION

Ecotones are transition zones between adjacent ecosystem types that contain conditions which represent a contrast of physicochemical properties, habitat structure, and biotic communities (Odum 1971, Naiman and Décamps 1990, Fagan et al. 2003). The concept of ecotones has been in the ecological and wildlife management literature for decades (i.e., Leopold 1933) and have been examined both empirically (Kolasa and Zalewski 1995) and theoretically (Shugart 1990). Ecotones are themselves considered a distinct habitat type and serve as major contact zones between ecosystems and their associated communities (Kolasa and Zalewski 1995). Ecotones often have relatively high biodiversity in landscapes, acting as species aggregators by containing species not only from the adjacent ecosystem types, but also species which may be found only within the ecotone itself (Winemiller and Leslie 1992, Walker et al. 2003, Traut 2005).

Springs (i.e., a place where groundwater emerges and is discharged onto the land surface) serve as ecotones that link multiple ecosystem types: terrestrial and aquatic habitats, and groundwater (hypogean) and surface water (epigean) habitats in multiple dimensions (i.e., longitudinal, lateral, vertical, and temporal) (Mori and Brancelj 2006, Cantonati et al. 2006, Scarsbrook et al. 2007, Cantonati et al. 2012). Environmental conditions found in the immediate vicinity of spring openings are characterized by reduced variability in physicochemical conditions, consistency in flow and persistence of water, and relatively discrete ecosystem boundaries (Odum 1957, Glazier 2012). Biological assemblages (biocenoses) inhabiting spring opening ecotones are often

composed of organisms representing a variety of habitat preferences and adaptations, including taxa solely restricted to the proximity of spring openings (i.e., crenobionts), more widespread epigeal taxa that are also found in spring influenced environments (sometimes called crenophiles, Cantonati et al. 2006), and taxa that have adaptations for and are associated with hypogean environments (Gerecke et al. 1998, Cantonati et al. 2012, Galassi et al. 2014, Stoch et al. 2016). Thus, spring ecotones may serve as important point of biodiversity in stream networks, by serving as habitats that contain assemblages composed of organisms representing a variety of morphological and physiological adaptations, life history strategies, and trophic ecologies (Cantonati et al. 2012, Kark 2013).

Ecotones also provide or facilitate ecosystem functions at a variety of spatio-temporal scales, serving as conduits and controllers of the movement of organic matter (OM), nutrients, and energy between adjacent ecosystems (Risser et al. 1995). In this context, spring ecotones represent a transition between ecosystems which have contrasting productivities and nutrient cycling regimes. Subterranean ecosystems are generally considered to be low productivity environments with limited resource availability for consumers and food webs should be dependent upon allochthonous inputs of surface-generated OM and nutrients (Poulson 1964, Hüppop 1985, but see Hutchins et al. 2016). In contrast, surface aquatic ecosystems are relatively more productive and have a greater diversity and abundance of resource types for consumers, including OM and nutrients from autochthonous and allochthonous origin (Gunn 2004). It has been hypothesized that spring ecotones are more resource limited than surface water dominated systems (Odum 1957, Tilly 1968), but to date there is little data on resource

availability and food web dynamics within spring ecotones. If spring ecotones are indeed relatively resource limited then there may be intense competition for food resources and space among taxa found within these habitats (Connell 1980, Luštrik et al. 2011). Co-occurrence of diverse groups of organisms from different environmental settings (e.g., epigeal, crenic, and hypogean species) in spatially confined spring opening ecotones may be due to their ability to avoid competitive exclusion through partitioning food resources (Hardin 1960, Hutchinson 1961, Schoener 1974). Mechanisms facilitating coexistence of diverse invertebrate communities in spring systems are not completely understood but factors such as resource partitioning due to specialized feeding habits might be influencing these patterns (Bowles et al. 2003, Hutchins et al. 2014, Hutchins et al. 2016). Thus, in a broader ecological context, springs ecotones may serve as model systems for understanding how resource partitioning among diverse assemblages containing related taxa allow for species coexistence and relatively high species diversity.

This study examined patterns in resource and habitat use in the macroinvertebrate communities found in spring ecotones in two karst spring systems, Comal and Finegan springs in central and west Texas, USA, respectively. I assessed resource use and trophic structure in communities found in spring run stream reaches and spring ecotones using stable isotopes of carbon and nitrogen ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$). I estimated niche overlap of closely related species found within spring ecotones at the study sites, but which are primarily associated with distinct ecosystem types (i.e., crenic, epigeal, and hypogean taxa). Using compound-specific stable isotopes in essential amino acids (CSIA-EAA), I then examined potential resource partitioning and niche overlap in riffle beetle species (Coleoptera: Elmidae) that co-occur in spring openings but are primarily associated with

distinct ecosystem types. Compound-specific stable isotope analysis is a potentially powerful analytical method which provides a stable isotope “fingerprint” of consumers that is relatively more detailed when compared to conventional bulk isotope analysis (Larsen et al. 2009, Bowes and Thorp 2015). I hypothesized that macroinvertebrates found within spring run reaches would exhibit differential patterns of resource use, dependent upon the taxon’s primary ecosystem or habitat type association. I predicted that primarily epigeal taxa would exhibit the greatest diversity in primary basal resource types (autochthonous and allochthonous resources), while hypogean and crenic taxa would exhibit a more limited range of basal resources. I also hypothesized that closely related taxa occupying the same spring ecotone would exhibit more specialized feeding strategies, partition resources, and have limited niche overlap. Understanding the trophic ecology of ecotones and how species may be partitioning resources on relatively small spatial scales ($\leq 1 \text{ m}^2$) will provide insight into basal resources supporting spring ecotones, and contribute information critical for effective conservation planning, captive breeding, and habitat restoration programs for biological communities associated with springs (Bowles and Arsuffi 1993).

MATERIALS AND METHODS

Study sites and field sampling

Invertebrate assemblages and OM were collected from two springs complexes, Comal Springs (29°43'5.32"N 98° 7'52.94"W) and Finegan Springs (29°53'58.45"N 100°59'51.17"W). Groundwater emerging from study sites is from the Edwards and Edwards-Trinity aquifers, which are large regional carbonate aquifers composed of Cretaceous limestones and dolomites (Green et al. 2014), covering about 87,283 km² of

the Edwards Plateau and Trans-Pecos region (Barker and Ardis 1996). This regional aquifer system is among the most diverse in the world in terms of obligate subterranean aquatic species (Longley 1981, Culver and Pipan 2009, Barr et al. 2015).

Comal Springs is in central Texas in the city of New Braunfels along the eastern edge of the Edwards Plateau where Edwards Aquifer groundwater discharges from springs. Comal Springs is the largest natural spring complex in Texas in terms of discharge, and is a spring complex with numerous discharge locations (>400 identified spring openings, W.H. Nowlin, *unpublished data*) occurring along a 1,300-m spring run (Gibson et al. 2008). This study focused on two sampling locations along the Comal Springs complex. Spring Run 3 (SR3) is one of the main headwater sites of the Comal River and has numerous springs entering a small channel ~50 m long (~2 m channel width, ~1 m maximum channel depth) that discharges into the larger Comal system. Springs in SR3 occur along channel margins, have partial canopy cover, and are composed of a diversity of spring opening types, including those composed of gravel substrates and larger rocky conduits. The second sampling location, Spring Island (SI), is located approximately 1 km upstream along the main Comal Springs spring run and has considerably less riparian cover, greater exposure to sunlight, and hence more periphyton cover relative to SR3. Spring openings in SI are widely distributed throughout this section of the reach and are generally more diffuse where water rises through gravel substrates.

Finegan Springs is located approximately 270 km west of Comal Springs along the southwestern edge of the Edwards-Trinity aquifer. The Finegan Springs complex is located along the Devils River in Devils River State Natural Area and is comprised of

numerous openings distributed throughout a ~250 m section along the base of a cliff adjacent to the Devils River, and spring openings consist of fractured bedrock, or discharge beneath talus and boulder slopes with large spaces between the rocks.

Live invertebrates used for bulk isotopic analysis (i.e., analysis of all biochemical forms of C and N stable isotopes in a sample) were collected at both Comal sites and from the Finegan Springs site. Organisms were collected from the areas within and in the immediate vicinity of spring openings (< 0.25 m of a spring opening) by a combination of hand picking, sweeps of small aquarium nets, and placing 0.2-m diameter drift nets (100 μ m mesh aperture size) over spring orifices for short (<24 h) periods of time, all sites were sampled multiple times between June 2015 and June 2017. Each sampling site contained numerous individual spring openings. I wanted to assess the general trophic ecology and niche dynamics within spring ecotones at each site rather than examine variation among individual spring ecotones within a given site, so organisms and OM collected from spring openings at each site were pooled together. Collected organisms were immediately placed in 50 ml high density polyethylene (HDPE) tubes containing water from the site for 1-2 hours to void gut contents prior to transport to the lab. I also collected several types of potential basal resources from sampling sites. I collected coarse particulate organic matter (CPOM) composed of instream detritus such as well-conditioned terrestrial leaves and smaller pieces of wood. At each sampling location, I selected 3-6 rocks for periphyton samples. Rocks were placed in plastic bags in a cooler in the field and transported to the lab for processing. Samples of fine particulate organic matter (FPOM, particulate material <64 μ m) were collected from both sampling locations at Comal Springs by collecting benthic materials from spring openings (rocks and

gravels), placing material into large plastic bags, and then putting bags in a cooler.

Material for FPOM was not collected from Finegan Springs because spring openings were found in fractured bedrock and conduits that had very little FPOM in the immediate vicinity of spring orifices. The aquatic moss *Amblystegium* was collected if present, moss was removed from rocks, placed into plastic bags in a cooler, and transported to the lab.

In the lab, animals used for bulk isotope analysis from a sampling site were identified to the lowest feasible taxonomic level (usually genus) and then sorted into single taxon groups and dried at 60°C for 48 h. In cases when there was not enough mass in an individual animal for bulk isotopic analysis, animals of the same taxon were pooled into composite samples consisting of two to ten individuals of similar sizes. A minimum of three replicate samples was prepared for each taxon. Samples were ground to a fine powder with a clean mortar and pestle. CPOM and *Amblystegium* samples were gently washed with Milli-Q water, dried at 60°C for 48 h, homogenized, and ground to a fine powder using a mill grinder or a mortar and pestle. Periphyton was removed from rocks by scrubbing the upper surface with a clean nylon bristle brush and washing material with Milli-Q water into a clean HDPE beaker. A portion of this slurry was filtered onto ashed Whatman GF/F filters. FPOM samples were rinsed through a 64 µm sieve with Milli-Q water and the particulate material passing through the sieve was retained and filtered onto ashed GF/F filters. For periphyton and FPOM samples, filters were dried at 60°C for 48 h, and ½ of the filters were then placed into a fuming HCl chamber for 8 hours to remove inorganic C (Pound et al. 2011). Fumed filters were used for ¹³C values and the unfumed filters were used for ¹⁵N analyses. All samples were placed into tin capsules and analyzed at the University of California – Davis Stable Isotope Facility. Analyses were conducted

using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Stable isotope ratios are reported as $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in the sample relative to the standards of Vienna Pee-Dee Belemnite for $\delta^{13}\text{C}$ and atmospheric air for $\delta^{15}\text{N}$. Standard deviations relative to standards for measurement of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ was $\pm 0.2\text{‰}$ & and $\pm 0.3\text{‰}$, respectively.

Compound specific isotope analysis of essential amino acids

I additionally used compound-specific isotopic analysis to complement bulk isotopic results. Despite widespread use and utility in bulk isotopes in food web studies (Fry 2006, Layman et al. 2012), ecological and metabolic factors may sometimes complicate interpretation (Post 2002, Fry 2006, Bowes and Thorp 2015). In particular, determination of the contribution of terrestrial and aquatic basal resources to consumers using bulk isotopes can be problematic (Boeckler et al. 2011). I examined compound specific isotopic values of essential amino acids (EAAs) (Larsen et al. 2009) to provide additional detail on the diet and niche partitioning of epigenic and crenic organisms. Amino acid C isotopic values ($\delta^{13}\text{C}_{\text{AA}}$) vary in regard to their synthesis (Abelson and Hoering 1961) and approximately half of AAs are only synthesized by bacteria, fungi and photoautotrophs. These EAAs are necessary and essential for life, can only be obtained by consumers through their diet, and are typically passed from food source to consumer without alteration of C skeletons or isotopic values (O'Brien et al. 2002, McMohan et al. 2010, Larsen et al. 2013). Thus, $^{13}\text{C}_{\text{AA}}$ values generated during biosynthesis can be distinguished between OM generated by bacteria, fungi, and photoautotrophs and the contribution of OM from these sources to consumers diets (Larsen et al. 2009, Larsen et al. 2013).

Analysis of $\delta^{13}\text{C}_{\text{AA}}$ is substantially more time consuming and costly than bulk isotope analysis, thus I elected to focus analytical efforts on two related species which co-occur in spring ecotones to determine if they are partitioning resources. I concentrated on two species of elmid beetles that occur in the Comal Springs: *Heterelmis comalensis* and *Microcylloepus pusillus* (Elmidae: Coleoptera). *M. pusillus* is a widespread North American epigeal elmid that is found in a diversity of well-oxygenated surface streams (Brown 1983). *H. comalensis* is an endemic crenic species whose distribution is limited to the Comal Springs and San Marcos spring systems and is listed as federally endangered under the US Endangered Species Act (USFWS 2007, Gibson et al. 2008). Despite these differences, both species co-occur within spring orifices in Comal Springs (Bowles et al. 2003). Indeed, previous research has hypothesized that the coexistence of these two species may be due to partitioning of food resources (Bowles et al. 2003), but there is virtually no data on the trophic ecology and resource use of these two species in the Comal system.

For $\delta^{13}\text{C}_{\text{AA}}$ analysis, I collected both elmid species from Comal Springs system by hand-picking. Live individuals were allowed to clear gut contents for ~1 hour in water from the site and brought back to the lab for further processing. I assessed $\delta^{13}\text{C}_{\text{AA}}$ in *H. comalensis*, *M. pusillus*, and their potential food sources: surface biofilms on CPOM materials and epilithic periphyton. Approximately 12-20 individuals of *H. comalensis* and *M. pusillus* were pooled together to make one sample. The top layer of material on CPOM was gently scraped from woody debris and well-conditioned leaves found at the site, and this scraped material was used in $\delta^{13}\text{C}_{\text{AA}}$ analyses. Animal tissues and biofilms (processed as slurries as described in the previous section) were acid-hydrolyzed in 1-2

mL of 6 N HCl at 110°C for 20 h to isolate the total free AAs. Samples were then evaporated to dryness under a gentle N₂ stream. Total free AAs were derivatized by esterification with acidified isopropanol followed by acylation with trifluoroacetic anhydride (Silfer et al. 1991) and were brought up in dichloromethane for analysis. For $\delta^{13}\text{C}_{\text{AA}}$ analyses, derivatized AAs were injected into a column in split mode at 250°C and separated on a DB-5 column (Agilent Technologies, Santa Clara, CA) in a Thermo Trace Ultra gas chromatograph (GC) at the University of California, Santa Cruz. Separated AA peaks were analyzed on a Finnegan MAT DeltaPlus XL isotope ratio monitoring mass spectrometer (IRM-MS) interfaced to the GC through a GC-C III combustion furnace (960°C) and reduction furnace (630°C).

Each sample type was analyzed in triplicate along with AA standards of known isotopic composition (Sigma-Aldrich Co., St. Louis, MO, USA). Standardization of runs was achieved using intermittent pulses of a CO₂ or N₂ reference gas of known isotopic value. Mean reproducibility of a laboratory algal standard across all individual AAs was ± 0.73 (± 1 SD) for ¹³C. For all consumers and sources, $\delta^{13}\text{C}$ values of twelve individual AAs was analyzed. These AA's included six EAAs [threonine (Thr), leucine (Leu), isoleucine (Ile), valine (Val), lysine (Lys) and phenylalanine (Phe)] and six non-essential AAs, glutamic acid (Glu), aspartic acid (Asp), alanine (Ala), proline (Pro), glycine (Gly), and serine (Ser). Although data were provided on a suite of non-essential AAs, I only present analyses for the EAA.

Data Analysis

Bulk isotope analysis, dietary mixing models, and niche modeling

To estimate contribution of different potential basal sources supporting invertebrate consumers at the two sites at Comal Springs and at Finegan Springs, I used the software package Stable Isotope Mixing Model in R ('simmr') (Parnell et al. 2010, Parnell et al. 2013, R Core Team 2017). 'simmr' utilizes a Bayesian statistical framework to estimate the proportional contribution of different potential basal resources to the diet of each consumer species in a food web (Parnell and Inger 2016). The mixing model utilizes Just Another Gibbs Sampling (JAGS) and runs a Markov Chain Monte Carlo function (MCMC) that produces likely values of the proportional contribution of each diet item to a consumer species. The model incorporates stable isotope values from the consumers, their potential food sources, and isotopic trophic enrichment factors (TEFs). The potential basal food resources used in models were CPOM, FPOM (except for Finegan Springs), and periphyton. TEFs were taken from a meta-analysis (Caut et al. 2009) and used the mean (\pm 1SD) trophic enrichment factors of $1.33\text{‰} \pm 0.454$ and $2.75\text{‰} \pm 1.637$ for ^{13}C and ^{15}N , respectively. Models were run for each consumer species in the food web at each of the three sites (SR3 and SI at Comal Springs, Finegan Springs) and model fitting was conducted with uninformed priors and each model was run for $n = 500,000$ iterations with an $n = 50,000$ burn-in. Prior to mixing model runs, stable isotope values for basal resources (CPOM, periphyton, and FPOM) were compared between the sampling locations at Comal Springs (SR3 and SI) using separate one-way ANOVAs to determine if there were differences in stable isotope values for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. All mixing model and statistical analyses were conducted in R version 3.3.3 (2017).

Using bulk stable isotope values of organisms, I examined potential resource partitioning and niche overlap between closely related species that occur within spring openings each site. I focused niche analyses at each sampling site on two main taxonomic groups that have multiple taxa occupying spring orifices, but species are thought to have different general habits: aquatic beetles (Coleoptera: Byrrhoidea) and amphipods. At the Comal Springs sites (SR3 and SI), I examined niche areas and proportional niche overlap in three aquatic beetle species: *M. pusillus*, *H. comalensis*, and *Stygoparnus comalensis*. As mentioned previously, *M. pusillus* is a cosmopolitan epigeal species and *H. comalensis* has a limited geographic range and is considered crenic, but *S. comalensis* (Coleoptera: Dryopidae) is a subterranean-adapted species with vestigial non-functioning eyes and is the only known subterranean dryopid species in the world (Barr and Spangler 1992). At the SI site, the *Stenelmis* sp. (Coleoptera: Elmidae) was included in niche analyses because it also occurred at that site, *Stenelmis* is a widespread epigeal elmid taxon (Merritt et al. 2008). The amphipod species I utilized from Comal Springs sites were the widespread epigeal *Hyaella azteca* (Amphipoda: Hyaellidae) and the subterranean adapted species, *Stygobromus pecki* (Amphipoda: Crangonyctidae). *S. pecki* is a relatively large-bodied, eyeless, and found in shallow subterranean habitats and spring interfaces (J.R. Gibson, USFWS, pers. comm.). At the Finegan Springs site, I focused niche analyses on the elmids *M. pusillus* and *Heterelmis glabra* (a crenic species in the Devils River, Barr et al. 2015). The amphipods analyzed at Finegan Springs were *H. azteca* and *Paramexiweckelia ruffoi* (Amphipoda: Hadziidae), an eyeless subterranean species with a geographic range limited to south central Texas (Holsinger 1992).

In order to define isotopic niche regions of each of the aforementioned species, I used the ‘nicheROVER’ package in R (ver. 1.0, Swanson et al. 2015). ‘nicheROVER’ utilizes a Bayesian inference framework and incorporates a measure of uncertainty in estimating the probabilistic (95% probability) niche regions (N_R) for species using raw $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for each consumer and performing $n = 1,000$ Monte Carlo draws (Swanson et al. 2015). Using the ‘nicheROVER’ package, I also calculated the degree of niche area overlap (percent overlap) between related species in isotopic space. Niche overlap estimates were calculated from $n = 1,000$ Monte Carlo draws, and the mean percent overlap and $\pm 95\%$ credible interval are reported. Percent niche overlap metrics between species are directional in that niche overlap estimates represent the probability that an individual from Species 1 will be found in the niche space of Species 2 and vice-versa (Swanson et al. 2015). All niche models were run with noninformative priors and niche analyses were run on each species group (beetles or amphipods) from each site (SI, SR3, and Finegan Springs).

Diet estimation using $\delta^{13}\text{CAA}$ - To construct models for predicting membership of unknown samples into known categories I performed linear discrimination analysis (LDA) with the R package ‘MASS’ (Venables and Ripley 2002). Unknown samples in analyses were the two elmids species and two basal resources (CPOM and periphyton) from Comal Springs. I utilized normalized ^{13}C values of six EAAs (Thr, Leu, Ile, Val, Lys, Phe) to compare EAA $\delta^{13}\text{C}$ patterns across groups (Larsen et al. 2013). Categorical groups in LDA models was a large data set of normalized $\delta^{13}\text{CAA}$ values for EAAs for bacteria, algae, fungi, and terrestrial plants extracted from the literature by Larsen et al.

(2009) and (2013). To test the null hypothesis that there was no difference in classification among the groups I applied Pillai's trace (MANOVA).

I additionally determined the relative contributions of EAAs from food sources to consumers using the software package FRUITS (Food Reconstruction Using Isotopic Transferred Signals, Version 3.0, <http://sourceforge.net/projects/fruits>) with normalized $\delta^{13}\text{C}_{\text{AA}}$ values (Fernandes et al. 2014, Fernandes 2015). FRUITS is a Bayesian dietary mixing model and was executed with BUGS (Bayesian inference Using Gibbs Sampling) that includes a system for determining the appropriate Markov chain Monte Carlo scheme based on Gibbs sampling. As dietary proxies, I used normalized $\delta^{13}\text{C}_{\text{AA}}$ of the most informative EAAs from consumers. I initially compared consumer $\delta^{13}\text{C}_{\text{AA}}$ values to those of basal resources collected from Comal Springs (CPOM and periphyton) and then to $\delta^{13}\text{C}_{\text{AA}}$ values from Larsen et al. (2009) and Larsen et al. (2013) for bacteria, algae, fungi, and terrestrial plants. Thus, these analyses will provide estimates of (1) the proportional contribution of periphyton versus CPOM and (2) the proportional contribution of different groups of basal producers (algae, bacteria, fungi, and terrestrial plants) to the diet of the two targeted elmids at Comal Springs. Offsets between food source and consumer were assumed to be negligible given that $\delta^{13}\text{C}$ values in these EAAs has little to no fractionation (Larsen et al. 2013, Gomez et al. 2018).

RESULTS

Bulk isotope analysis, dietary mixing models, and niche modeling

At the Comal Springs site, I examined food webs at the SR3 and SI sites separately because, the sites have contrasting environmental conditions and spring hydrogeomorphic settings. At the SR3 site, I collected and analyzed bulk isotopes for 12 taxa from spring

orifices, which included a mix of crenic, epigean and hypogean habits (Fig. 3.1 A). Epigean taxa were the most species rich group (*M. pusillus*, *H. azteca*, *Psephenus* sp., *Tarbia granifera*, *Elimia* sp., *Melanoides tuberculata*, baetid mayflies, and *Argia* sp.), followed by crenic species (*S. pecki* and *H. comalensis*), and one hypogean species (*S. comalensis*) at SR3 spring orifices. At the SI site, I collected and analyzed 14 taxa (Fig. 3.1 B). Most of the same taxa at SI also occurred at SR3, but SI community did not have baetid mayflies and instead contained several other epigean taxa (*Stenelmis* sp., and heptageniid and leptophlebiid mayflies). Bulk isotopic values varied among the three *a priori* identified basal food resources (i.e., periphyton, CPOM, and FPOM) for spring ecotone food webs at each site (SR3 $\delta^{13}\text{C}$: $F_{2,16}=21.10$, $p < 0.0001$, SR3 $\delta^{15}\text{N}$: $F_{2,18}=20.11$, $p < 0.0001$; SI $\delta^{13}\text{C}$: $F_{2,13}=22.81$, $p < 0.0001$, SI $\delta^{15}\text{N}$: $F_{2,13}=7.81$, $p < 0.01$). However, isotopic values of basal organic matter sources did not significantly differ between the two sampling locations except for FPOM which was approximately $\sim 1\%$ more enriched in $\delta^{15}\text{N}$ at SR3 (periphyton: $\delta^{13}\text{C}$: $F_{1,9}=0.018$, $p = 0.90$, $\delta^{15}\text{N}$: $F_{1,8}=1.28$, $p = 0.29$; CPOM: $\delta^{13}\text{C}$: $F_{1,16}=0.008$, $p = 0.93$, $\delta^{15}\text{N}$: $F_{1,16}=0.52$, $p = 0.48$; FPOM: $\delta^{13}\text{C}$: $F_{1,2}=0.001$, $p = 0.98$, $\delta^{15}\text{N}$: $F_{1,4}=306$, $p < 0.0001$). At both Comal Springs sites, $\delta^{13}\text{C}$ in periphyton ($\bar{x} \pm 1$ SE) was more deplete ($-32.62 \pm 0.2\%$) when compared to CPOM ($-29.48 \pm 0.3\%$) and FPOM ($-26.35 \pm 0.3\%$). Similarly, $\delta^{15}\text{N}$ values of periphyton were most deplete ($3.48 \pm 0.2\%$), while CPOM ($5.02 \pm 0.2\%$) was intermediate and FPOM ($6.31 \pm 0.3\%$) were enriched.

Members of the SR3 food web exhibited a range in basal food resources, with one group of consumers, (*H. azteca*, *Psephenus* sp., adult and larval *M. pusillus*, Baetidae, and Helicopsychidae) primarily supported by periphyton-based OM (contribution to diet

$\geq 50\%$,Table 3.1). Bulk isotopic values for another group of consumers indicated that they were likely feeding on biofilms associated with CPOM and FPOM sources ($\geq 50\%$ of diet from combined CPOM and FPOM), which notably included *H. comalensis* (crenic) and *S. comalensis* (hypogean). The epigean snail fauna at SR3 (*Elimia* sp., *T. granifera*, and *M. tuberculata*) and the damselfly *Argia* had relatively elevated $\delta^{15}\text{N}$ values and a greater contribution of CPOM and FPOM sources to their diet. Snails in stream systems often exhibit somewhat elevated $\delta^{15}\text{N}$ values (e.g., Pound et al. 2011) because they may be less selective grazers than other algivorous invertebrate groups, such as *Psephenus* (Anderson and Cabana 2007). *S. pecki* had the greatest $\delta^{15}\text{N}$ value in the SR3 ecotone community, indicating that it is likely predatory and consumes other invertebrates. In addition, mixing model analysis indicated that *S. pecki* derived a majority of its OM ultimately from CPOM and FPOM sources (42% and 36% contribution, respectively).

The SI food web was similar to SR3, with the same groupings of organisms and their primary OM sources (Fig. 3.1 B, Table 3.1). However, a greater number of taxa appeared to rely upon periphyton-derived OM (10 taxa had $\geq 50\%$ contribution of periphyton OM to diet) when compared to SR3. As observed at SR3, larval and adult *H. comalensis* and adult *S. comalensis* diets were associated with CPOM and FPOM and derived $>60\%$ of their diet from these OM sources. Again, *S. pecki* was the top consumer in the SI spring ecotone food web, presumably feeding on other invertebrates. However, *S. pecki* derived a substantially greater portion of its OM from periphyton (49% contribution, 95% CI = 13-83%) when compared to *S. pecki* populations at SR3.

At Finegan Springs, 12 crenic, epigean and hypogean taxa were collected from spring ecotones and analyzed for bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (Fig. 3.1 C, Table 3.1). Values for

$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ differed between periphyton and CPOM ($\delta^{13}\text{C}$: $F_{1,15}=108.1$, $p < 0.001$, $\delta^{15}\text{N}$: $F_{1,15} = 25.56$, $p < 0.001$), in that periphyton $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were more deplete ($-34.56 \pm 0.4\text{‰}$ and $0.65 \pm 0.3\text{‰}$, respectively) than CPOM ($-29.6 \pm 0.2\text{‰}$ and $3.08 \pm 0.2\text{‰}$, respectively). Members of the Finegan Springs ecotone community exhibited differential use of basal food resources, with one group composed of *Psephenus* sp. (adults), *M. pusillus* (adults and larvae), *Argia* sp., *H. azteca*, Naucoridae, Dytiscidae, and helicopsychid caddisfly larvae relying periphyton based OM (contribution to diet $\geq 50\%$, Table 1). Remaining members of the food web utilized biofilms on CPOM largely, including *H. glabra* (adults and larvae), *Elimia* sp., *M. tuberculata*, and the caddisfly *Phylloicus* sp. (larvae). Two members of the food web, *P. ruffoi* and stratiomyid (soldier fly) larvae had $\delta^{13}\text{C}$ values substantially more enriched than other consumers in the food web ($\sim 4\text{‰}$ more enriched) and the closest basal resource (CPOM, $\sim 6\text{‰}$ more enriched). This suggests that both of these taxa may be utilizing a dietary resource that has an enriched $\delta^{13}\text{C}$ value but was not collected and analyzed at this site (perhaps FPOM). However, repeated sampling at spring sites indicated that there were no other obvious basal resources present at these sites and quantities of FPOM were low to non-existent, thus I do not know the potential “missing” basal OM source that was not sampled from the ecotone.

Percent niche overlap estimates indicated that there is little to no overlap ($\leq 1\%$ for all overlap estimates) among the co-occurring phylogenetically related species in Comal Springs and Finegan Springs (Table 3.2 and Table 3.3). Aquatic beetle taxa at Comal and Finegan systems exhibited little to no niche overlap, even when taxa were utilizing a similar overall resource (i.e., CPOM and FPOM; Table 3.1). Similarly, co-occurring

amphipod species at both Comal sites and Finegan Springs showed < 0.2% overlap in N_R (Table 3.3).

$\delta^{13}C_{AA}$ analyses and Comal Springs elmids

Compound-specific essential $\delta^{13}C_{AA}$ values from the literature data indicated that normalized values of the six amino acids examined in this study (Leu, Phe, Lys, Val, Ile, and Thr) significantly differed when the AAs from the four major producer groups are compared (fungi, bacteria, algae, and terrestrial plants) (Fig. 3.2 A). Normalized $\delta^{13}C_{AA}$ values differed among the broad producer groups for Leu ($F_{3, 89} = 195.8$, $p < 0.001$), Phe ($F_{3, 89} = 25.58$, $p < 0.001$), Lys ($F_{3, 89} = 65.72$, $p < 0.001$), Val ($F_{3, 89} = 43.27$, $p < 0.001$), Ile ($F_{3, 89} = 31.37$, $p < 0.001$), and Thr ($F_{3, 89} = 43.68$, $p < 0.001$), indicating that normalized $\delta^{13}C_{AA}$ values for this group of EAAs could be used to determine the origin (bacteria, algae, fungi, or terrestrial plants) of basal food resources for the two focal elmid species in Comal Springs. When the normalized $\delta^{13}C_{AA}$ values basal food resources from Comal (periphyton, CPOM biofilms) are examined, normalized $\delta^{13}C_{AA}$ values significantly differed among basal resources for Leu ($F_{1, 7} = 11.02$, $p < 0.02$), Phe ($F_{1, 7} = 29.1$, $p < 0.01$), Val ($F_{1, 7} = 19.29$, $p < 0.01$) and Ile ($F_{1, 7} = 12.65$, $p < 0.01$), but did not differ among sources for lys ($F_{1, 7} = 1.74$, $p = 0.229$) and Thr ($F_{1, 7} = 0.177$, $p = 0.687$) (Fig. 3.2 B). In addition, the elmid species, *H. comalensis* and *M. pusillus* differed in their $\delta^{13}C_{AA}$ values for Leu ($F_{1, 6} = 10.28$, $p = 0.018$) and Lys ($F_{1, 6} = 27.8$, $p < 0.01$), but did not differ for Thr, Val, Ile, and Phe ($F_{1, 6} < 0.997$, $p > 0.357$ for all) (Fig. 3.2 C).

Overall, the LDA model was a good fit for the data (Pillai's trace = 2.2099, $F_{18, 258} = 40.09$, $p < 0.001$). The first discriminant axis separated fungi from algae, plants and bacteria, and the second discriminant axis separated bacteria from fungi, algae and plants.

However there was an overlap between clusters of algae and terrestrial plants. In the LDA, literature-derived data were correctly classified with a 97.8% probability. When both the literature and Comal Springs data were included in the LDA, Comal Springs periphyton were classified to the region occupied by bacteria and algae and CPOM was assigned to an area occupied by algae and fungi. When *H. comalensis* and *M. pusillus* were plotted in LDA space, *M. pusillus* was more strongly associated with a clear photoautotrophic signal (CPOM, periphyton, and algae) and *H. comalensis* was categorized in a more bacterial $\delta^{13}\text{C}$ EAA region (Fig 3.3).

When $\delta^{13}\text{C}_{\text{AA}}$ values were used in the isotopic mixing model (i.e., FRUITS), the proportional contribution of different basal food resources to the diets of the two elmid species generally agreed with the LDA. FRUITS ordination models were run with two source data sets: one was conducted with basal resources from Comal Springs (periphyton and CPOM) and the other with basal $\delta^{13}\text{C}$ EAA values from major phylogenetic groups from the literature. For the first model run with Comal Springs basal resources, the most informative EAAs Leu, Ile, Phe, and Val were selected because these AAs were significantly different among the basal resources (Fig 3.2 B). For the second model run, with basal resources from literature EAAs Leu, Ile, and Thr were selected based on the AAs driving the discriminant separation (i.e., those with the highest absolute coefficients in the LDA; Larsen et al. 2013, Gómez et al. 2018) (Fig 3.3).

The $\delta^{13}\text{C}_{\text{AA}}$ mixing model indicated that *M. pusillus* received a majority of their diet from periphyton (~69%) while CPOM contributed a smaller fraction (Fig 3.4 A). In contrast, *H. comalensis* received 90% of their diet from periphyton, with the remaining percent from CPOM (9%) (Fig. 3.4 B). When models were run with $\delta^{13}\text{C}_{\text{AA}}$ values from

the literature for the different producer groups, the output presented a different view of the basal resources for the two species (Fig 3.4 C-D). The dietary mixing model estimated that *H. comalensis* derived a majority of its $\delta^{13}\text{C}_{\text{AA}}$ from bacteria (~68%), whereas fungi, algae, and plants contributed the remainder (7 – 18% each). *M. pusillus* had a much more equivalent contribution of the various basal sources to its $\delta^{13}\text{C}_{\text{AA}}$ signature, with bacteria (~32%), fungi (~28%), and algae (~22%) making the largest contributions.

DISCUSSION

In the present study, I found that communities at spring orifices were composed of taxa exhibiting multiple primary habits (e.g., epigeal/crenophilic, obligate crenic, and hypogean/subterranean) which coexist in a spatially discrete and relatively confined ecosystem type. I also observed that invertebrate communities found within spring ecotones rely upon a diverse set of OM sources of autochthonous and allochthonous origin and that phylogenetically related consumers appear to be partitioning basal OM resources (i.e., niche partitioning). My results indicate that several obligate crenic and hypogean species (i.e., *H. comalensis* and *S. comalensis*) exhibited a close dietary association with terrestrial surface-derived OM (e.g., Simon et al. 2003). Thus, results from this study indicate that OM inputs can play a central role in determining not only the physicochemical conditions of springs, but also the community composition and biological productivity of biotic spring ecotone communities (Lowe and Likens 2005, Reiss and Chiffard 2017).

Input and deposition of terrestrial plant material to stream and flowing water ecosystems can play an important role in the structure and function of macroinvertebrate

communities (Wallace et al. 1999, Hoffman and Hering 2000, Benke and Wallace 2003). In addition to providing a substrate for grazable microbial biofilms (e.g., Eggert and Wallace 2007), wood and leaf materials can serve as habitat (microhabitat sites) and can create larger-scale instream habitats through flow alteration (snags and CPOM accumulation areas, Benke and Wallace 2003). Colonization and use of terrestrial CPOM and woody material by macroinvertebrates is dependent upon several factors, including the type of CPOM, its state of decay, and the species-specific traits and requirements of the macroinvertebrates in question (Magoulick 1998, Collier and Halliday 2000). Two species of spring orifice associated elmids (*H. comalensis* at Comal Springs and *H. glabra* at Finegan Springs) derived a majority of its diet from CPOM and FPOM materials. In addition, two subterranean species at Comal Springs (*S. pecki* at the SR3 site and *S. comalensis* throughout Comal Springs) derived most of their OM from terrestrial sources. Previous work in the Edwards Aquifer region has found that inputs of terrestrial OM from the surface support subterranean and spring orifice taxa, but that the relative importance of terrestrial OM to consumers at a particular site depends upon the hydrogeomorphic setting and spatial location of the site within the aquifer (Hutchins et al. 2016). Findings of this study are consistent with those of Hutchins et al. (2016), in that terrestrial OM sources are relatively important to crenic and subterranean taxa at the Comal Springs site. Thus, my data indicates that surface – subsurface connections that facilitate inputs and flows of allochthonous terrestrial C are critical for the persistence and stability spring ecotone food webs (e.g., Huxel et al. 2002).

The relative importance of allochthonous terrestrial inputs to aquatic food webs varies with environmental setting, such as stream geomorphology and the degree of

canopy cover (Minshall 1978, Connors and Naiman 1984, England and Rosemond 2004). In this study, the relative importance of terrestrial OM varied between sites for some consumers in the Comal Springs system. Using the natural abundance of $\delta^{15}\text{N}$ in Comal Springs consumers, the eyeless subterranean amphipod, *S. pecki* was the top invertebrate predator in spring orifices at both SR3 and SI. However, the relative importance of terrestrial versus periphyton OM differed between the sites for *S. pecki*: periphyton contributed 22% of the OM to *S. pecki* at SR3, but contributed 49% of OM to *S. pecki* at SI. This change is likely related to differences in the environmental conditions at SR3 and SI in that SR3 has more extensive canopy cover and the riparian vegetation that extends all the way to the stream bank and spring orifices, whereas SI is an open canopy environment with little adjacent terrestrial vegetation. Furthermore, it appears as though this shift in the relative importance in OM sources for *S. pecki* largely tracked that of its likely primary prey item. Assuming TEFs (i.e., isotopic enrichment per trophic transfer) of 2.75‰ for ^{15}N and 1.33‰ for ^{13}C (Caut et al. 2009), it is likely that *S. pecki* is feeding largely upon the epigeal amphipod *H. azteca* at both sites (Fig. 3.1 A and B). Percent contribution of periphyton to the diet of *H. azteca* similarly shifted to a greater reliance on CPOM materials between sites (70% at SR3 to 81% at SI). Other studies have also observed that eyeless subterranean amphipod species will consume epigeal amphipod taxa when they occur in the same habitat (Simon et al. 2003). However, this study also found that the environmental setting of spring orifice ecotones has consequences for the relative importance of different OM sources that cascade to top trophic level consumers.

Identification of isotopically distinct sources is a key component in the successful use and application of isotopic mixing models (Fry 2006, Phillips et al. 2014). In the

present study, the subterranean amphipod *P. ruffoi* ($-23.4 \pm 0.3\text{‰}$) and soldier fly larvae ($-24.09 \pm 0.1\text{‰}$) at Finegan Springs had more enriched $\delta^{13}\text{C}$ values when compared to the *in situ* basal resources (CPOM: $-29.6 \pm 0.2\text{‰}$ and periphyton: $-32.62 \pm 0.2\text{‰}$) indicating that these organisms are likely feeding on a relatively isotopically enriched OM source which was not identified and collected at the study site. It is possible that these organisms could be deriving dietary OM from processed and degraded CPOM. Decomposition of CPOM can result in FPOM materials with enriched $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, due to the preferential use of the ^{12}C and subsequent fractionation (Engle & Mako 1993, Johnson et al. 2018). At the Comal Springs site, CPOM ($-29.48 \pm 0.3\text{‰}$) was isotopically more deplete than the collected FPOM source ($-26.35 \pm 0.3\text{‰}$), indicating that processing of materials leads to an enrichment of OM. Assuming that processing and decomposition of CPOM at Finegan Springs undergoes similar isotopic enrichment ($\sim 3\text{‰}$) and that there is a $\delta^{13}\text{C}$ TEF of 1.33‰ a consumer and their food source (Caut et al. 2009), and it is plausible that FPOM serves as a dietary source for *P. ruffoi* and soldier fly larvae in the Finegan Springs system. As stated previously, I did not collect FPOM from Finegan because spring openings were fractures in bedrock with no obvious FPOM. However, inclusion of FPOM as a basal resource at Finegan springs might be able to further elucidate the dietary requirements of *P. ruffoi* and soldier fly larvae in future studies.

Species inhabiting low-productivity environments can exhibit a high degree of specialization (i.e., morphological and physiological traits) and niche partitioning that reduces competition for limited resources (Correa and Winemiller 2014, Hutchins et al. 2016, Francois et al. 2016). This study clearly demonstrates resource partitioning among co-occurring and phylogenetically related species within spring ecotones. Estimation of

the percent niche space overlap using bulk stable isotope data indicated that the aquatic beetle species in Comal Springs ($n = 4$ species) and at Finegan Springs ($n = 2$ species) showed $<1\%$ overlap in their respective niche areas. In addition, amphipod taxa at Comal Springs and Finegan Springs ($n = 2$ species at each site) also showed remarkably little niche overlap ($\leq 1\%$ overlap). There was a considerable amount of trophic and feeding niche diversity in spring ecotone food webs, indicating a variety of foraging and feeding strategies (e.g., selective herbivores, non-selective herbivores/omnivores, CPOM and FPOM consumers, and predators). Furthermore, I observed an almost complete lack of niche overlap when phylogenetically related taxa at a site occupied the same general trophic guild. For example, both *S. comalensis* and *H. comalensis* in the Comal Springs system obtain a majority of their diet from use of CPOM and FPOM, but niche overlap estimates between the two species indicate that they exhibited $\leq 1\%$ overlap. Thus, there also appears to be a great deal of niche specialization in spring ecotone spaces, even within a given trophic guild. Previous studies have also found that multiple phylogenetically related invertebrate taxa in relatively resource limited systems can occupy the same habitat space and exhibit niche partitioning related to slight differences in mouthpart morphology (Hutchins et al. 2014). In addition, permanently flowing spring systems often contain a great deal of microhabitat structural complexity (Cantonati et al. 2012), which may account for some of the apparent niche partitioning and stable coexistence in low resource environments (e.g., Trontelj et al. 2012).

Use of bulk isotopes and $\delta^{13}\text{C}_{\text{AA}}$ for the two co-occurring elmids species at Comal Springs (*H. comalensis* and *M. pusillus*) provided different perspectives on niche partitioning in spring ecotone systems. Bulk isotope dietary mixing models indicated that

H. comalensis derived a majority of its resources from terrestrially-derived OM, whereas *M. pusillus* relied more on periphyton. Dietary mixing models using $\delta^{13}\text{C}_{\text{AA}}$ data for *H. comalensis* and *M. pusillus* present a more complex and potentially contrasting picture. Mixing models using the *in situ* basal resources (periphyton and CPOM biofilms) indicated that *H. comalensis* derived ~90% of its diet from periphyton in contrast to mixing model results using bulk isotopes. However, dietary mixing models of the elmid species using basal producer data from the literature indicated that *H. comalensis* derived most of its $\delta^{13}\text{C}_{\text{AA}}$ from bacteria and that *M. pusillus* had a more equivalent mix of dietary sources derived from bacteria, fungi, algae, and terrestrial plants. This outcome is likely related to the complex and heterogeneous nature of biofilms in aquatic systems. Aquatic biofilms are complex communities composed of multiple organism types and OM including algae, bacteria, fungi, protists, and detritus (Wetzel 1983). In addition, my removal of biofilm materials from the CPOM surfaces also likely collected some terrestrial plant material and associated amino acids. Indeed, when periphyton and CPOM biofilms from Comal Springs are plotted in LDA space with literature producer data (Fig. 3.3), epilithic periphyton are largely distributed in the bacterial region and CPOM biofilms are positioned in an area intermediate to algal and bacterial regions. These results demonstrate difficulties working with biofilms that are an amalgam of multiple basal producer types that have different $\delta^{13}\text{C}_{\text{AA}}$ profiles. Regardless, the analysis of *H. comalensis* and *M. pusillus* diets using literature data as sources clearly indicate that these species likely exploit different types of basal resources to varying degrees and have limited feeding niche overlap.

This study demonstrates that diverse assemblages of macroinvertebrate in spring ecotones use a diversity of resources and effectively partition niche space. Spring-influenced lotic ecosystems often exhibit relatively high biological diversity and contain numerous endemic fauna, but these systems also face numerous natural and anthropogenic impacts including drought, groundwater extraction, introduction of non-native fauna, and introduction of pollutants (Bowles and Arsuffi 1993, Crowe and Sharp 1997, Earl and Wood 2002). Some of the ecotone specialists examined by this study are listed as federally endangered in the US and have ranges restricted to just a few sites (i.e., *S. comalensis*, *S. pecki*, and *H. comalensis*). It is also critical to note that these species (including subterranean fauna) largely acquire dietary OM from terrestrial subsidies (CPOM and FPOM). Due to the relatively small size of spring systems, natural and anthropogenic perturbations can potentially affect their hydrology, and lead to its disconnection from adjacent riparian vegetation, thereby reducing availability of allochthonous resources to communities. Thus, I suggest that maintaining and restoring riparian zones around some spring ecotones may be critical for the conservation and management of these unique habitats and their biological communities.

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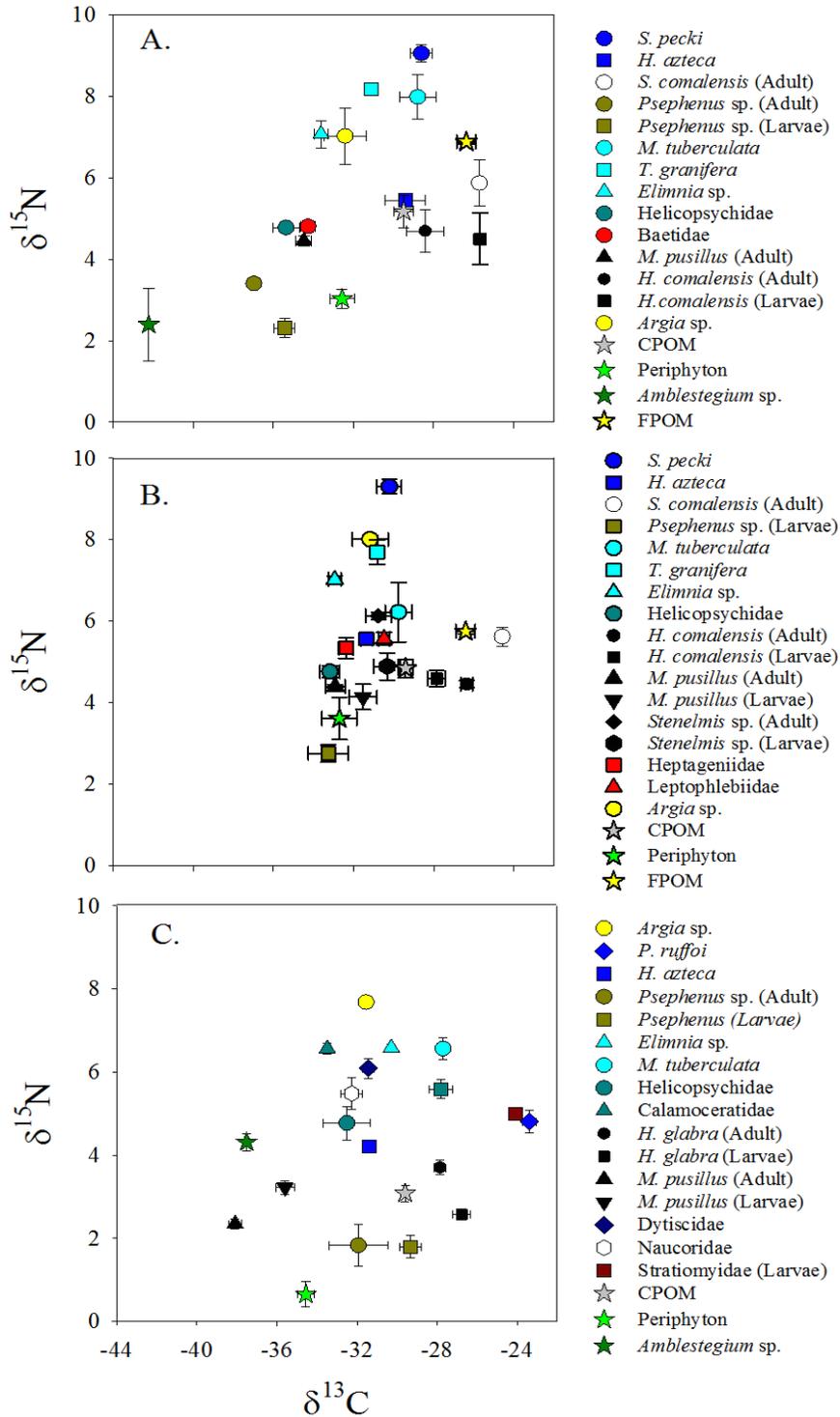


Figure 3.7: Stable isotope ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) biplot for Spring Run 3 (A), Spring Island (B), and Finegan Springs (C). Each point represents the mean of each species and bars represent ± 1 SE

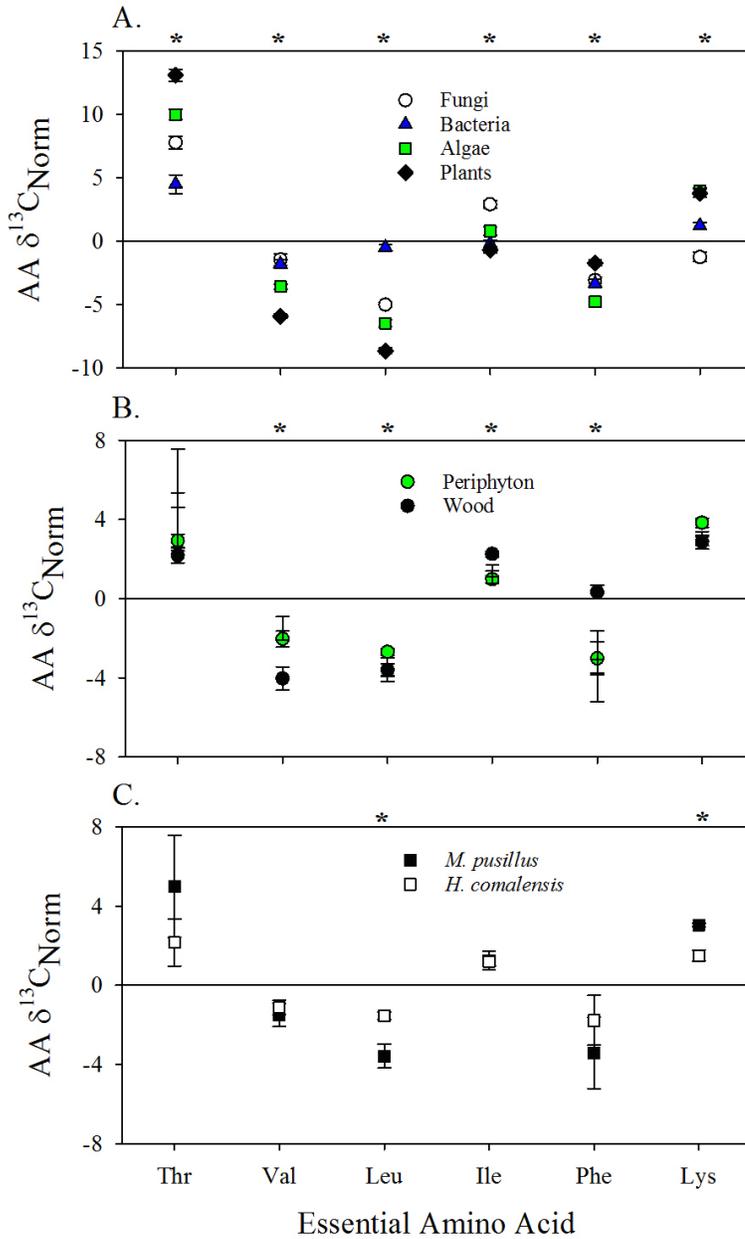


Figure 3.8: Normalized essential amino acid $\delta^{13}\text{C}$ values for basal sources from the literature (A), basal food resources from Comal (B), and *H. comalensis* and *M. pusillus* (C). Each point represents a mean of $n = 3$ or 4 replicate analyses and bars are ± 1 SE * along the top of each panel represent a significant difference exists between sources or consumers for each amino acid

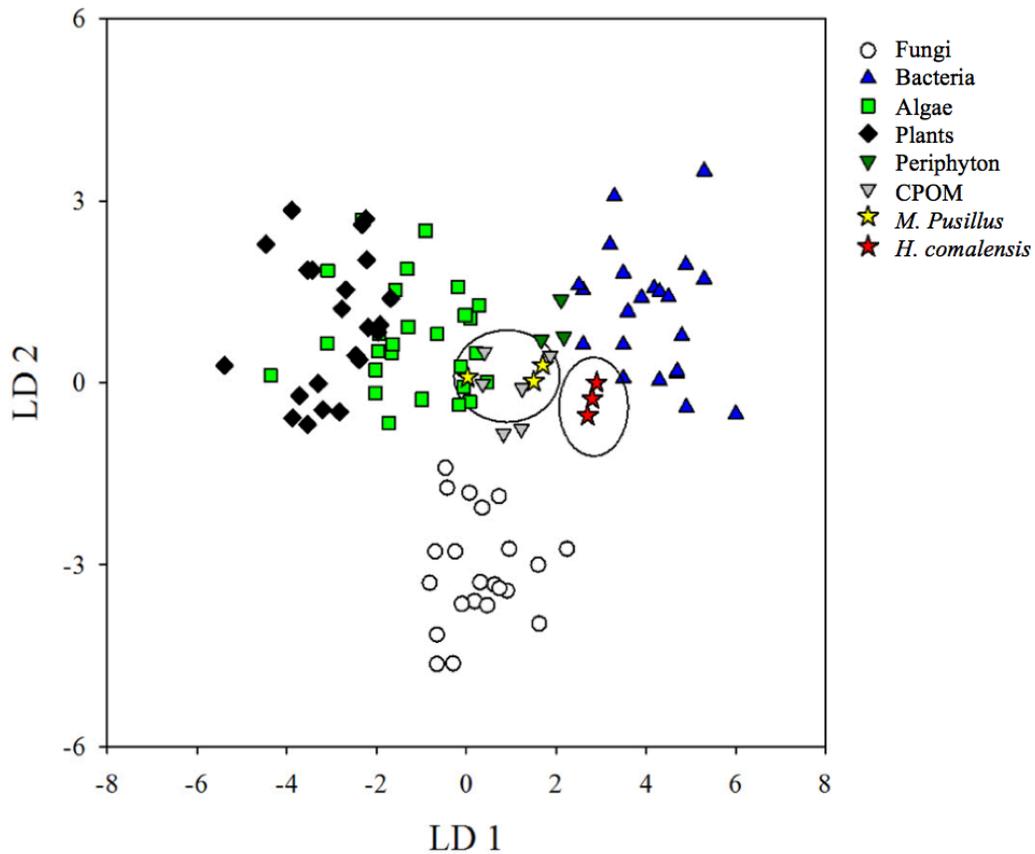


Figure 3.9: Linear discriminant analysis figure (LDA) graph portraying normalized $\delta^{13}\text{CAA}$ values for literature basal resources (fungi, bacteria, algae and plants), Comal Springs basal resources (periphyton, leaves, and wood), and *H. comalensis* and *M. pusillus* from the Comal system. Clusters of the points for the Comal basal resources are circled to illustrate their ordination in biplot space. The amino acids driving the discriminant separation (i.e., those with highest absolute coefficients) were leucine (6.99), isoleucine (6.14), and threonine (6.11) while phenylalanine (6.04) and lysine (6.03), valine (5.99) had smaller effects.

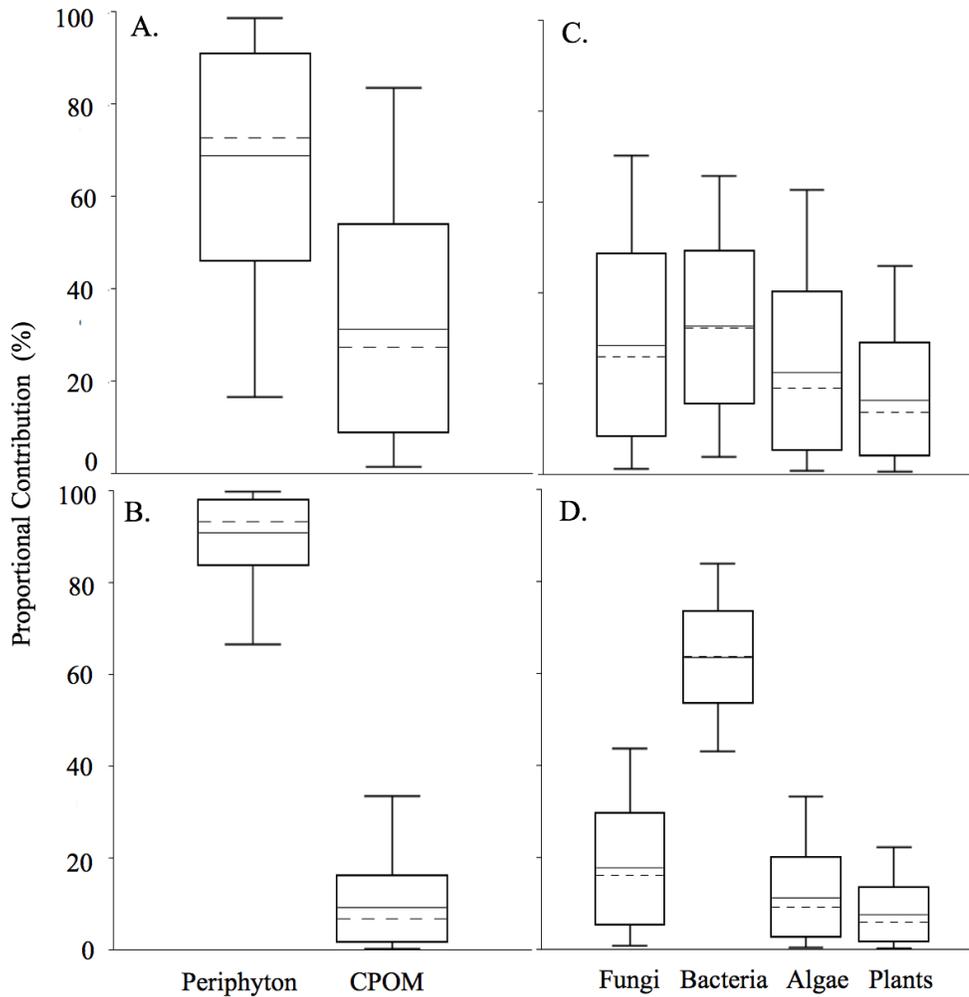


Figure 3.10: FRUITS model output for *H. comalensis* (A, C) and *M. pusillus* (B, D) using *in situ* organic matter sources (CPOM and periphyton) (A, B) and outputs for runs utilizing literature data for phylogenetically-defined producer groups (fungi, bacteria, algae, and plants) (C, D). Boxes represent a 68% credible interval (corresponding to the 16th and 84th percentiles) while the whiskers represent a 95% credible interval (corresponding to the 2.5th and 97.5th percentiles). The horizontal continuous line represents the estimated mean while the horizontal dashed line represents the estimated median (50th percentile)

Table 3.1: Bayesian mixing model outputs for Spring Run 3 (SR3) and Spring Island (SI) at Comal Springs and Finegan Springs, with the percent contribution of basal resources (mean and 95% credible interval values) to each consumer species that was sampled.

Abbreviations in parentheses: A=Adult, L=Larvae

Taxon	Periphyton	CPOM		FPOM
		Mean (\pm 95% CI)		
Comal Springs (SR3)				
<i>Stygobromus pecki</i>	22 (4-48)	42 (6-84)	36 (6-68)	
<i>Hyalella azteca</i>	64 (35-91)	21 (2-54)	15 (2-29)	
<i>Heterelmis comalensis</i> (L)	26 (0-57)	28 (0-60)	46 (0-86)	
<i>Heterelmis comalensis</i> (A)	41(2-81)	36 (3-81)	23 (4-89)	
<i>Microcylloepus pusillus</i> (A)	87 (67-97)	8 (1-25)	5 (1-16)	
<i>Stygoparnus comalensis</i>	23 (3-76)	26 (3-76)	51 (5-89)	
<i>Psephenus</i> (L)	62 (10-99)	23 (2-75)	15 (2-51)	
<i>Psephenus</i> (A)	49 (5-92)	29 (3-80)	22 (2-67)	
<i>Tarebia granifera</i>	51 (14-86)	28 (3-67)	21 (3-53)	
<i>Elimia</i> sp.	60 (24-89)	25 (3-62)	15 (2-38)	
<i>Melanoides tuberculata</i>	30 (4-64)	40 (4-84)	30 (5-62)	
<i>Helicopsychidae</i>	82 (53-97)	11 (1-36)	7 (7-52)	
<i>Baetidae</i>	82 (46-96)	11 (1-41)	7 (1-22)	
<i>Argia</i> sp.	55 (9-90)	28 (3-75)	18 (2-50)	
Comal Springs (SI)				
<i>Stygobromus pecki</i>	49 (13-83)	31(0-60)	20 (0-47)	
<i>Hyalella azteca</i>	81 (64-97)	14 (0-31)	5 (0-13)	
<i>Heterelmis comalensis</i> (L)	31 (1-52)	35 (0-73)	34 (1-57)	
<i>Heterelmis comalensis</i> (A)	10 (0-24)	30 (0-53)	60 (39-81)	
<i>Microcylloepus pusillus</i> (L)	54 (14-94)	27 (0-58)	18 (0-47)	
<i>Microcylloepus pusillus</i> (A)	81 (47-100)	13 (0-41)	6 (0-21)	
<i>Stygoparnus comalensis</i>	16 (0-50)	22 (0-57)	62 (13-100)	
<i>Stenelmis</i> sp.(L)	59 (29-90)	27 (0-54)	13 (0-35)	
<i>Stenelmis</i> sp. (A)	61 (31-91)	27 (0-52)	12 (0-33)	
<i>Psephenus</i> (L)	67 (33-99)	22 (0-52)	11 (0-25)	
<i>Tarebia granifera</i>	64 (39-87)	26 (0-50)	10 (0-60)	
<i>Elimia</i> sp.	66 (36-96)	23 (0-49)	10 (0-31)	
<i>Melanoides tuberculata</i>	46 (19-76)	33 (0-64)	20 (0-41)	
<i>Helicopsychidae</i>	86 (62-100)	10 (0-30)	4 (0-13)	
<i>Heptageniidae</i>	69 (31-99)	20 (0-51)	11 (0-35)	

Table 3.1. Continued.

<i>Leptophlebiidae</i>	62 (37-89)	27 (0-51)	11 (0-28)
<i>Argia sp.</i>	51 (14-93)	30 (0-60)	19 (0-46)
Finegan Springs			
<i>Heterelmis glabra</i> (A)	12 (0-39)	88 (47-56)	--
<i>Heterelmis glabra</i> (L)	44 (0-34)	56 (64-75)	--
<i>Microcyloepus pusillus</i> (L)	80 (51-100)	20 (0-49)	--
<i>Microcyloepus pusillus</i> (A)	91 (75-100)	9 (0-25)	--
<i>Psephenus</i> (L)	36 (1-75)	64 (25-99)	--
<i>Psephenus</i> (A)	72 (40-100)	28 (0-60)	--
<i>Dytiscidae</i>	57 (36-78)	43 (22-64)	--
<i>Paramexeweckelia ruffoi</i>	41 (0-73)	59 (27-98)	--
<i>Hyalella azteca</i>	61 (49-73)	39 (27-51)	--
<i>Phylloicus sp.</i>	14 (0-35)	86 (66-100)	--
<i>Helicopsychidae</i>	57 (30-88)	43 (14-70)	--
<i>Melanoides tuberculata</i>	5 (0-14)	95 (86-100)	--
<i>Elimia sp.</i>	39 (27-50)	61 (50-73)	--
<i>Naucoridae</i>	63 (36-90)	39 (1-64)	--
<i>Argia sp.</i>	63 (49-76)	37 (24-51)	--
<i>Stratiomyidae</i>	38 (0-69)	62 (31-99)	--

Table 3.2: Results of the probabilities of niche overlap among riffle beetles and dryopid beetle at Comal Springs - Spring Run 3 (SR3) and Spring Island (SI), and at Finegan Springs. The mean probability indicates the probability of Species A niche being found within the niche of Species B. The probability of overlap is indicated by mean and credible intervals (2.5%, 97.5%). The niche overlaps were calculated using $\alpha = 0.95$

Species A	Species B	% Mean Probability (Credible Interval), $\alpha=0.95$
Comal Springs (SR3)		
<i>Heterelmis comalensis</i>	<i>Microcyloopus pusillus</i>	0.04 (0, 0)
	<i>Stygoparnus comalensis</i>	0.28 (0, 3)
<i>Microcyloopus pusillus</i>	<i>Heterelmis comalensis</i>	0.00 (0, 0)
	<i>Stygoparnus comalensis</i>	0.00 (0, 0)
<i>Stygoparnus comalensis</i>	<i>Heterelmis comalensis</i>	0.06 (0, 1)
	<i>Microcyloopus pusillus</i>	0.00 (0, 0)
Comal Springs (SI)		
<i>Heterelmis comalensis</i>	<i>Microcyloopus pusillus</i>	0.49 (0, 2)
	<i>Stygoparnus comalensis</i>	0.02 (0, 0)
	<i>Stenelmis sp.</i>	0.05 (0, 0)
<i>Microcyloopus pusillus</i>	<i>Heterelmis comalensis</i>	0.20 (0, 2)
	<i>Stygoparnus comalensis</i>	0.00 (0, 0)
	<i>Stenelmis sp.</i>	0.00 (0, 0)
<i>Stenelmis sp.</i>	<i>Heterelmis comalensis</i>	0.01 (0, 0)
	<i>Microcyloopus pusillus</i>	0.00 (0, 0)
	<i>Stygoparnus comalensis</i>	0.09 (0, 1)
<i>Stygoparnus comalensis</i>	<i>Heterelmis comalensis</i>	0.02 (0, 0)
	<i>Microcyloopus pusillus</i>	0.00 (0, 0)
	<i>Stenelmis sp.</i>	0.60 (0, 2)
Finegan Springs		
<i>Heterelmis glabra</i>	<i>Microcyloopus pusillus</i>	0.00 (0,0)
<i>Microcyloopus pusillus</i>	<i>Heterelmis glabra</i>	0.00 (0,0)

Table 3.3: Results of the probabilities of niche overlap among amphipods at Comal Springs - Spring Run 3 (SR3) and Spring Island (SI), and at Finegan Springs. The mean probability indicates the probability of Species A niche being found within the niche of Species B. The probability of overlap is indicated by mean and credible intervals (2.5%, 97.5%). The niche overlaps were calculated using $\alpha = 0.95$

Species A	Species B	% Mean Probability [Credible Interval], $\alpha=0.95$
Comal Springs (SR3)		
<i>Hyaella azteca</i>	<i>Stygobromus pecki</i>	0.10 (0, 0)
<i>Stygobromus pecki</i>	<i>Hyaella azteca</i>	0.01 (0, 0)
Comal Springs (SI)		
<i>Hyaella azteca</i>	<i>Stygobromus pecki</i>	0.01 (0,0)
<i>Stygobromus pecki</i>	<i>Hyaella azteca</i>	0.00 (0,0)
Finegan Springs		
<i>Hyaella azteca</i>	<i>Paramexiweckelia ruffoi</i>	0.00 (0,0)
<i>Paramexiweckelia ruffoi</i>	<i>Hyaella azteca</i>	0.00 (0,0)

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4. METABOLIC RESPONSES TO LONG-TERM FOOD DEPRIVATION IN SUBTERRANEAN AND SURFACE AMPHIPODS

INTRODUCTION

Subterranean ecosystems are traditionally thought to be energy-limited systems because of spatiotemporal patchiness of food resources due to a lack of *in situ* autotrophic production and intermittent allochthonous (surface-generated organic matter) inputs (Poulson 1964, Hüppop 1985, Venarsky et al. 2014, but see Hutchins et al. 2016). It has been hypothesized that hypogean (subterranean) adapted organisms living in low energy habitats can withstand long periods of resource shortage and show increased starvation resistance due to reduced metabolic rate (Hüppop 1985, Hervant et al. 1997, Hervant et al. 1999, Hervant and Renault 2002, Issartel et al. 2010). Subterranean biologists have long thought that subterranean ecosystems are more energy limited than photosynthetically based surface ecosystems (Poulson and Lavoie, 2000), but there is recognition that some subterranean systems, such as caves with bat populations and guano production (Ferreira and Martins 2000, Gnaspini 2005), relatively open cave systems with allochthonous terrestrial organic matter (OM) inputs (Culver and Poulson 1971), or systems with chemoautotrophic production (Engel 2007, Porter et al. 2009, Hutchins et al. 2016) are not as strongly energy limited. In these relatively energy-rich subterranean environments, it is hypothesized that selection pressure on organisms to exhibit reduced metabolic rates should be relaxed (Culver and Poulson 1971, Spicer 1998, Riesch et al. 2011).

There are multiple lines of evidence that hypogean invertebrates in subterranean systems have evolved behavioral, physiological, and metabolic adaptations that allows

them to persist in low energy habitats and survive for extended time periods during food deprivation (Hervant et al., 1997, 1999, Hervant and Renault 2002, Issartel et al. 2010). Experiments comparing responses of hypogean invertebrates to those of related epigean taxa to long-term food deprivation have indicated that hypogean organisms have higher amounts of stored energy reserves (e.g., glycogen, triglycerides, and proteins) and utilized these reserves at slower rates than related epigean taxa (Hervant et al. 1997, Hervant et al. 1999, Hervant and Renault 2002, Mezek et al. 2010). In these experiments, epigean invertebrates (i.e., amphipods and isopods) exhibit immediate, linear, and simultaneous declines in energy reserve forms when exposed to periods of prolonged food deprivation (Hervant et al. 1999, Hervant and Renault 2002). In contrast, hypogean amphipods and isopods respond to prolonged food deprivation in successive phases, with an immediate but slower rate of glycogen depletion, followed by utilization of proteins, and then use of lipids (Hervant et al. 1999, Hervant and Renault 2002). However, how cosmopolitan this metabolic response to prolonged food deprivation is among hypogean fauna from different subterranean conditions remains largely unknown. In addition, identifying how organisms from subterranean systems respond to food deprivation will improve our understanding of organisms' ability to withstand nutritional stress and survivability in a diversity of energy-limited biotopes.

The Edwards Aquifer (central Texas, USA) is a karst limestone aquifer that is one of the most biodiverse groundwater ecosystems in the world in terms of stygobionts (i.e., obligate subterranean aquatic organisms) (Longley 1981, Bowles and Arsuffi 1993, Ourso and Horring 2000, Hutchins 2017). Organic matter (OM) dynamics in the Edwards Aquifer are spatially diverse, that is much of the aquifer is supported by spatiotemporally

patchy inputs of allochthonous terrestrial detritus, but sites around a saline – freshwater interface are supported by *in situ* chemolithoautotrophic production (Engel 2009, Hutchins et al. 2016). Previous researchers have hypothesized that organisms at sites in the Edwards Aquifer that are supported by chemolithoautotrophic production would have the selection pressure for reduced metabolic rates and differential patterns of energy utilization reduced or absent (Bishop et al. 2014). However, there is also a vertical spatial gradient in resource availability present within the aquifer; hypogean organisms that inhabit shallow phreatic environments and spring opening ecotones are more likely to have greater access to terrestrial OM sources and therefore greater resource availability (Nair et al. *in prep*). Indeed, spring ecotone environments are considered “windows” to the subterranean world, and present unique environments to investigate the ecology and evolution of groundwater organisms (Galassi et al. 2014).

Spring ecotones often contain unique and diverse assemblages composed of surface, crenic (spring obligates) and hypogean taxa (Cantonati et al. 2012). The Edwards Aquifer contains one of the most diverse subterranean amphipod assemblages on the planet, both in terms of the taxonomic and functional diversity (Holsinger 1967, Longley 1981, Hutchins et al. 2014, Hutchins et al. 2016, Hutchins 2017). Edwards Aquifer spring ecotones frequently contain shallow phreatic subterranean amphipod species that, while having morphological adaptations to subterranean existence, such as the absence of eyes and the presence of long appendages (Holsinger 1967), feed largely on surface derived OM sources (Nair et al. *in prep*). One such Edwards Aquifer species, *Stygobromus pecki* (Peck’s cave amphipod; (Amphipoda: Crangonyctidae) is listed as a federally endangered species (United States Fish and Wildlife Service 1997) and is

endemic to two major spring systems in the aquifer (i.e., Comal and Hueco springs) (Holsinger 1967, Gibson et al. 2008). The presence and persistence of *S. pecki* at spring ecotone sites in the Edwards Aquifer, and its clear morphological adaptation to subterranean existence, presents an opportunity to examine the physiological responses and potential adaptations of a subterranean organism living in a more energy-rich environment.

In the study presented here, I experimentally examined the energy utilization and metabolic responses of *S. pecki* to long-term food deprivation. For comparison, I conducted a parallel experiment on a related epigean amphipod, *Synurella* sp. in the same family (Crangonyctidae). I also compared basal metabolism of both species by measuring O₂ consumption rates. The purpose of this study was to examine metabolic and food deprivation responses in a subterranean adapted organism that exists in a more energy rich environment in order to assess the hypothesis that subterranean organisms in more energy-rich environments experience relaxation of selective pressures on stygomorphic metabolic adaptations.

MATERIAL AND METHODS

Site description and organism collection

Live individuals of *S. pecki* were collected from spring openings at Comal Springs (29°43'5.32"N, 98°7'52.94"W). Comal Springs (city of New Braunfels, Comal County) is located along the eastern edge of the Edwards Plateau and is the largest spring complex in Texas. The Comal Springs system discharges groundwater from the Edwards Aquifer from > 400 spring openings and is one of the principle locations for *S. pecki*. *S. pecki*

were collected from the immediate vicinity of spring openings by a combination of hand picking and sweeps of small aquarium nets. The surface-dwelling amphipod *Synurella* were collected using the same techniques from perennially flowing streams (30°35'53.8"N, 95° 7'42.6"W) that receive groundwater inputs from the Gulf-Coast Aquifer (city of Coldspring, San Jacinto County). Although *Synurella* were collected from spring run portions of a small stream, their population is not restricted to immediate spring openings and is distributed throughout the entire spring run system. Coldspring is located ~300 km to the northeast of Comal Springs. *Synurella* collected at the Coldspring site are morphologically consistent with *S. bifurca*, but there is a great deal of cryptic diversity within the genus that has yet to be resolved (JR Gibson, USFWS). Thus, I hereafter refer to this species as as *Synurella*. Live animals were placed in high-quality coolers filled with site water and were brought back to the Freeman Aquatic Biology building (FAB) at Texas State University.

Once animals were in the lab, they were acclimated to lab conditions in a large plastic flow-through chamber with an ambient water temperature of 23°C and DO >8 mg/L. Water supplying the Wet Lab came from the Edwards Aquifer. The flow through chamber contained pre-cleaned limestone river cobbles, polyester mesh, and well-conditioned conditioned wood and leaf material. Both the species were fed *ad libitum* (typically weekly) with dense culture fish food (Pentair Dense Culture Food, F2A) prior to the start of experiments. Previous studies examining life history and mating of *S. pecki* have successfully used these housing systems and feeding frequencies (Nowlin et al. 2015). Animals used in experiments were not differentiated based on sex due to the potential stress and mortality associated with sex determination. However, gravid females

were excluded from the study in order to exclude the differences in biochemical composition between gravid and non-gravid females, and gravid females and males.

Estimation of basal metabolic rates

After acclimating both species to lab conditions for a two-week period, I estimated basal metabolic rates by measuring O₂ consumption rates under acclimated laboratory conditions. Oxygen consumption rates of fed individuals were estimated using Qubit systems OX1LP-30 dissolved oxygen (DO) cuvettes with Clark cell type polarographic oxygen sensor (Qubit Systems, Kingston, ON, Canada). An individual test subject was placed in a cuvette filled with 5-mL of water and was allowed to acclimate to the chamber for 30 minutes prior to recording O₂ changes of the chamber. After acclimation, DO concentration was recorded at 30-second intervals for 15 minutes. Cuvettes are externally jacketed with a water flow through system in order to maintain thermal stability. All experiments were carried out in a dark room. Oxygen consumption rates were obtained by regressing the change in DO concentration over the 15 min observation period. O₂ consumption rates were divided by wet weight (ww) of an individual (in g) to obtain mass-specific rate of O₂ consumption (mg O₂/g ww/h). O₂ consumption rates were estimated for $n = 5$ fed individuals for each species.

Food deprivation and metabolic responses

To assess biochemical changes and utilization of potential energy reserves in amphipods during extended periods of food deprivation, I experimentally examined change in whole-body metabolites in both species. Changes in metabolites during food deprivation was estimated at regular sampling intervals during food deprivation over 90 days for *S. pecki*

(metabolites measured on days 0, 15, 30, 60, and 90) and over a 30 days for *Synurella* (metabolites measured on days 0, 15, and 30). These species-specific food deprivation time periods were based a pilot study that assessed mortality during an extended food deprivation period for *S. pecki* and *Synurella*.

Before the start of experiments, animals were acclimated to lab conditions, maintained, and fed as above for ~1 month. At the start of experiments, individuals of each species were separated into two treatments: fed (receiving regular feeding) and unfed (food deprived). Individuals in unfed treatments were not fed during the experimental period, and in the fed treatments individuals were fed on a weekly basis with dense culture fish food pellets. For both species, each treatment contained $n = 75$ individuals. Experimental set ups for each treatment consisted of five PVC manifolds which were connected via nylon tubing to flow-through holding chambers made from 20-cm long and 1.91 cm diameter PVC pipes; each manifold was connected to three holding chambers. Each holding chamber was internally separated into five sections using 1000 μm nylon mesh. Each section within a holding chamber held an individual amphipod; amphipods were segregated from each other in order to prevent cannibalism (Nowlin et al. 2015). Water was continuously fed into each manifold from the lab water supply and into and through holding chambers to ensure volume was replaced every 2-3 minutes. On each sampling date, a randomly selected individual amphipod from each of the three holding chambers attached to a manifold were removed for analysis. Because of sample mass requirements for biochemical analysis, these three individuals were pooled prior to analysis (a pilot study was conducted and determined that analyses required $n = 3$ individuals to exceed method detection limits). Thus, on each sampling date each

treatment had $n = 5$ analytical replicates (each replicate consisting of three randomly selected individuals from a single manifold set up). Animals were checked weekly for mortality; animals were not replaced in cases of mortality.

Metabolite analysis

Whole-body metabolites were estimated by analyzing five samples (comprised of 3 individuals per sample) from each treatment on each sampling date (Day 0, 15, 30, 60 and 90 days for *S. pecki* and day 0, 15, and 30 for *Synurella*). Once removed, animals were immediately frozen at -80°C in clean 2 mL microcentrifuge tubes. Before the body metabolites were assayed, animals were thawed and wet weight (ww) of each individual was determined (in mg). Animal tissue was homogenized in phosphate buffer solution (pH =7.4) for protein and total carbohydrate analysis. Proteins were analyzed using Coomassie (Bradford) protein assay kit (Thermo-Scientific) and total carbohydrates were analyzed using the total carbohydrate assay kit (Cell Biolabs, STA-682). Lipids were extracted using a modified procedure by Folch et al. (1957) and quantified (unsaturated fatty acids only) using the lipid quantification kit (Cell Biolabs, STA-613). All assays were performed using a spectrophotometer at 25°C .

Data analysis

For the observations on basal metabolic rates, difference in mass-specific O_2 consumption rates between species was assessed using one-way ANOVA. For the food deprivation experiment, mass specific metabolite content of species was compared on Day 0 (immediately prior to the start of food deprivation) using one-way ANOVA. The effect of food deprivation on mass specific metabolite concentration of each species was

assessed with repeated measures ANOVA, which provides the effect of the experimental treatment (Fed *versus* Unfed), time (Days 15 and Day 30 dates for *Synurella*, Days, 15, 30, 60, and 90 for *S. pecki*), and the treatment \times time interaction. Data were examined for normality, homoscedasticity, and sphericity (repeated measures ANOVA) prior to analyses. If data did not meet assumptions, they were either \ln or square root transformed. Significance for all tests was inferred at $p \leq 0.05$ and all analyses were performed in the R platform (version 3.5.0, R Core Team, 2018).

RESULTS

O₂ consumption rates

Basal metabolic rates differed between *S. pecki* and *Synurella* ($F_{1,8} = 16.02$, $p < 0.005$; Fig. 4.1). Mass-specific O₂ consumption rates of *S. pecki* (0.10 ± 0.03 mg g⁻¹ ww h⁻¹) were significantly lower (on average ~10x lower) than those observed for *Synurella* (1.0 ± 0.22 mg g⁻¹ ww h⁻¹).

Effects of food deprivation on metabolite content

Prior to the start of experiments (Day 0), the mass-specific content of some metabolites differed between the two species (Fig. 4.2 A – C). Mass-specific protein content did not differ between species ($F_{1,8} = 1.46$, $p = 0.261$). However, mass-specific total carbohydrates was higher in *S. pecki* ($F_{1,8} = 43.83$, $p < 0.001$) and lipid content was higher in *Synurella* ($F_{1,8} = 7.40$, $p = 0.026$).

During the period of food deprivation, total carbohydrate content of *S. pecki* did not differ between Fed and Unfed treatments ($F_{1,4} = 0.356$, $p = 0.583$; Fig 4.2 A). However, in both treatments, total carbohydrate content of animals declined with time ($F_{3,12} =$

19.89, $p < 0.001$), but the rate of carbohydrate reduction did not depend upon treatment (Time \times Treatment: $F_{3, 12} = 0.456$, $p = 0.718$). In contrast, food deprivation in *Synurella* led to an immediate reduction in total carbohydrates ($F_{1, 8} = 17.44$, $p = 0.003$; Fig 4.2 A). Total carbohydrate content in *Synurella* varied temporally (Time: $F_{1, 8} = 15.61$, $p = 0.004$), but this temporal variation was dependent upon the specific experimental treatment (Time \times Treatment: $F_{1, 8} = 10.11$, $p = 0.013$).

Protein content of *S. pecki* differed between Fed and Unfed treatments, with fed animals having higher protein content ($F_{1, 8} = 19.28$, $p = 0.023$; Fig 4.2 B). Protein content varied through time in both treatments ($F_{3, 24} = 3.17$, $p = 0.043$), but there was no interdependence between treatment and time ($F_{3, 24} = 0.316$, $p = 0.813$). Food deprivation of *Synurella* led to an immediate reduction in protein content ($F_{1, 8} = 8.07$, $p = 0.022$; Fig 4.2 B), but mass-specific content did not vary temporally after 15 days of food deprivation (Time: $F_{1, 8} = 5.17$, $p = 0.0517$; Time \times Treatment: $F_{1, 8} = 2.55$, $p = 0.149$).

Lipid content of *S. pecki* did not differ between treatments during the food deprivation period ($F_{1, 8} = 2.51$, $p = 0.152$; Fig 4.2 C). Lipid content of *S. pecki* varied through time in both treatments ($F_{3, 24} = 4.43$, $p = 0.01$), but this variation did not depend upon treatment (Time \times Treatment: $F_{3, 24} = 0.481$, $p = 0.698$). For *Synurella*, lipid content was higher in the Fed treatment during the starvation period ($F_{1, 8} = 15.66$, $p = 0.004$; Fig 4.2 C). However, after the start of the food deprivation period, *Synurella* lipid content did not vary temporally (Time: $F_{1, 8} = 2.95$, $p = 0.124$; Time \times Treatment: $F_{1, 8} = 2.91$, $p = 0.127$).

DISCUSSION

Capacity to withstand periods of low to no food supply depends on the presence of endogenous reserves and metabolic responses that ensure efficient utilization of stored metabolites (Hervant et al. 1997, Hervant et al. 1999, Fuglei et al. 2000, Hervant et al. 2001, Issartel et al. 2010). Previous studies have reported species-specific differences in storage and utilization of metabolites in crustaceans during long periods of food deprivation (Hervant et al. 1999, 2001, Hervant and Renault 2002, Sacristán et al. 2016). In the current study, the two related hypogean and epigean amphipod species exhibited markedly different physiological strategies to deal with low food availability. The lower overall metabolic rates observed in *S. pecki* (in comparison to *Synurella*) is in accordance with numerous published studies which have found that hypogean organisms have lower metabolic activity when compared to surface relatives (Hervant et al. 1997, Hervant et al. 1998, Spicer 1998, Hervant and Renault 2002, Simčič et al. 2005, Mezek et al. 2010). In addition, presence of greater total carbohydrate stores in *S. pecki* (relative to *Synurella*) on Day 0 prior to start of food deprivation is in line with previous studies that have compared metabolic responses of epigean and hypogean taxa (Hervant et al. 1999, 2001, Hervant and Renault 2002). Storage of greater energy stores in hypogean species is thought to be indicative of adaptation to low energy subterranean conditions so that individuals can more continuously fuel metabolic needs for longer periods of low food availability.

In the present study, I observed that lipid reserves were substantially higher in the epigean amphipod *Synurella*, when compared to *S. pecki*. In contrast, Hervant et al. (1999) found that that the subterranean amphipods, *Niphargus virei* and *N.*

rhenorhodanensis had higher stored lipids and carbohydrates (as glycogen) than surface amphipods (*Gammarus fossarum*). Contrasting results from Hervant et al. (1999) and this study could be due to differences in species-specific adaptations to food limitation resulting from different ways of life and metabolic needs (Danielopol and Rouch, 1991). In addition, species-specific differences in storage and utilization of body reserves may occur due to differences in thermal adaptations, type and quality of food consumed, feeding history and life-cycle strategy, as well as their biosynthesis from either dietary fatty acids or dietary proteins, carbohydrates or lipids (Lahdes et al. 2010, Pond 2012). The two species which Hervant et al. (1999) studied (*N. virei* and *N. rhenorhodanensis*) are typically found in deeper phreatic habitats than *S. pecki*, which is a spring opening ecotone specialist. Thus, it is possible that differences between the findings of this study and that of Hervant et al. (1999) could come from the evolutionary history, specific habitat associations, and access to food resources of the specific fauna used in experiments.

Although I did not observe higher amounts of stored lipids in *S. pecki* when compared to *Synurella*, the present study found differences between epigeal and hypogean species in terms of utilization and depletion of various energy reserves during starvation. Starvation leads to changes in the body composition of animals (Gibert and Mathieu 1980, Barclay et al. 1983). The relative importance of different metabolite reserves depends on the duration of starvation as well as species-specific differences in metabolism and regulation (Hervant et al. 1999, Caruso et al. 2008). During starvation, crustaceans must meet energy demands by regulating enzymatic activities to access energy reserves (i.e., hydrolysis of proteins to amino acids, glycogen to glucose, and

triglycerides to free fatty acids), while ensuring cell integrity (Sanchez-Paz et al. 2006). In the present study, *Synurella* demonstrated a monophasic response to food deprivation, characterized by an immediate linear decrease in all energy reserves including lipids. In contrast, the only metabolite that differed between Fed and Unfed treatments during starvation of *S. pecki* were proteins. Thus, it appears that *S. pecki* accessed proteins as an energy reserve during a 90-day starvation period. Similarly, Hervant et al. (1999) observed that hypogean amphipods significantly utilized their protein reserves before other sources (i.e., carbohydrates and lipids) during periods of food deprivation. Thus, initial utilization of protein energy reserves during periods of starvation may be a cosmopolitan feature of hypogean amphipod metabolism, but further comparative studies are required.

In the present study, carbohydrate reserves in *S. pecki* were depleted in both Fed and Unfed populations in the lab. The mechanisms behind the loss of carbohydrate reserves in the Fed treatment is not understood. However, it could be associated with the stress of being held in captivity in a confined space for a long period of time. Similarly, Gibert and Mathieu (1980) observed that a hypogean amphipod, *N. rhenorhodanensis* that were fed and kept under conditions very close to their natural environment in the lab, showed significant reduction in carbohydrate reserves within a month of being held in captivity.

In the study presented here, *S. pecki* had lower energetic requirements (i.e., basal metabolic rates), greater total carbohydrate reserves, and lower rates of lipid use during starvation when compared to a surface relative. Cumulatively, these findings indicate that *S. pecki* maintains an efficient strategy for survival in unpredictable and harsh

environment with sporadic food availability. *S. pecki* is closely related to deeper phreatic amphipod species (Ethridge et al. 2013), and it is hypothesized that their ancestors invaded freshwater subterranean systems during the late Cretaceous or early Cenozoic (Holsinger 1967). The current metabolic adaptations to persistence in a deeper phreatic low-energy environments observed in *S. pecki* is consistent with their longer-term evolutionary history, rather than their current distribution in and around surface spring opening ecotones. Indeed, *S. pecki* serves as the top invertebrate predator in spring opening and likely feeds on surface invertebrates, including the epigeal amphipod *Hyalella azteca* (Nair et al. *in prep*). In addition, *S. pecki* detects and actively avoid light, presumably to avoid predation (Nowlin et al. 2015, Worsham et al., *in prep*). The suite of subterranean metabolic characteristics exhibited by *S. pecki* may be an adaptation to deeper phreatic low resource conditions that is not easily modified even though *S. pecki* now lives in shallow groundwater – surface water interfaces.

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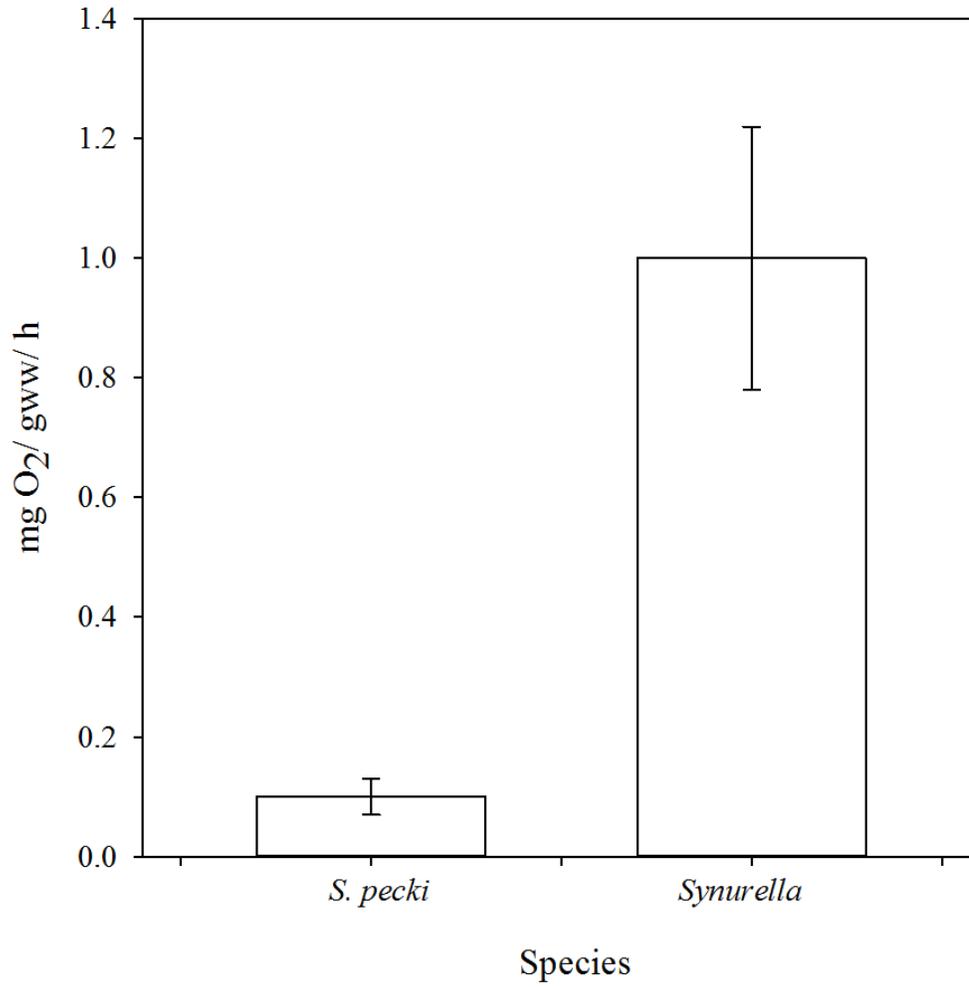


Figure 4.1: Oxygen consumption in darkness for *Stygobromus pecki* and *Synurella* sp. at ambient temperature of 23°C. Values are means \pm standard error (SE) for n = 5 animals; ww = wet weight.

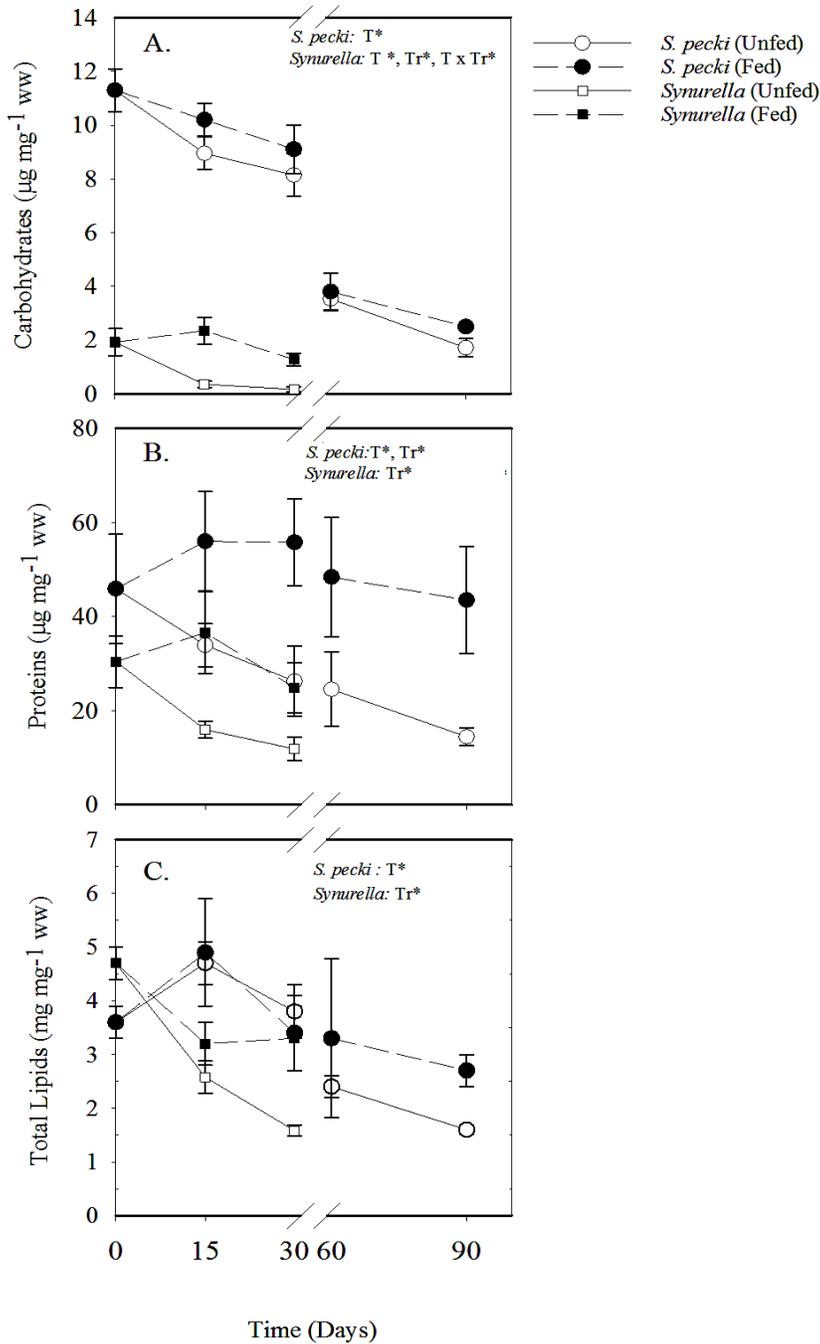


Figure 4.2: Changes in the levels of body metabolites in *Stygobromus pecki* and *Synurella* sp: Carbohydrates (A), Proteins (B), Lipids (C) during long-term food deprivation at 23°C in darkness. Values are means \pm S.E.M. for n = 5 replicates. *p*-values for the main effects of Treatment, Time and the Time x Treatment interaction are presented. * indicates significance at $\alpha < 0.05$

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5. CONCLUSIONS

Spring ecotones links multiple ecotones (groundwater and surface waters, aquatic and terrestrial systems), have a microhabitat-mosaic structure, exhibit high temporal stability of distinct physicochemical conditions, and have communities composed of organisms exemplifying taxonomical and functional diversity (Ward 1989, Scarsbrook et al. 2007, Cantonati et al. 2012). Although almost 60 years has passed since crenobiology was recognized as a special field of limnology (Botosaneanu 1963), research on the biology and ecology of spring systems have been infrequent and localized. Consequently, crenobiology is an under-investigated field. Given the potential role of spring systems in biodiversity of stream networks and the maintenance and persistence of freshwater flows, the conservation and protection of these habitats have implications for both biodiversity and ecosystem functioning. Exploitation of spring systems due to groundwater extraction and habitat destruction is likely to increase in the near future due to anthropogenic climate change and increasing water demands by human societies. Therefore, an integrative approach to understand spring systems and their biological communities is imperative for the conservation. The three studies in this dissertation provide critical information on ecology, evolutionary biology, and food web dynamics of macroinvertebrates that inhabit spring orifice ecotones.

In Chapter 1, I investigated the responses of two spring-associated and surface water associated species to acute and longer-term chronic temperature exposures in the insect family Elmidae. Results from this study support the hypothesis that crenic species (*H. comalensis* and *H. glabra*) have more stenothermal tolerance profiles when compared to more widespread species (*H. vulnerata* and *M. pusillus*). However, I also found that

temperature and DO tolerances differed between the two crenic species and this variation could be in response to specific environmental characteristics at their respective occurrence sites. Overall, I found that a wide range of environmental tolerances can be present in species within a single family of insects that co-occur within a biogeographic region, but that environmental tolerances are primarily related to the main habitat type in which each species occurs. *H. comalensis* is federally listed as endangered under the United States Endangered Species Act and has a geographic distribution primarily limited to Comal Springs (Comal County, Texas). This study is the first to provide clear environmental tolerance data for temperature and DO for this species and my results suggest that reduction in spring flow from prolonged drought and/or increased pumping could potentially reduce water quality of their occupied habitat and will likely impact their fitness and survival. However, there is a clear need to perform a risk assessment analysis for *H. comalensis* for various drought and pumping scenarios (Newman and Unger 2003).

In Chapter 2, I assessed resource use and trophic structure of communities within spring ecotones using stable isotopes of carbon and nitrogen ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and amino acid-specific stable isotopes ($\delta^{13}\text{CAA}$). Invertebrate communities within spring ecotones relied upon OM of autochthonous and allochthonous origin and phylogenetically related consumers within the same food web appear to be finely partitioning basal OM resources. In addition, isotopic data indicate that several crenic and hypogean species exhibited a close dietary association with terrestrial surface-derived OM, but co-occurring species did not have substantial overlap in diet even though they were largely using the same OM source. Results from this study indicate that allochthonous OM inputs can play a central

role in determining not only the hydromorphological conditions, but also community composition and trophic interactions of biotic spring ecotones (Lowe and Likens 2005, Reiss and Chiffard 2017). These results also suggest that maintaining and restoring riparian vegetation connections around spring ecotones may be critical for these unique habitats and their biological communities.

In Chapter 3, I experimentally examined energy reserve utilization and metabolic responses of the subterranean-adapted ecotone specialist amphipod *S. pecki* to long-term food deprivation. I also conducted a parallel experiment on a related epigeal amphipod, *Synurella* in the same family (Crangonyctidae). Experiments were conducted to test the hypothesis that subterranean organisms (i.e., *S. pecki*) in more energy-rich environments (i.e., spring orifice ecotones) will experience relaxation of selective pressures on stygomorphic metabolic adaptations (e.g., slow metabolism and differential use of energetic reserves). This study did not find support for this hypothesis, as the hypogean species exhibited markedly different physiological strategies than the epigeal species to deal with low food availability. *S. pecki* had lower energetic requirements (i.e., lower basal metabolic rates), greater total carbohydrate reserves, and lower rates of lipid use during starvation when compared to a surface relative. Overall, these findings indicate that *S. pecki* maintains an efficient strategy for survival in deeper phreatic low-energy environments. Thus, its metabolic strategy is more consistent with their longer-term evolutionary history, rather than their current distribution in spring orifice ecotones. However, *S. pecki* shows some adaptation to shallow phreatic and spring opening living in that it forages on surface invertebrates around spring openings (Nair et al. *in prep*) and

detects and actively avoids light, even though it is eyeless (Nowlin et al. 2015, Worsham et al., *in prep*).

Taken together, the three studies contained this dissertation demonstrate that spring ecotones systems support diverse assemblages of macroinvertebrate that use a diversity of resources that effectively partition niche space and utilize a variety of basal OM sources. In addition, spring orifice ecotones contain endemic and endangered ecotone specialist species with unique subterranean adaptations and narrow tolerances to environmental stressors. This dissertation represents a substantial and novel contribution and provides a substantial amount of data and findings on an understudied ecosystem type and group of organisms (ecotone specialists). When the body of knowledge in this dissertation is viewed from an applied perspective, my results provide information which is directly and immediately usable for resource managers and conservation officials with regard to environmental tolerances of endangered and at risk species as well as the maintenance of habitat conditions within and around spring ecotones.

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