

NATURAL HISTORY STUDIES ON THE COMAL SPRINGS RIFFLE BEETLE

(HETERELMIS COMALENSIS)

THESIS

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	v
LIST OF TABLES	viii
LIST OF FIGURES	ix
ABSTRACT.....	x
CHAPTER	
I. INTRODUCTION	1
Family Elmidae	1
Biogeography of the Genus <i>Heterelmis</i>	2
Description	5
Habitat	6
Threats	9
Conservation Efforts	10
II. MATERIALS AND METHODS	12
Sampling	12
Association with Springs	13
Microhabitat Preference	14
Experiment 1: Well Water versus Re-circulated water	16
Experiment 2: Light	16
Experiment 3: Temperature	16
Experiment 4: Flow	17
Experiment 5: CO ₂	17
Statistical Analyses	18

Pupation Requirements	19
Number of Instars	22
III. RESULTS.....	23
Association with Springs	23
Microhabitat Preference	23
Experiment 1: Well Water versus Re-circulated water	24
Experiment 2: Light	24
Experiment 3: Temperature	25
Experiment 4: Flow	25
Experiment 5: CO ₂	26
Pupation Requirements	28
Number of Instars	28
IV. DISCUSSION.....	29
Association with Springs	29
Microhabitat Preference	31
Pupation Requirements	37
Number of Instars	40
Conservation Implication	41
Conclusion	43
LITERATURE CITED	59

LIST OF TABLES

Table	Page
1. Spring experiment – Abundance of <i>H. comalensis</i> with distance from the spring	44
2. Spring experiment – Analysis with the Friedman test for four spring locations in spring run 2, 3, and Landa Lake	44
3. Spring experiment – Measurements of water quality at the four spring locations used for analysis.....	44
4. Preference experiments – Results of statistical analyses of preference experiment data	45
5. Pupation experiment – Analysis of data from different substrates tested in pupation experiment.	46
6. Water quality measurements taken at Plum Creek near Luling, Texas	46

LIST OF FIGURES

Figure	Page
1. Distribution of <i>Heterelmis</i> species	47
2. Distribution of <i>H. comalensis</i> , <i>H. vulnerata</i> , <i>H. glabra</i> , <i>H. obesa</i> , and <i>H. stephani</i> in the United States.....	48
3. Location of spring runs and Landa Lake in New Braunfels, Texas.....	49
4. Sampling locations of <i>H. comalensis</i> and spring experiment locations at spring runs 2, 3, and Landa Lake	50
5. Association of <i>H. comalensis</i> with spring discharge in four springs.....	51
6. Water preference for <i>H. comalensis</i> and <i>H. vulnerata</i>	52
7. Light preference for <i>H. comalensis</i> and <i>H. vulnerata</i>	53
8. Temperature preference for <i>H. comalensis</i> and <i>H. vulnerata</i>	54
9. Flow preference for <i>H. comalensis</i> and <i>H. vulnerata</i>	55
10. CO ₂ preference for <i>H. comalensis</i> and <i>H. vulnerata</i>	56
11. Pupation results for different substrate type tested	57
12. Frequency distribution of head capsule measurements of <i>H. comalensis</i> larvae.....	58

ABSTRACT

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The Comal Springs riffle beetle (*Heterelmis comalensis*) is an endangered species inhabiting springs of the Edwards Aquifer. It is only known to exist in Comal and San Marcos springs and is threatened by decreased water quality and quantity, and habitat destruction. We tested *H. comalensis*' restriction to spring outlets and assessed preference for several habitat variables in an effort to describe the determinants of the species' narrow range and provide information for protection of water quality and habitat. Furthermore, we tested substrate requirements needed for the larvae of *H. comalensis* to successfully pupate and investigated the number of larval instars. To test the association of beetles with the spring outlets we buried long cotton strips in the spring openings.

After four weeks we collected adults and larvae from the strip and recorded their distance from the opening. We then set up preference experiments, testing five different water quality parameters (well water, flow, CO₂, temperature, and light) to find out which factors restrict this species to the spring outlets. We conducted all preference experiments with *H. comalensis* and *H. vulnerata*, a more widespread species that occurs in shallow streams, to compare preferences between the two. To test substrate types for pupation we set up aquaria with pupation chambers containing seven different substrate types, supplied with constant flow and food resources. The number of larval instars was determined by peaks in a frequency distribution, based on measurements of larval head widths. We found a significant association of beetles with the springs. The preference testing showed significant preferences for well water, low flow, elevated CO₂, temperatures around 23 °C, and darkness. We did not detect significant differences between substrate types in our pupation experiment, likely due to inadequacies of habitats provided. We found *H. comalensis* to have seven instars, within the typical range of elmids species. We concluded that *H. comalensis* is restricted to a very narrow habitat at the springs and prefers water quality associated with the Edwards Aquifer.

CHAPTER I

INTRODUCTION

The Comal Springs riffle beetle (*Heterelmis comalensis*) is a federally endangered species that occurs in Comal and San Marcos springs in Texas, outlets of the Edwards Aquifer, a unique groundwater system. It is one of three aquatic invertebrates listed as endangered species, living in or around the springs (United States Fish and Wildlife Service, 1997). The distribution of *H. comalensis* is limited to the springs, which poses the question of why this species is unable to populate a wider habitat and geographical range. *Heterelmis vulnerata*, a related species, is more widespread and occurs in shallow streams across Texas and in parts of Oklahoma. It is not spring-adapted and seems to be able to disperse and colonize a larger range.

Family Elmidae

Heterelmis comalensis belongs to the family Elmidae (Order Coleoptera) with nearly 100 species in North America. Riffle beetles, like all coleopterans, are holometabolous insects that have a larval, pupal, and adult life stage. Eggs are most likely glued to the underside of submerged wood, rocks, or plant stems. Their larvae are aquatic with 5-8 instars, depending on the genus. The larval stage can last 6-36 months (White and Roughley, 2008). Generally, development is faster with higher temperatures;

southern populations might have a shorter larval stage than northern populations. Smaller species need less time to develop, thus have fewer instars than large species (Brown, 1987). They are believed to be collector-gatherers and scrapers that feed on algae and detritus. Examination of guts of some elmids species indicated a diet of periphytic and filamentous algae and detritus for the adults, and detritus for the larvae (Elliot, 2008). Pupation usually occurs out of the water, near the surface in soil or under rocks or wood. Some species of riffle beetles are capable of flying soon after they emerge from the pupal stage; others seem to be incapable of flying. Once the young adults enter the water they may not be able to survive out of it for more than a few hours (Seagle, 1980; Brown, 1987). Most adult elmids are aquatic. They respire through a plastron, an airspace on the ventral surface of the beetle. This airspace is made possible by a dense coating of hydrofuge hairs allowing oxygen to diffuse directly from the surrounding water (Bosse, 1979; Resh et al., 2008). This allows the adults to stay submerged throughout their lives but also makes them dependent on a relatively high amount of dissolved oxygen (DO) in the water, otherwise, at low DO levels, oxygen would diffuse out of the plastron into the water. Because of this, riffle beetles can typically be found in well-aerated streams and rivers, especially in shallow riffles or rapids. Riffle beetles often move to different locations by drifting, a mechanism used to find pupation sites or escape poor environmental conditions (Brown, 1987).

Biogeography of the Genus *Heterelmis*

Within the family Elmidae, the genus *Heterelmis* includes at least 19 neotropical species described to date, most of which occur in South and Central America (Manzo,

2005). *Heterelmis dubia*, *H. pubipes*, and *H. limnoides* can be found in streams in Brazil. *Heterelmis longula* and *H. tarsalis* occur in Mexico. *Heterelmis simplex* occurs in Peru, and was later described from Guatemala as well. A subspecies, *H. simplex codrus*, can be found in Trinidad and Tobago (Brown, 1972). A large number of *Heterelmis* species, including *H. concexicollis*, *H. impressicollis*, *H. lucida*, *H. pusilla*, *H. apicata* and *H. neglecta*, were described from Ecuador. *Heterelmis neglecta*, much later, was also described from streams in Bolivia. Two species were described with ranges from South America up to Texas. *Heterelmis obscura* has been found in Guatemala, Costa Rica, and Mexico. *Heterelmis obesa* was first described from Peru and has later been collected from streams in Guatemala, Arizona, New Mexico, and Texas. A subspecies, *H. obesa plana*, was described from two locations in Mexico (Brown, 1972). Several unidentified specimens of *Heterelmis* sp. were reported from Mexico, Costa Rica, Panama, Bolivia, Brazil, and Argentina (Flowers, 1991; Buss et al., 2002; Dinger et al., 2005; Manzo, 2005; Tomanova et al., 2006) (Figure 1). Species found in the southern United States include *H. glabra*, *H. vulnerata*, *H. obesa*, *H. stephani*, and *H. comalensis*. *Heterelmis glabra* is mostly found in Arizona and west Texas, but specimen have also been found in Mexico and Peru (originally described as *H. acicula*). *Heterelmis obesa* occurs in Arizona, New Mexico and Texas. *Heterelmis stephani* is an endemic species from springs in southern Arizona. *Heterelmis vulnerata* is a widespread species, reported from 21 counties in Texas and 11 counties in Oklahoma (Brown, 1972; McCulloch, 1986; Levine, 1999; Tolley, 2000; Gonzales, 2008).

Heterelmis comalensis is only known to occur in two counties, Hays and Comal, in Texas. Most known populations exist at Comal springs, only one has been confirmed

from San Marcos springs (Gibson et al., 2008) (Figure 2). It is most closely related to *H. glabra*, and more distantly related to *H. vulnerata* (Gonzales, 2008). There are several reasons why species like *H. comalensis* can be endemic. The species could have originated in the spring, and never dispersed, its range could have shifted in locality, or it is now present in only a small part of its former range (Lomolino et al., 2009). Recent studies suggest speciation due to isolation, which separated *H. comalensis* from its sister species *H. glabra* approximately 0.5 million years ago (Gonzales, 2008). In contrast to its related species *H. vulnerata*, *H. comalensis* does not occur in streams but seems to be restricted to spring sources. It has non-functional wings and was rarely found to drift (Bosse et al., 1988; Norris, 2002; Gibson et al., 2008). Flight- and drift-dispersed *Heterelmis* species have wider distributions due to their dispersal abilities and are more likely to colonize new regions and habitats. Limited dispersal abilities are one possible reason for *H. comalensis*' small range. Other reasons for a limited range include barriers to dispersal, or environmental factors (biotic or abiotic) (Lomolino et al., 2009).

Heterelmis comalensis could be restricted to spring sources by physical and chemical factors like temperature, flow, or specific water quality at the springs. Possible biotic factors include certain predators outside the springs, food sources inside the springs, or competition with other species that keep them from expanding their range further downstream. A species niche is defined a multidimensional space with a habitat optimum, usually in the center, and less suitable conditions at the edges of the niche. Habitat specialists, including *H. comalensis* have specific and narrow environmental tolerances and tend to have lower population densities, which makes them more susceptible to extinction when habitat conditions are altered. The question of why *H.*

comalensis is endemic to Comal and San Marcos springs has not been fully answered. Physical or chemical habitat quality, biotic factors or dispersal abilities, or combinations of these, likely contribute to its limited range. Historical events, such as droughts or varying temperatures might have pushed *H. comalensis*' ancestor into spring habitats, where populations became isolated.

Description

Heterelmis comalensis was first described in 1988. It is a small aquatic beetle of the family Elmidae. It has an elongated body with elytra slightly wider than prothorax. The length of the body averages between 1.7 and 2.1 mm, with the females being slightly larger than the males. This species' color ranges from light to dark brown. *Heterelmis comalensis* has short, non-functional flight wings. It is not known if this species pupates above the water line, like other elmids, and enters the water soon after emergence. Either way, it is unlikely for the adults to spend much time out of the water after pupation (Bosse et al., 1988). The specific pupation requirements for *H. comalensis* are unknown, but habitat characteristics suggest pupation below the waterline. While elmids generally pupate above the waterline, some populations of this species do not have habitat above the water available. The springs of Landa Lake are covered by several feet of water.

Both, adults and larvae of *H. comalensis* live and feed under water, often living alongside each other. They are unable to swim but rather crawl slowly on the substrate. The larvae have gills that can be expanded and contracted to increase ventilation in case of short-term lower oxygen levels. Both of them are scrapers that occur in the benthos and on waterlogged wood, as well as other woody debris, presumably feeding on fungus

and algae (Brown, 1987). Predators of *H. comalensis* are not known but may include *Eurycea* salamanders and larger invertebrates such as dytiscid beetles and *Stygobromus* amphipods, but it has been shown that elmids in general don't seem to be the most delectable food for predators like fish and larger invertebrates. They are often ignored as prey items or even spit out when accidentally ingested (White, 1989). Much of this beetle's life history, like the number of larval instars, is still unknown. Genetic work done by Gonzales in 2008 revealed that populations of *H. comalensis* contained higher levels of genetic variation and exhibited greater differentiation than populations of the more widespread *H. vulnerata*. Populations of *H. comalensis* at Comal Springs also appear to be significantly isolated from each other despite relatively little geographic isolation. However, *H. comalensis* populations do not appear to have suffered a loss of genetic variation usually expected with small population sizes (Gonzales, 2008).

Habitat

Heterelmis comalensis is an endemic species with populations at Comal Springs, located in Comal County, and San Marcos Springs, in Hays County, Texas. Our description of the beetle's habitat will focus on Comal Springs in Landa Park, New Braunfels, Texas. The Comal Springs system is the largest in Texas with an annual flow of approximately 8 m³/sec. (U.S. Fish and Wildlife Service, 2007). The springs issue from Lower Cretaceous limestones of the Edwards Aquifer (Brune, 1981). There are four major spring outlets and spring runs in this area that flow into Landa Lake, named Comal 1, 2, 3, and 4. *Heterelmis comalensis* has been found at all the spring runs except 4. Many smaller springs are located along the spring runs and in Landa Lake, which are also

suitable habitats for this species. Spring run 1, 2, and 3 do not have the same size, appearance, canopy and riparian cover, substrate composition and vegetation. Comal Spring run 1 ranges from 9-13 m in width on a length of 230 m long before flowing into Landa Lake. Comal Spring run 2 ranges from 2-3 m in width and enters a shallow wading pool after 66 m. Comal Spring run 3 ranges in width from 4.5-8 m and enters Landa Lake after 130 m. Comal 1 has the largest discharge with an approximate average of 0.6 m³/sec, Comal 2 has the smallest average discharge of about 0.3 m³/sec. (Bowles et al., 2003). Lower flows have been reported for the year 2009, where discharge was estimated to be only 0.215 m³/sec. at Comal 1, 0.158 m³/sec. at Comal 2, and 0.2 m³/sec. at Comal 3 (BIO-WEST, 2010). These conditions of lower flow could be problematic for *H. comalensis* and other endangered species that live at Comal Springs and depend on sufficient water quantity and quality (U.S. Fish and Wildlife Service, 2007; BIO-WEST, 2010). Landa Park and the spring runs are situated in the City of New Braunfels, and parts of the spring runs have been channelized and modified.

Heterelmis comalensis is mostly found in the substrate of the direct spring area, in the gravel area, on woody debris or roots, and under rocks (Gibson et al., 2008). Springs of the Edwards Aquifer in New Braunfels are characterized by a relatively high CO₂ concentration of approximately 30-40 mg/L, and a constant temperature of approximately 23 °C all year. Dissolved oxygen ranges from 4-6 mg/L, always above 50% saturation (BIO-WEST, 2010). These conditions change slightly with distance from the spring. *Heterelmis comalensis* seems to be adapted to these specific spring conditions because it is generally not found anywhere further away from the spring. It is unknown why it is restricted to springs. In a previous study conducted on the habitat of *H. comalensis*,

Bowles et al. found a poor correlation to water depth, current velocity, and the distance downstream from the primary spring outlets. They suggested that *H. comalensis* is randomly distributed in the spring-runs and does not seem to be restricted to the direct spring-outlet (Bowles et al., 2003). Gibson et al. found *H. comalensis* primarily in or near spring outlets, but microhabitat preferences still are not known (Gibson et al., 2008). A flow study conducted in 2002 showed the tendency of the beetle to move toward the current, which may be a factor restricting them to the spring outlets. However, the results of this study were not sufficient to be statistically testable (BIO-WEST, 2002). Substrates found in all spring runs are silt, sand, gravel, and cobble (8-128 mm). Vegetation most commonly found here are filamentous algae, fanwort, water hyssop, dwarf Hygrophila, yellow water-lily, Illinois pondweed, delta arrowhead, and wild celery. Some of the spring runs have a greater diversity of vegetation, while spring run 2 is almost vegetation free, only moss and filamentous algae can be found there. Bowles et al. found that some type of vegetation was present in 70% of samples but could not find a statistical association of *H. comalensis* with a specific plant species (Bowles et al., 2003). In personal sampling in 2011 *H. comalensis* was often found in habitats with no macrophytes. Sampling in 2009 showed that numbers of beetles increased in the lake bottom spring upwellings at Spring Island and declined at Spring Run 3 and West Shoreline of Landa Lake. Spring upwellings at the lake bottom are less susceptible to droughts and provide a more stable habitat for the beetle.

Threats

The biggest threats for *H. comalensis* are decreased water quality and quantity. A decrease of water quality includes changes in temperature, pollution and DO. A decreased water quantity ultimately leads to habitat reduction and siltation (Arsuffi, 1993). Pollution with substances such as soap and other detergents can be a particular problem in combination with low water levels. These surfactants can become more concentrated and can be fatal to riffle beetles because it makes it impossible for them to maintain a plastron (Bosse, 1979). Decreasing water levels are caused by drought and over-pumping. The Edwards Aquifer and the springs that issue from the aquifer are the main source of groundwater for the region. People have an increasing need for water, especially in the summer. This can cause a decrease in flow from the springs. Comal Springs ceased to flow during a drought in 1956, when the springs were dry for almost six months (Brune, 1981). *Heterelmis comalensis* might have survived this drought by burrowing down into the moist substrate or moving deeper within the aquifer via springs. It is unclear if this survival strategy would work for longer periods of no spring flow. The beetles need at least some degree of moisture to keep their plastron intact. During declining spring flows, water depths decrease, which can lead to higher temperatures and lower oxygen concentrations. A relatively high concentration of DO is vital for aquatic invertebrates with plastron respiration, including *H. comalensis* (Arsuffi, 1993). The channelization of the spring runs decrease general habitat quality of springs and may destroy potential pupation sites in the riparian zone. Recreation and wading during the summer months lead to damaged riparian and benthic habitats. Due to these threats, this species could potentially suffer a severe population reduction or even extinction (Arsuffi,

1993). Because *H. comalensis*' existence is threatened by all these factors, it was listed as endangered by the United States Fish and Wildlife Service (U.S. Fish and Wildlife Service, 1997). Together with the Texas Parks and Wildlife Department, they developed baseline ecological information about the species. A large portion of the beetle's characteristic habitat, however, is still unknown (Bowles et al., 2003)

Conservation Efforts

Yearly monitoring of *H. comalensis* for population fluctuations is reported by the Edwards Aquifer Authority (EAA, http://www.edwardsaquifer.org/display_document_cat.php?cID=1). Beetles are collected using cotton cloth lures, a passive capturing method that has proven highly effective over past years and is the least harmful to the beetle (Gibson et al., 2008). The San Marcos National Fish Hatchery and Technology Center (NFHTC) keeps a population of *H. comalensis* in refugium and efforts are being made towards captive propagation (Fries, 2003). The species can survive for a long time in captivity and has produced hundreds of eggs and larvae over the past few years, but few successful pupations have been observed (Gibson, pers. comm., 2011). Brown reports successful rearing of elmids in aerated aquaria containing sand, rocks, waterlogged wood, and aquatic plants in a set-up that allows for air spaces between rocks as potential pupation sites. He recommends using air bubblers under rocks or wood to create a current in the water. He seemed to have varying success with pupations, depending on the species. The right air moisture level seems vital for the pupae; if humidity is too high, fungi can grow and destroy the pupae; if it is too low, pupae could dry out (Brown, 1973).

Finding the right requirements for pupation will be necessary for successfully rearing the species to support conservation efforts. Questions regarding these issues include: Where do they pupate? How long is pupation and do they need airspaces? There is not much known about the microhabitat preferences of *H. comalensis*, which is needed for management of this endangered species and habitat conservation. Which specific conditions do the adults require for survival and how far from the spring source are they able to live? This information can help estimate the impacts of low flow on the Comal Springs populations.

With this study we attempt to answer critical questions about *H. comalensis*' natural history: how closely is *H. comalensis* associated with the spring openings? Which microhabitat variables do adults prefer? Which substrate conditions are needed for *H. comalensis* to pupate in captivity? And how many instars does the species have?

This study will provide information about this endangered species' life and provide knowledge needed for conservation management. We propose that *H. comalensis* will pupate in rocky substrate, similar to the substrate found at the Comal springs. We expect it to prefer water quality conditions similar to spring conditions as opposed to stream and re-circulated water. The species *H. vulnerata* will likely prefer stream water to well water. This would confirm that *H. comalensis* is indeed restricted to spring environments due to certain water quality parameters. In the same context we hypothesize that in our spring experiment *H. comalensis* will be found in and close to the spring outlet and in decreasing numbers at further distance from the spring.

CHAPTER II

MATERIALS AND METHODS

Sampling

We conducted all experimental work at the San Marcos National Fish Hatchery and Technology Center (NFHTC). The NFHTC is part of the U.S. Fish & Wildlife Service, its goal is to recover species listed under the endangered species act, restore native aquatic populations, mitigate for fisheries lost, and provide fish to benefit Tribes and National Wildlife refuges (<http://www.fws.gov/fisheries/nfhs/overview.htm>). They currently provide refugia for endangered and threatened species of the Edwards Aquifer and associated springs, including the Comal Springs riffle beetle (*H. comalensis*), the fountain darter (*Etheostoma fonticola*), Texas Blind Salamander (*Typhlomolge rathbuni*), San Marcos Gambusia (*Gambusia georgei*), San Marcos Salamander (*Eurycea nana*), and Texas Wild Rice (*Zizania texana*) (Gibson, pers. comm., 2012).

We collected adults of *H. comalensis*, needed for experimental work, in spring run 3 in Landa Park in New Braunfels, Texas in June 2011 (Figure 3 and 4). We used the cotton cloth lures method developed by Gibson et al. (2008) as a passive collection method less harmful to the beetle than net sampling. Beetles were collected by placing cotton cloth squares in the springs as lures that will be retrieved several weeks later. Fungal, bacterial, and algal growth that the adults and larvae seemingly feed on, develops

on the cotton. Beetles can easily be picked off the cloth or transported in a container staying on the cloth (Gibson et al., 2008). We brought back 102 adults to the NFHTC, which were kept in aquaria supplied with well water and re-circulated water. We collected adults of *H. vulnerata*, needed for preference experiments, in June 2011 from Plum Creek in Luling, Texas by examining the bottom of logs and large pieces of wood. One hundred adults were brought back to the NFHTC and kept in an aquarium supplied with re-circulated water. Both aquaria contained rocks, Anaqua (*Ehretia anacua*) leaves, and cotton strips as food supply.

Association with Springs

To explore how closely *H. comalensis* is associated with direct spring openings, we quantified distance traveled away from spring openings as a function of water quality parameters. For this experiment we buried 6 cotton cloth strips (100 x 6 cm) in spring runs 2, 3 and Landa Lake (Figure 3 and 4) so that one end would be right over the spring outlet, and the other end furthest away from the spring. This approach was modified from the cotton cloth lure method, in which the cloth provided a medium for nutritional growth, which attracted beetles so they could be easily picked off (Gibson et al., 2008). All strips were buried in early December 2011 to minimize recreational disturbance common during the summer. We used gravel around the strips to create similar substrate conditions in all springs. We retrieved all strips four weeks later. We picked off all beetles and larvae and reported their distance from the spring. We measured temperature, conductivity, pH, DO, CO₂, flow, and depth, at the spring opening and at one meter away

from it, using a HYDROLAB model MS5 (Hydrotech), CO₂ test kit model PCO-DR (LaMotte), and a FLO-MATE 2000 portable flow meter (Marsh McBirney).

Data were analyzed with program R, Package *stats* version 2.11.1 (<http://www.r-project.org>) using the Friedman test, a nonparametric two-way analysis of variance, to detect significant differences between distances from the spring outlet. The Friedman test utilizes sums-of-squares, based on ranked observations. It is most powerful when the number of treatments is five or more. For the purpose of analyzing several different distances from the spring, the total distance of one meter was separated into sections of 20 cm, which were considered treatments. Multiple comparison values were calculated by hand (Conover, 1999).

Microhabitat Preference

We conducted a series of choice-test experiments to look at microhabitat variables (including well water versus re-circulated water, light, temperature, flow, and CO₂) and estimate preference of adult *H. comalensis* for a range of treatments. For comparison with the endangered *H. comalensis*, we also conducted these experiments with *H. vulnerata*, a related species not adapted to spring conditions, which occurs rivers and streams across Texas. *Heterelmis vulnerata* occurs in the near vicinity of *H. comalensis*, but the two species are not sympatric. With this comparison we can assess the difference between the two species, which might provide clues as to why *H. vulnerata* is more widely distributed than *H. comalensis*. We ran all preference tests in three aquaria of size 119.5 x 8 x 10 cm with three drainage holes on each side evenly spaced over the length of the aquarium. This created different sections throughout the aquarium that were connected to each other

so the beetles could move without barriers. We put mesh lining on the bottom to provide a substrate for beetles. Ten beetles were randomly assigned and placed in the middle of each of the three aquaria at the same time. We ran each experiment for three days, with exception of the CO₂ experiment, which ran for two days. In test trials, these amounts of time proved to be sufficient for the beetles to choose a section. The experiment manipulating CO₂ was run for only two days due to the danger of overexposure to CO₂. Initial trials showed a great vulnerability of beetles and increased mortality to overexposure for more than two days. When starting each test we measured water quality using a HYDROLAB model MS5 (Hydrotech) and CO₂ test kit model PCO-DR (LaMotte). Parameters measured were temperature, conductivity, pH, DO, and CO₂. This was done to ensure that water quality was similar in all sections, with exception of the condition being manipulated. After each trial was run for the allotted time, we counted beetles in each section and removed them from the aquarium. After the first run, we switched the orientation of treatment levels to the opposite side of the aquarium to account for other possible factors that might influence the beetles' movement. The same 10 beetles were put in the same aquarium for the second run. After the second run, beetles were put back into the refuge aquarium and 10 new beetles picked out for each aquarium to do third and fourth runs. Due to the limited amount of beetles available, re-sampling of beetles was possible. We ran one control experiment in three aquaria for each species (30 beetles each total), without treatment. The control aquaria were submerged in re-circulated water and did not show differences between aquaria sides.

Experiment 1: Well Water versus Re-circulated Water

For this experiment, we supplied one side of the aquaria with slow flowing well water and the other one with slow flowing re-circulated water from the hatchery. Both sides had a flow of 5 ml/sec. to avoid creating turbulence. Our measurements indicated two sections in this set-up: half of each aquarium contained well water, the other half re-circulated water. A small area of mixed water might have formed in the middle but wasn't measureable. When assessing beetles position in the aquaria, we considered them to be in one of the two treatments, well water or re-circulated water.

Experiment 2: Light

For this experiment, the aquaria were partially submerged to allow water exchange. We covered one half of the aquaria with black pond liner to create darkness on one side. Over the other half we placed a light with three full spectrum 6700 Kelvin white compact fluorescent lamps (Hamilton Technology) to create conditions similar to bright daylight. The beetles' position was quantified as being in light or in dark.

Experiment 3: Temperature

For the temperature preference test, we set up two large reservoirs, one on each end of the aquaria. Reservoirs were made of PVC pipe with a diameter 30.5 cm, approximately 1 m in height to establish sufficient water pressure to supply the aquaria. They had drainage on the bottom end connecting to pipes, one pipe ending in each aquarium. One reservoir was supplied with cold water of approximately 18 °C regulated by a cooling unit; the other reservoir contained two submerged aquarium heaters to raise

temperature to 27 °C. This created four sections throughout the aquarium: one with 27.0-25.5 °C, one with 25.5- 22.5 °C, one with 23.0-19.5 °C, and one with 19.5-18.0 °C. In each run, the temperatures of the different sections varied slightly within these ranges due to a lack of precision in regulating water pressure and flow to each aquarium, but these slight variations were not considered significant.

Experiment 4: Flow

To introduce fast flowing water on one end of the aquaria, we built a hose attachment out of a 2.5 cm PVC pipe in the width of the aquarium. Holes (2 mm) spaced approximately 5 mm apart on one side of the attachment provide a consistent stream of water over the whole width of the aquarium. To eliminate a strong drainage flow, the aquaria were partially submerged which allowed a gradual decrease of flow from one side to the other. We measured flow with the FLO-MATE 2000 portable flow meter (Marsh McBirney) throughout each section, and identified the following sections: one with flow of 0.12-0.05 m/sec (25% of the aquarium length) and one with 0.04-0.01 m/sec. (75% of the aquarium length).

Experiment 5: CO₂

We used the same two water reservoirs used in the temperature test for the CO₂ test as well. CO₂ was bubbled into one of the tanks to create a stable CO₂ concentration supplying the aquaria. The other tank had air bubbling in to increase DO and provide a more pronounced gradient. All three aquaria were partially submerged to allow some water exchange and create a more even gradient. With this set-up, three clear sections

were established: one with 120-160 mg/L, one with 12-25 mg/L, and one with 4-6 mg/L CO₂. Again, concentrations could differ slightly between runs, which was reflected by the range of values reported. We were not able to create equal sized sections in this experiment, high CO₂ accounted for 25%, medium CO₂ for 50%, and low CO₂ for 25% of the aquarium length. Hydrogen Ion Concentration (pH) was correlated with CO₂ and thus different between sections. This test did not distinguish between a preference for CO₂ or pH, in nature these two are correlated most of the time.

Statistical Analyses

We analyzed the results of the well water experiment, light experiment, temperature experiment, and control with the Quade test, a non-parametric, rank-based, two-way analyses of variance (Conover, 1999). The test utilizes sums-of-squares to detect differences between blocks. It is similar to the Friedman test but more powerful with a small number of treatments. Most of the results violated the assumptions of normality because beetles chose one specific section or were very evenly distributed across sections. The different sections were considered treatments. Significant differences between sections were determined by multiple comparisons. The results of the flow experiment and CO₂ experiment we analyzed with replicated G-tests of goodness-of-fit to account for different-sized sections (McDonald, 2009). The test analyzes differences between observed and expected numbers and also allows for data of several samples to be pooled. Differences between sections were determined by their chi-square component ratios of observed to expected numbers. One dataset (n=10) of the *H. vulnerata* flow experiment was excluded from the replicated G-test due to opposite proportions to all

other datasets. This allowed us to analyze the remaining datasets in one combined analysis without having to analyze all of them separately. The excluded dataset was analyzed separately with Chi-square goodness-of-fit and Yates correction of continuity for small datasets (McDonald, 2009). All analyses using the Quade test were done in program R, Package *stats* version 2.11.1 (<http://www.r-project.org>). Multiple comparison values were calculated by hand (Conover, 1999). Due to the limited number of available adults of *H. comalensis*, we used the same beetles when switching sides of treatments, thus the replicated tests were not independent. To account for this we treated these two sets of replicates as separate experiments. Runs with the same treatment sides but different sets of beetles were combined, which resulted in two sets for each preference test. Total numbers for each set can differ slightly because we excluded dead beetles from the analyses.

Pupation Requirements

To explore substrate requirements for pupation of *H. comalensis*, we assessed pupation success as a function of substrate type. Two aquaria, each with dimensions of 130 x 19.5 x 19.5 cm were fitted with 21 containers, built out of PVC pipe, with a diameter of 3.2 cm and a height of 5 cm, with an end-cap forming a cup. Each container was supplied with water from the bottom flowing through the container and over the rim at the top of the container, simulating water flow conditions at the springs. All containers were connected and supplied with both well water and re-circulated water from a common reservoir (3 m fiberglass tank) on top of which the aquaria sat. Well water was pumped from the Edwards Aquifer, which also supplies the springs where the beetles

occur. We used re-circulated water, in addition, as backup in case of insufficient well water supply. The water draining out of the containers was pumped through a heater/chiller unit and maintained a temperature of approximately 21 °C, which was as close to spring conditions as we were able to maintain. In each container, we placed a mesh bag (5.5 x 20.5 cm) to hold substrate and larvae. We used seven substrates types: (1) sand (<1 mm in diameter), (2) rocks (1-2 cm in diameter), (3) sand and rocks mixed in equal proportions, (4) sand mixed with three medium size Anaqua (*Ehretia anacua*) leaves, (5) rocks mixed with three medium sized Anaqua leaves, (6) sand mixed with rocks and leaves, and (7) cotton cloth strips (8 x 3 cm). Cotton cloth strips have been used in previous trials as a nutrition source and pupation has been observed in mesh bags with cotton cloth lures retrieved from Comal Springs. We cleaned all substrates and placed four cotton strips of equal size (8 x 3 cm) in each bag other than the cotton strip-only bag to provide a consistent food source for the larvae. Once bags were inserted into the PVC containers, water slowly flowed through the bottom half and the top half was exposed above the water line. We covered the aquarium with mesh to allow air-circulation and avoid high humidity forming in the aquarium. Each aquarium held three sets of the seven substrate types. We separated the aquaria into three sections, one for each set of substrates. Within each section we placed the substrates in random order, assigned by a random number generator, to avoid bias due to the position in the aquarium. In each bag we placed larvae of three different size groups (produced in refuge aquarium): two small (1-3 mm), two medium (3-5 mm), and two large (5-9 mm). All larvae were placed below the water line. The tops of the bags we tightly closed with bag clips.

Preliminary experiments indicated that frequent checking created disturbance and did not allow pupation. Consequently, the pupation experiment was conducted without disturbance. We ran one test with one aquarium for a four-month period and a second test for five months for two aquaria to see if it would allow for more pupation. In the second test, we also used six fresh cotton strips per bag instead of four to provide more opportunity for food. The same substrates were used after being cleaned thoroughly. We replaced leaves. Due to a limited availability of larvae the same ones were used for the second test and each bag had only 5-6 larvae haphazardly distributed between bags. We checked for pupation by carefully spreading out the substrate and looking for larvae, pupae or adults above and below the waterline. The bag would then be rinsed out to make sure no larvae remained. Larvae, pupae, and adults were counted and then put back into the refuge aquarium.

We analyzed the data using the Friedman test for non-parametric data (Conover, 1999). The results of the pupation experiment included a large number of zeros and only three substrates that actually yielded results, these numbers violated the assumptions of normality. The seven substrates were considered as treatments, the three sections within each aquarium were considered replicates. Because we had to use some of the same larvae for both tests, they had to be analyzed separately. We conducted the analysis using program R, Package *stats* version 2.11.1 (<http://www.r-project.org>). Multiple comparison values were calculated by hand (Conover, 1999).

Number of Instars

To find out the number of instars we measured head capsule widths of *H. comalensis* larvae preserved at the NFHTC that had been produced in refuge between 2005 and 2007. We measured all head capsule widths with a Nikon DS-5M scope camera. We measured at the widest point of the head, which was right behind the eyes. For each larva, we made three measurements and then calculated the average for a final value. We then determined the number of larval instars by using the size-frequency distribution method (Steedman and Anderson, 1985; Phillips, 1997). Larval instars can be identified in a histogram based on peaks in frequency of sizes.

CHAPTER III

RESULTS

Association with Springs

With this experiment we assessed spring association of *H. comalensis* by placing a one-meter long cotton strip in six springs. Only four of the six springs tested contained individuals of *H. comalensis* and were used for analysis. Adults and Larvae showed a significant association with the direct spring environment (Friedman test, p -value=0.01089, $n=68$). We found significant differences between different distances from the spring. Abundance was highest within 20 cm of the spring outlet and decreased gradually with distance from the spring. We found no adults or larvae more than 80 cm away (Table 1 and 2; Figure 5).

Microhabitat Preference

With the microhabitat preference experiments we tested five environmental conditions in an aquarium set-up to find and compare preference of *H. comalensis* and *H. vulnerata*. The control test showed no significant differences between aquarium sides for either species. Numbers were exactly evenly distributed for *H. comalensis* (Quade test, $p=1$, $n=30$). For *H. vulnerata*, numbers were almost evenly distributed (Quade test, $p=0.4226$, $n=30$) (Table 4).

Experiment 1: Well Water versus Re-circulated Water

For the experiment designed to assess preference for well-water or re-circulated water, we found that *H. comalensis* showed a clear preference for well water, which has water quality most similar to spring water. Because we used the same beetles for different test runs we analyzed to datasets separately to avoid issues with independence. Dataset 1 showed a significant difference between treatments (Quade test, $p=0.0056$, $n=59$). Dataset 2 also showed significant differences (Quade test, $p=0.0043$, $n=60$) (Table 4).

Heterelmis vulnerata seemed to have a higher preference for re-circulated water, which has water quality similar to stream water. Analysis of dataset 1 showed no significant difference between treatments (Quade test, $p=0.1537$, $n=59$). Dataset 2 showed a significant difference between treatments (Quade test, $p=0.0043$, $n=60$) (Figure 6; Table 4).

Experiment 2: Light

For the experiment designed to assess preference for light versus dark habitat, we found a preference for darkness in both species. Exact same proportions were found for both datasets of *H. comalensis*. Both datasets showed significant differences between treatments (Dataset 1: Quade test, $p=0.0034$, $n=60$; Dataset 2: Quade test, $p=0.0034$, $n=60$). (Table 4).

Heterelmis vulnerata chose darkness in almost the same numbers as *H. comalensis*. Both datasets showed significant differences between treatments (Dataset 1: Quade test, $p=0.0034$, $n=60$; Dataset 2: Quade test, $p=0.0029$, $n=59$). (Figure 7; Table 4).

Experiment 3: Temperature

For the experiment designed to assess preference for different temperatures, we found that *H. comalensis* preferred temperatures between 22.5 and 25.5 °C in one dataset. Dataset 1 showed significant difference between treatments (Quade test, $p=0.0061$, $n=59$). Dataset 2 did not show significant differences (Quade test, $p=0.1435$, $n=59$) (Table 4).

Heterelmis vulnerata had similar preferences. Analysis of dataset 1 showed no significant differences between treatments (Quade test, $p=0.3247$, $n=60$). Dataset 2 showed significant differences between treatments (Quade test, $p=0.0009$, $n=58$). Sections 2 (25.5- 22.5 °C) was not different from section 1 (27.5-25.5 °C) but showed significant differences to sections 3 (23.0-19.5 °C) and 4 (19.5-18.0 °C) (Figure 8; Table 4). This suggested a preference for temperatures between 22.5-25.5 °C in one dataset for this species as well.

Experiment 4: Flow

For the experiment designed to assess preference for different flow conditions, we found that *H. comalensis* mostly preferred to be in low flow. Dataset 1 showed significant differences between observed and expected numbers under random distribution for all treatments (G-test, $p=0.0039$, $n=59$). The high flow section showed the greatest difference from expected numbers. Beetles seemed to avoid this section. In low flow, numbers were higher than expected, which indicated a preference for this treatment. Numbers in dataset 2 were exactly as expected in a random distribution (G-test, $p=1$, $n=60$). No preference was observed for either flow condition (Table 4).

Analysis of dataset 1 for *H. vulnerata* showed significant differences between observed and expected numbers (G-test, $p=0.0004$, $n=50$). The high flow section showed the greatest difference from expected numbers. *Heterelmis vulnerata* was avoiding this section. In low flow observed numbers were higher than expected, which indicated a preference for this treatment. One single test run had to be excluded from the G-test in order to pool all remaining test runs. This separately analyzed dataset showed a significant difference between observed and expected numbers (Chi-square test, $p=0.0285$, $n=10$). Preference in this dataset conflicted with data from other trials, beetles preferred the high flow section over the low flow section.

Dataset 2 showed significant differences between observed and expected numbers (G-test, $p=0.0002$, $n=60$). Again, the high flow section showed the greatest difference from expected numbers and observed numbers were higher than expected in low flow, which indicated a preference for this treatment (Figure 9; Table 4).

Experiment 5: CO₂

For the experiment designed to assess preference for different CO₂ concentrations, we found that *H. comalensis* showed similar preferences in both datasets. Dataset 1 showed significant difference between observed and expected numbers in a random distribution for all treatments (G-test, $p=0.0031$, $n=60$). The low CO₂ section (4-6 mg/L) showed the greatest difference from expected numbers. Beetles avoided this section. Numbers were higher than expected in the medium CO₂ section (12-25 mg/L), which indicated a preference for this treatment. The high CO₂ section (120-160 mg/L) had the smallest difference from observed numbers, which we didn't consider as being

different. Dataset 2 also showed significant difference from expected numbers (G-test, $p=0.0002$, $n=60$). Section differences were similar with the low CO₂ section having the greatest difference from expected numbers. Numbers in medium CO₂ were higher than expected, and slightly higher than expected in high CO₂. This indicated avoidance of low CO₂ and a slight preference for medium CO₂ (Table 4).

For *H. vulnerata* the replicated G-test indicated slightly different results. Dataset 1 showed significant difference between observed and expected numbers in a random distribution for all treatments (G-test, $p<0.0001$, $n=60$). The low CO₂ section showed the greatest difference from expected numbers, with numbers being lower than expected. Beetles avoided this section. Numbers were higher than expected in medium CO₂, which indicated a preference for this treatment. The high CO₂ section had the smallest difference from expected numbers, with numbers being slightly lower. This section was also avoided by the beetles. Dataset 2 also showed significant differences from expected numbers (G-test, $p<0.0001$, $n=60$). Section differences were similar to dataset 1 with the low CO₂ section having the greatest difference from expected numbers. Numbers were higher in medium CO₂, and lower in high CO₂. This indicated avoidance of low and high CO₂ and a slight preference for medium CO₂ (Figure 10; Table 4).

Pupation Requirements

Because we used the same larvae for both tests they were analyzed separately. Test one (one aquarium run for four months) showed no significant difference between substrate types (Friedman test, $p=0.42$, $n=3$). We found two teneral adults in sand and one dead pupa in sand with leaves (Fig. 7). In test two (two aquaria run for five months) we found one dead pupa and one dead adult in rocks with leaves, and one live pupa in sand with leaves in aquarium one. In aquarium two we found one dead adult in sand with leaves and one dead pupa and one dead adult in rocks with leaves (Figure 11). The analysis for test two showed no significant difference between substrate types (Friedman test, $p=0.1247$, $n=6$). However, the results indicate a slight preference for sand and rock substrates in combination with leaves even though not all repetitions of these substrate types showed pupation (Table 5).

Number of Instars

Measurements of head capsule widths for a total of 208 *H. comalensis* larvae showed sizes ranging from 0.13 mm to 0.46 mm. Peaks in frequency of sizes indicate larval instars (Steedman and Anderson, 1985; Phillips, 1997) as shown in figure 12. We identified seven instars at the following sizes: I) 0.013, II) 0.17 mm, III) 0.21 mm, IV) 0.25, 0.26 mm, V) 0.32, 0.33 mm, VI) 0.4, 0.41, 0.42 mm, VII) 0.44, 0.45 mm. (Figure 12). The smallest instar is represented by only one measurement of 0.013 mm. This size group was under represented, most likely due to collecting bias. It is a particularly small and thus difficult to capture instar.

CHAPTER IV

DISCUSSION

Association with Springs

Heterelmis comalensis' association with habitats at the Comal springs and San Marcos springs has been well documented in literature (Bosse et al., 1988; Bowles et al., 2003; Gibson et al., 2008), but their narrow restriction to spring openings had not yet been thoroughly explained. Our study found a clear association of the beetles with the springs, which indicates they are not distributed across spring runs, as found by Bowles et al., but rather restricted to an area of 80 cm from the springs (Bowles et al., 2003). They found a poor correlation of beetle abundance and distance downstream from the primary spring outlets (Bowles et al., 2003). They did, however, use a sampling resolution of 1 m², which is likely to be too large to actually detect microhabitat differences or preferences (Gibson et al., 2008). Beetles can be found not just in the primary spring outlets, but also in smaller spring outlets along the spring runs, which may not have been detected in Bowles et al.'s study. The federal register describes the surface waters associated with Comal springs and San Marcos springs as *H. comalensis*' critical habitat and identifies the potential failure of spring flow as a primary threat to the species' survival. Drought or over-pumping of groundwater could lead to loss of critical habitat (U.S. Fish and Wildlife Service, 2007). It has been speculated that beetles may be able to

move back into spring openings during low flow periods (Bowles et al., 2003), but how far they would actually be able to retreat is not known. Our light preference results, however, show a clear movement toward dark areas--a finding that supports the possibility of retreat into the spring opening to some degree. Typical subterranean elmids (e.g. *Neoelmis sketi*, found in caves in Ecuador) have missing or reduced eyes as an adaptation to subsurface environments (Spangler, 1996). *Heterelmis comalensis* does not possess characteristics of such adaptation and is unlikely to be able to live deep in the springs on a long-term basis. When moving away from its optimal habitat, deeper into the spring outlets, *H. comalensis* could be faced with suboptimal conditions that may include new predators and competitors, which may allow the species to survive for a certain amount of time, but not thrive. Subterranean species at Comal Springs, including the omnivorous Peck's cave amphipod (*Stygobromus pecki*) could be potential predators. Light might also play an essential role for certain life stages and restrict population survival in subterranean habitats. Outside the spring opening, *H. comalensis*' restriction is most likely a result of narrow water quality tolerances. Water quality at the springs during sampling was similar to water quality measurements reported by BIO-WEST's annual report (BIO-WEST, 2010), except for DO, which was higher in our measurements. At the spring locations tested, flow declined with distance from the spring outlet but was difficult to measure within the substrate, where beetles normally occur. The substrate didn't seem to be a restricting factor, since we embedded the cotton strip in same-sized gravel over the whole length. However, that does not mean that the substrate type at the springs does not matter. Other substrate types like silt would provide unsuitable habitat conditions for this species (RECON Environmental et al., 2011) and

thus would simply add another restricting factor. Water quality was very similar across the one meter testing area, except for CO₂. CO₂ declined slightly with distance from the spring, however, differences were minimal (Table 3). Temperature did not show significant change within one meter of the spring, indicating that temperature might not be the most significant factor contributing to *H. comalensis*' restriction, but other specific water quality parameters or other unknown factors, which will be discussed below.

Microhabitat Preference

Heterelmis comalensis' clear preference for well-water over re-circulated water supports the findings of our spring experiment. Well water at the fish hatchery is pumped from the Edwards Aquifer, which also supplies Comal Springs, thus is very similar in water quality. Re-circulated water at the hatchery is well water that has been degassed, which lead to changes in CO₂, pH, and DO. It is more similar to stream water, which goes through a similar process under natural conditions. Spring water is characterized by a higher concentration of CO₂, a lower concentration of DO, and a slightly lower pH. Our measurements of well-water used in our experiment mostly concur with measurements taken at the springs (BIO-WEST, 2010; Table 3), with exception of DO, which was slightly higher, compared to spring condition. Because there was no difference in substrate or biotic factors, these water quality parameters are likely to contribute to the beetle's habitat limitations. Nutritional growth correlated with water type could be a factor but was not evident in the aquaria. It is possible that food sources like algae, fungi, and bacteria restrict the beetle's range to spring outlets. In tentative food trials we saw a preference for fungi and possibly bacteria growing in well water over fungi and bacteria

growing in re-circulated water. We let growth develop on cotton rags in both water types and, after eliminating the difference in water type, let the beetles choose a food source. The growth that developed in darkness on the well-water side was most often preferred. This could suggest a restriction to springs due to a dependence on spring adapted, heterotrophic food sources. However, these food trials were just observational and did not result in sufficient data for analysis.

The beetle's attraction to potentially heterotrophic food sources in the springs is also supported by our light preference experiments where beetles choose to be in darkness. Many aquatic insects are negatively phototactic and actively avoid light (Ward, 1992). This also applies to elmids; they can often be found on the underside of rocks or wood (Elliot, 2008). *Heterelmis comalensis*' light preference shows that darkness is not a factor keeping the beetles from moving into the springs or burrowing into the substrate. Their affinity to hide concurs with observations from the wild; they are mostly found in the substrate and in spring outlets. However, light could be essential for other phases of the life cycle, such as pupation and emergence (Ward, 1992). This is the case with most aquatic insects. If this is also true for *H. comalensis*, then retreat into spring openings seems unlikely for extended periods.

Temperature preference suggests that *H. comalensis* is somewhat adapted to the temperatures at the springs. One of the datasets did not show a significant preference, which means *H. comalensis* might not necessarily be restricted to temperature ranges as narrow as expected. At the Comal springs, temperatures are constant at approximately 23 °C (BIO-WEST, 2010). We expected *H. comalensis* to have narrow temperature tolerances and a preference for temperatures close to 23 °C, which is often true for

spring-adapted species (Ward, 1992). Examples of other elmids adapted to constant temperatures at springs include the endemic *Microcylloepus thermarum* or *Zaitzevia thermae*, which occur in warm springs in Nevada and Montana, respectively (Brown, 1987; Herbst, 1996; Stagliano et al., 2007). Spring sources often provide stable habitat conditions with constant temperatures and are known to be a dependable refuge for aquatic organisms. They allow sensitive species to avoid seasonal extremes of temperatures (Herbst, 1996). In this light, *H. comalensis*' adaptation to constant spring temperatures seems likely. One reason for insignificant results in one dataset could be slight inconsistencies with temperature ranges in different section. The exact repetition of temperatures was difficult to control and temperatures were slightly overlapping in a few tests. Even though differences between sections were insignificant in one dataset, abundance was highest in two sections that were closest to spring temperature (19.5-25.5 °C).

Heterelmis comalensis did not show preference for high flow, but instead showed preference for low flow in one dataset. In contrast to our results, a variable flow study conducted by the Edwards Aquifer Authority found a preference for beetles to be in and move towards the current, as well as move downwards (BIO-WEST, 2002). The movement towards flow might be a response to water quality. Elmids are often found in shallow riffles with rapidly flowing water, and require well-aerated water (Bosse, 1979; Brown, 1987; Elliot, 2008). *Heterelmis comalensis* is mostly found in interstitial spaces between substrate particles, where flow is lower. Elmids do not swim, but instead cling to the substrate. Being physically exposed to high flow increases the chance of being swept downstream away from optimal habitat. Nevertheless, habitat quality is highly dependent

on sufficient flow from the spring. A high DO concentration is maintained by the exchange of gases between atmosphere and water through turbulence, which is particularly crucial considering that DO is generally lower in source areas (Horne and Goldman, 1994). Elmids are dependent on high DO because of their plastron respiration (Bosse, 1979; Brown, 1987). Their oxygen consumption and survival are determined by the rate of oxygen diffusion into the plastron; thus lower oxygen can result in less oxygen diffusing into the plastron or actually diffusing out of the plastron if DO in surrounding waters is lower than concentrations within the plastron (Resh et al., 2008). Other water quality factors that might be important to habitat quality, like temperature and CO₂, are also maintained by sufficient discharge from the Aquifer. A decrease in discharge would thus result in a decrease of habitat size. If *H. comalensis* would in fact be dependent on a spring-adapted food source in form of bacterial, fungal, and algal growth, then adequate flow from the springs would indirectly affect beetles through maintenance of nutrition. *Heterelmis comalensis* is mostly found in gravel and seems to be absent from sand and silt - substrates typically associated with low flow areas (Bowles et al., 2003). Gravel and cobble are essential substrate types for the maintenance of beetle populations (U.S. Fish and Wildlife Service, 2007). Constant flow conditions keep the substrate stable and allow development of habitat structure and nutritional growth (Ward, 1992). In one dataset *H. comalensis* did not show a preference for either treatment, DO might not have been sufficient in the low flow section in some tests, which is a possible explanation for why that area wasn't chosen.

We found *H. comalensis* to prefer CO₂ concentration lower than spring concentrations (between 30 and 40 mg/L). With our set-up, it was somewhat difficult to

control CO₂, we were not able to create the exact concentration found at the springs, which *H. comalensis* might have chosen. The actual concentration of 12-25 mg/L they chose was closest to spring conditions. As we expected, they avoided low CO₂, which could provide evidence for spring adaptation due to CO₂ concentration. As mentioned above, food in the form of bacteria or fungi might be a factor correlated with higher CO₂ concentrations and might have influenced the beetle's choice. Identifying different growth on cotton strips in well water or re-circulated water could aid in answering that question. Interestingly enough, beetles mostly chose medium and high CO₂, even though DO was higher in low CO₂. Perhaps DO was sufficient in high CO₂ areas, otherwise this could have been a restricting factor due to their dependence on well-aerated water. Our results clearly demonstrate that CO₂ provides an essential habitat feature and is favored over DO.

The stream adapted *H. vulnerata* did not show a strong preference for well water or re-circulated water, with only one data set showing significant preferences for re-circulated water. In contrast to the related *H. comalensis*, *H. vulnerata* seems to have less narrow water quality tolerances. The difference in preference for water types demonstrates that these two species may in fact inhabit different habitats due to water quality.

Heterelmis vulnerata also preferred to be in the dark. Elmids typically hide under rocks and in the substrate. This serves as protection from predators; feeding on the surface of rocks and other structures typically occurs at night (Ward, 1992). In this regard both species exhibited typical behavior.

Surprisingly, *H. vulnerata* showed a preference for spring-like temperatures in one dataset and no preference for a temperature in the other. Temperatures in streams fluctuate during the year, with lower temperatures in winter and higher temperatures in summer. *Heterelmis vulnerata* might be more tolerant to lower temperatures but still seems to prefer higher temperatures when given the choice. Development and growth is faster in higher temperatures (Brown, 1987) and reproduction typically occurs in summer when water temperatures are higher. Temperatures between 22-25 °C promote incubation of eggs and hatching in elmids (Huryn et al., 2008). Beetles might have chosen higher temperatures for reproductive reasons or developmental advantages.

Just like *H. comalensis*, *H. vulnerata* mostly preferred the low flow area, likely for the same reasons. DO was still high in low flow; in the wild beetles depend on high turbulence for aeration. Being exposed to high flow results in drifting, which is a mechanism mostly used to escape poor environmental conditions (Brown, 1987). Our results show that beetles remain in low flow if environmental conditions are suitable. Often, the underside of rocks or logs and interstitial spaces where beetles hide are naturally less exposed to high flow. One test showed opposite preference, where most beetles were found in the high flow section. Since we found this result in only one test, it might have been a result of set-up bias. If flow wasn't consistent, beetles could have chosen the high flow side when they would normally avoid this area.

We found *H. comalensis* and *H. vulnerata* to prefer the same CO₂ concentration (given the range), even though *H. comalensis* inhabits the springs with CO₂ concentrations between 30 and 40 mg/L, and *H. vulnerata* inhabits shallow streams with CO₂ concentrations of about zero (Table 6). We expected *H. vulnerata* to choose low

CO₂, but it did in fact avoid it, just like *H. comalensis*. This could be interpreted as contradictory to the assumption of *H. comalensis*' spring restriction due to CO₂ but most likely just demonstrates *H. vulnerata*'s wider tolerances. But even though we found the preference of *H. comalensis* and *H. vulnerata* in general to be similar, it was noticeable that *H. comalensis* was in fact found in high CO₂, in greater numbers than *H. vulnerata*, which indicates greater tolerance for extreme CO₂ values. Unfortunately our results do not indicate if *H. vulnerata* might even prefer concentrations similar to the springs because we were not able to create those exact conditions. If they would in fact prefer similar concentrations it would demonstrate that habitat separation of both species does not differ due to CO₂. A test with CO₂ concentrations more similar to spring conditions might show differences between the two species. More testing needs to be done in this regard.

Pupation Requirements

Even though substrate choice was not significantly different than random given our small sample sizes and the very low rate of pupation generally, two of the three substrates chosen contained leaves and two contained sand. These results are somewhat surprising considering substrates at the springs tend to be gravel, and leaves can only be found at some of the springs. Most members of the family Elmididae are known to pupate above the water line in moist sand, humus, moss, under debris, rocks, or similar material, others simply wait in shallow water until the water level drops to pupate (Brown, 1987; Elliot, 2008). The question remains if *H. comalensis*' final instar larvae somehow drift to pupation sites outside of the springs in order to pupate or if they are able to pupate under

water within the substrate of the spring opening. If larvae drift to pupation sites outside the spring opening, young adults would have to find their way back to the springs, which seems unlikely considering their non-functional wings. Most elmids have a short flight period after emergence before they enter the water (Seagle, 1980; Brown, 1987; Elliot, 2008), however, *H. comalensis* seems to be a species incapable of flying (Bosse et al., 1988). Young adults would have to crawl back to the springs, which seems equally unlikely considering their narrow environmental tolerances. Springs located at the shoreline of spring runs 1, 2, and 3 provide opportunity for pupation in the substrate above the water line but springs located in Landa Lake do not. They are covered by several feet of water. Considering spring locations and dispersal abilities of *H. comalensis*, pupation below water line seems more probable. They are also known to be non-seasonal (Bowles et al., 2003), which would complicate pupation with distance to the spring due to fluctuating temperatures. It has been shown that riffle beetles of other families, like *Psephenus murvoshi*, living in springs successfully pupate under water. It is possible that pre-pupal larvae somehow trap and retain an air-bubble around them, much like the plastron of an adult (Brown, 1987). Perhaps *H. comalensis* uses this or a similar method to pupate in the springs without having to emerge. It seems unlikely that larvae travel up to 50 m to reach the riparian zone of Landa lake. Pupae in our experiment were often found below the waterline; however, they might have shifted in location during pupation check. We also noticed that pupae appeared to be flowing on the water surface when separated from the substrate, possibly due to air trapped in their pupal case or tracheal air sacs. Perhaps, larvae pupate in small airspaces within the substrate or in air bubbles caught under rocks. When checking the different substrate types for pupation we

often noticed air bubbles attached to the substrate, which demonstrated the existence of air spaces within submerged substrate.

Pupations were mostly unsuccessful in previous studies conducted at the NFHTC (Fries, 2003), and only occurred in very small numbers in our experiment. For future research on pupation requirements, the set-up would benefit from improvement. Temperature in our experiment was held constant around 21 °C. We suspect that chances of pupation generally may be higher with temperatures around 23 °C, as found in their natural habitat. With current possibilities at the hatchery, we were not able to hold temperatures constant at that degree. Food could also be a factor that limited pupation. Perhaps pupation chambers need to be supplied with more cotton strips for food or more leaves. Finding specific nutritional requirements might improve the set-up. The duration of pupation tests also plays a significant role. More time could allow more opportunity for pupation, however, without sufficient food supply it also decreases survival of larvae and emerged adults. We noticed a greater number of dead larvae, pupae, and adults after the five-month test. Emergence could be dependent on certain light conditions (Ward, 1992). Our pupation aquaria were covered with pond liner to avoid excessive algal growth; perhaps altered light conditions would improve pupation success. For our set-up, we had to use well-water and re-circulated water in combination, to ensure stable water supply. Using only well water with higher CO₂ might improve the chances for pupation, perhaps beetles need these specific environmental characteristics found at springs to successfully pupate. Generally we were not able to determine one substrate as suitable for pupation. Small substrates like sand did not seem to inhibit pupation, although it has to be noted that flow at the springs is faster than in our tests and probably would not allow for

airspace to form as well as they did in our condensed sandy substrates. We noticed large deposits of scale, especially in sand, that kept sand particles from moving and airspaces intact. This might not be the case at the springs; gravel (which is similar in size to the rocks we used in our experiment) is less likely to shift. Due to a higher probability of shifting, sandy substrates provide poor sites for attachment less organic material as food (Huryn et al., 2008).

Number of Instars

Heterelmis comalensis is a member of the family Elmidae, which can have larval periods lasting six to 36 months, with 5-8 instars (Brown, 1987). Studies show that larval periods can last up to 48 months for some species including *Stenelmis crenata* and *Macronychus glabratus* (LeSage and Harper, 1976). In other cases larval development can be quick, with the larval period only lasting six months with seven instars in the laboratory for *Stenelmis sexlineata* (Phillips, 1997; Elliot, 2008). Development is generally faster at higher temperatures. The constant, warm temperature at the springs could support a shorter larval period for *H. comalensis*. We found the species to have seven instars, which is within the typical range for elmids. The first instar is underrepresented, likely due to inadequate sampling. Its size is 0.13 mm, most larvae of that size were probably overlooked. The largest instar was underrepresented as well. Due to their inability to pupate in captivity, mature larvae of the last instar die in larger numbers compared to other instars, which might explain their small numbers. Our analysis did not determine the length of each instar. Instar duration and growth rates need

to be determined in future studies to accurately estimate length of larval period for this species.

Conservation Implications

The federal register lists the spring outlets of the Comal and San Marcos springs as the beetle's critical habitat, specifically Landa Lake, the spring runs, and the upstream portion of Spring Lake. These areas have characteristics necessary for life-history functions, like high quality water, temperatures around 23 °C, DO between 4.0 and 10.0 mg/L, food supply in form of leaves and roots, and gravel or cobble substrate free of sand or silt (U.S. Fish and Wildlife Service, 2007). Furthermore, a reduction of spring flow and drying of spring runs are factors limiting survival of the species due to the species respiration (U.S. Fish and Wildlife Service, 2007). Considering our results, it becomes apparent, that *H. comalensis* does inhabit a very restricted area of the springs and is dependent on relatively specific water quality. This is of special concern because beetles might not be able to retreat to standing water in the streambed, in the event of spring flow ceasing. Pools are unlikely to be spring fed and may develop oxygen deficits and other adverse conditions, which will be unsuitable habitat for the beetle due to its dependency on high DO and good water quality. Other beetle species are known to burrow deep into the substrate during dry periods (Ward, 1992). *Heterelmis comalensis* seems to be more likely to move back into spring openings, however, that might result in crowding of populations.

The excellent water quality of the Edwards Aquifer and spring outlets are vital for the beetle's long-term survival. The Edwards Aquifer Recovery Implementation Program

(EARIP) has the goal of maintaining silt-free habitat through continued spring flow, riparian zone protection, and recreation control in areas designated as critical habitat. Moreover, aquifer water should be maintained with high water quality, not to exceed a 10% deviation from historically recorded water quality. The program also suggests the implementation of riparian habitat of spring run 3 and western shoreline (RECON Environmental et al., 2011). *Heterelmis comalensis* is dependent on sufficient flow from the aquifer; their specific water quality requirements can only be met with constant flow. The EARIP recommends an average long-term discharge of 6.3713 m³/s (225 cfs), and a short-term minimum of 0.84951 m³/s (30 cfs) (RECON Environmental et al., 2011). Because of *H. comalensis*' dependence on water quality associated with the aquifer, preventing the springs from going dry is most important for the species survival. The necessary amount of discharge depends on water quality changes expected with lower flow. Higher flow most certainly improves *H. comalensis*' chances of survival. Even if beetles might be able to retreat into the springs during low flow periods, it could greatly affect abundance and decrease chances of long-term survival. Endemic habitat specialists like *H. comalensis* are very unlikely to find and colonize new habitat; the destruction of existing habitat can lead to population reduction or even extinction (Lomolino et al., 2009). EARIP models predict the probability of a drought as dry as the one in 1956 as 1.6% in any given year in the near future. In addition, climate change models predict a long-term drying of climate in Texas (Mace and Wade, 2008). This will not just decrease recharge of the Aquifer but also increase water demands for the region. Such an increase in water demand will cause water levels to drop and threaten spring flow even during less intense and shorter duration droughts (Longley, 1995). The increased probability of

ceasing spring flow will pose a threat to *H. comalensis*' survival. Flow research studies to test *H. comalensis*' reaction to changes in flow are currently in planning by the EARIP (RECON Environmental et al., 2011), and will increase knowledge about movement or retreat in an actual spring run. We consider this research much needed, our flow experiment did not show great variation of water quality normally associated with changes in spring flow.

Conclusion

Our study supports the idea that *H. comalensis* is generally restricted to spring outlets and dependent on spring water with narrow water quality parameters that include temperatures close to 23 °C, and elevated CO₂. Furthermore, beetles preferred to be in darkness and low flow, typical behavior shown by elmids as a strategy to avoid predation and unintentional drift. Ceasing spring flow would greatly impact this endemic species' wellbeing and decrease chances of survival. We were not able to determine favorable substrate types for pupation. Few pupations occurred, many of which did not survive. An improved set-up, specifically water quality and food supply, might increase pupation success. We found seven larval instars for *H. comalensis*; a number similar to other elmids species, which typically have 5-8 instars.

Table 1. Spring experiment – Abundance of *H. comalensis* with distance from the spring. Shown are mean and standard error. Distance is shown in distance categories, as used for analysis.

Spring	Abundance with distance to spring					N
	0-20 cm	21-40 cm	41-60 cm	61-80 cm	81-100 cm	
1	8	2	1	2	0	13
2	15	9	11	3	0	38
3	4	1	0	0	0	5
4	9	2	0	1	0	12
Total	36	14	12	6	0	68
Mean	9.0	3.5	3.0	1.5	0	
Standard Error	0.758	0.988	1.067	0.527	0	

Table 2. Spring experiment – Analysis with the Friedman test for four spring locations in spring run 2, 3, and Landa Lake.

	N	Friedman χ^2	Degrees of Freedom	P-value
Analysis	68	13.0811	4	0.01089

Table 3. Spring experiment – Measurements of water quality at the four spring locations used for analysis. Shown are measurements at the spring outlet and one meter from the spring outlet.

Water Quality	Water Quality Measurements							
	Spring outlet				1 meter distance			
	1	2	3	4	1	2	3	4
Temperature (°C)	23.32	23.24	23.37	23.59	23.36	23.26	23.37	23.47
Conductivity (S/cm)	0.5	0.5	0.49	0.49	0.49	0.49	0.49	0.49
pH	7.26	7.25	7.26	7.26	7.26	7.24	7.26	7.27
DO (mg/L)	5.39	5.46	5.41	5.44	5.38	5.46	5.37	5.45
CO ₂ (mg/L)	40	40	35	40	35	35	30	30
Flow (m/sec)	0.02	0.36	0.22	0.03	0.03	0.21	0.05	0.02
Depth (cm)	9.1	15.2	7.6	73.2	8.5	15.2	15.2	70.1

Table 4. Preference experiments – Results of statistical analyses of preference experiment data. Data was analyzed with the Quade test and the G-test. Shown are the total number, test statistic values, degrees of freedom and p-value for *H. comalensis* and *H. vulnerata*. One test of the flow experiment for *H. vulnerata* was analyzed separately with a Chi-Square goodness-of-fit test (shown as excl.).

Treatment (dataset)	Analysis									
	<i>H. comalensis</i>					<i>H. vulnerata</i>				
	N	F	G-value	Df _n /Df _d	P	N	F	G-value	Df _n /Df _d	P
Control	30	0	-	1/2	1	30	1	-	1/2	0.4226
Well/Recirculated water (1)	59	21.617	-	1/5	0.0056	59	2.8242	-	1/5	0.1537
(2)	60	24.5	-	1/5	0.0043	60	24.5	-	1/5	0.0043
Light/Dark (1)	60	27.222	-	1/5	0.0034	60	27.222	-	1/5	0.0034
(2)	60	27.222	-	1/5	0.0034	59	29.4	-	1/5	0.0029
Temperature (1)	59	6.1605	-	3/15	0.0061	60	1.2564	-	3/15	0.3247
(2)	59	2.0976	-	3/15	0.1435	58	9.3914	-	3/15	0.0009
Flow (1)	59	-	8.332	1	0.0039	50	-	12.663	1	0.0004
excl.	-	-	-	-	-	10	-	4.8	1	0.0285
(2)	60	-	0	1	1	60	-	13.919	1	0.0002
CO ₂ (1)	60	-	11.544	2	0.0031	60	-	24.489	2	<0.0001
(2)	60	-	17.613	2	0.0002	60	-	28.367	2	<0.0001

Table 5. Pupation experiment – Analysis of data from different substrates tested in the pupation experiment. Data was analyzed with the Friedman test. Shown are the number of pupations (N), mean, standard error, test statistic value, degrees of freedom and p-value. Only substrate types that yielded pupations are shown on table.

Statistic	Pupation test			
	1		2+3	
	Sand	Sand+Leaves	Sand+Leaves	Rocks+Leaves
N	2	1	2	4
Mean	0.667	0.333	0.333	0.667
Standard Error	0.816	0.577	0.365	0.516
Friedman χ^2		6		10
Df		6		6
P		0.4232		0.1247

Table 6. Water quality measurements taken at Plum Creek near Luling, Texas. Measurements were taken in June 2011 around noon. Measurements were taken with a portable Hydrolab and CO₂ titration.

Location	Water Quality Measurements				
	Temperature (°C)	Conductivity (S/cm)	pH	DO (mg/L)	CO ₂ (mg/L)
Plum Creek	21.99	1.58	8.39	6.12	0

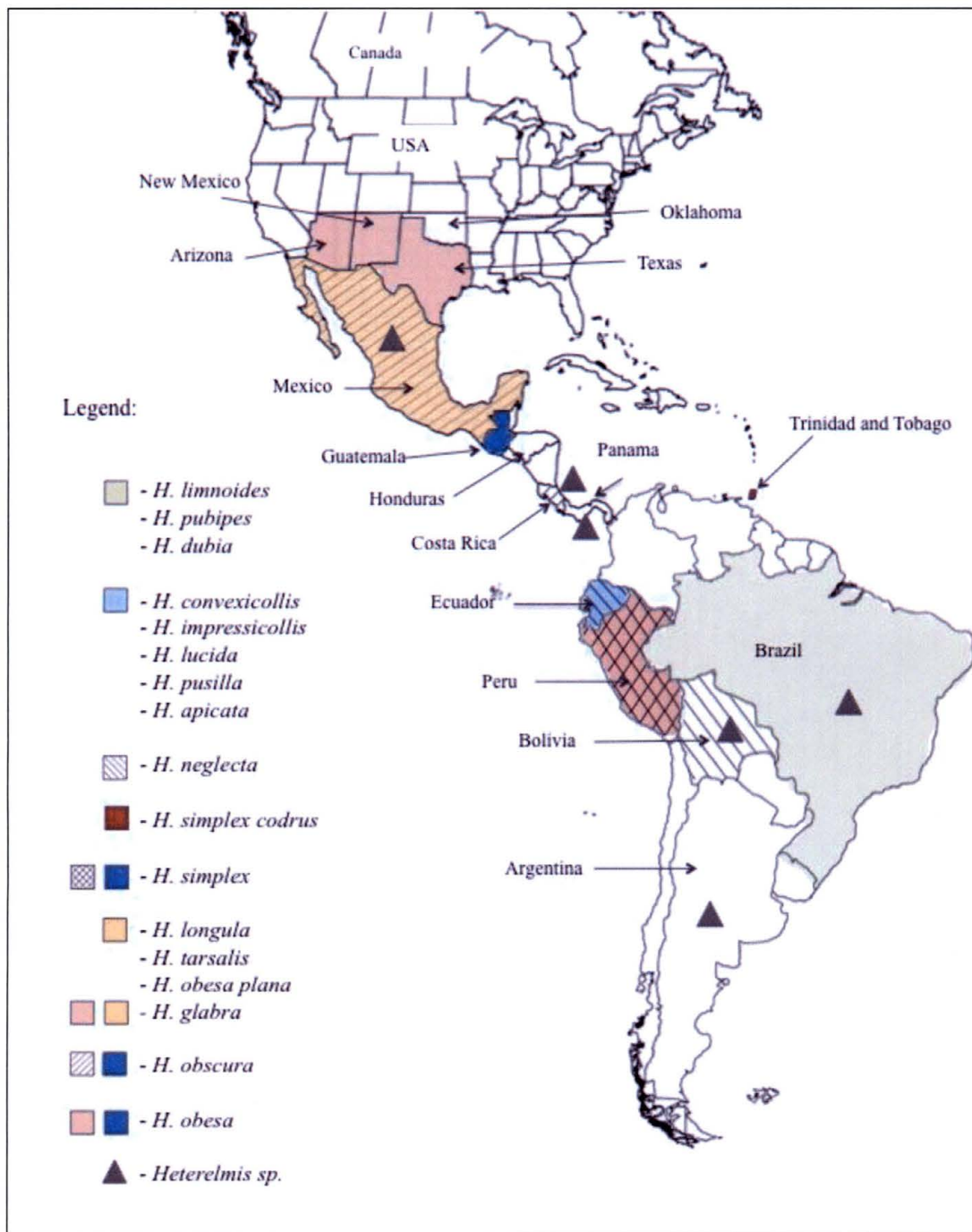


Figure 1. Distribution of *Heterelmis* species. Shown is the general distribution by country throughout South America, Central America and southern parts of the United States of America. Excluded are *H. comalensis*, *H. vulnerata*, and *H. glabra* (shown in separate graph). Unidentified specimens are indicated as *Heterelmis sp.*

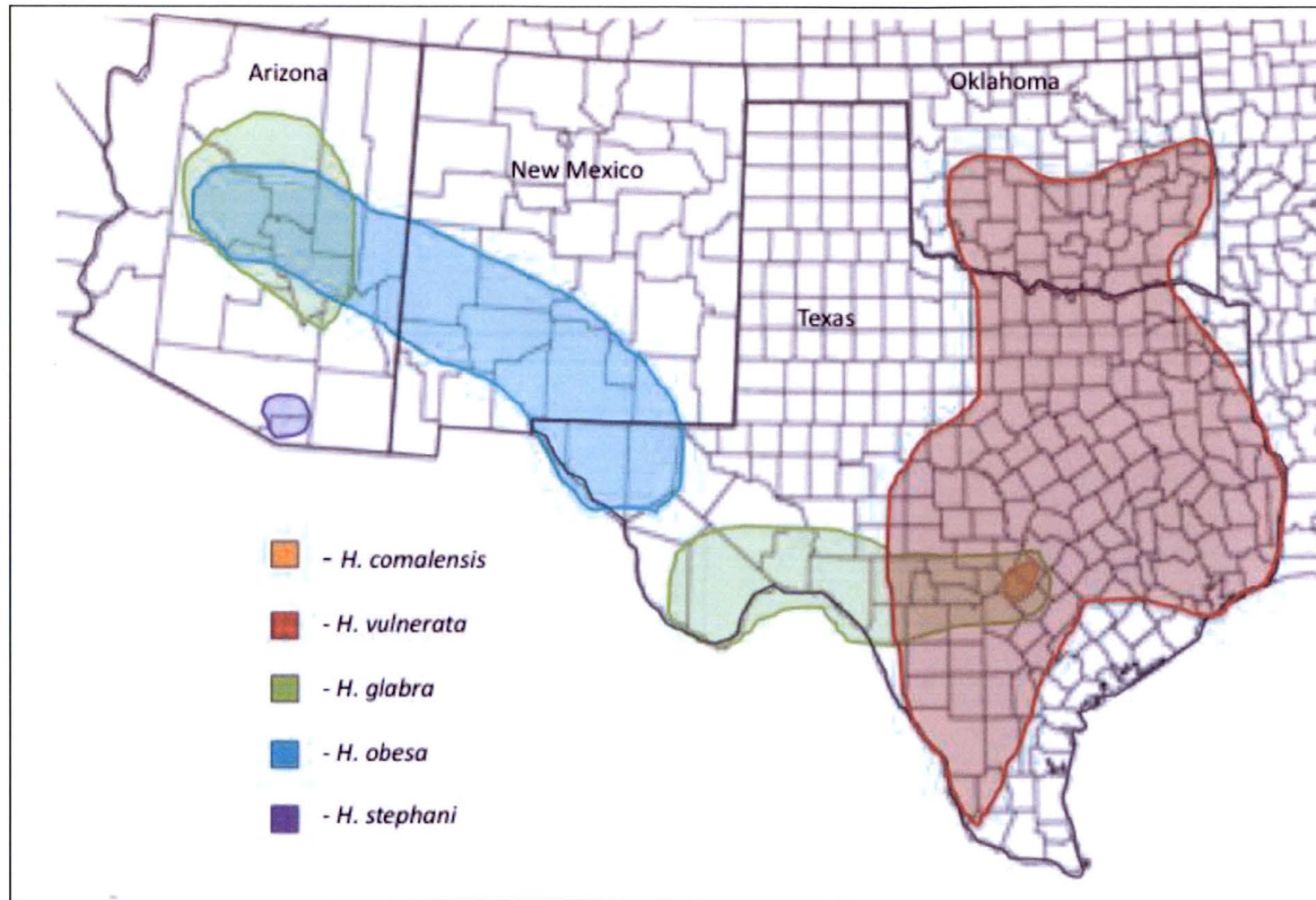


Figure 2. Distribution of *H. comalensis*, *H. vulnerata*, *H. glabra*, *H. obesa*, and *H. stephani* in the United States. Shown are species ranges based on known populations.

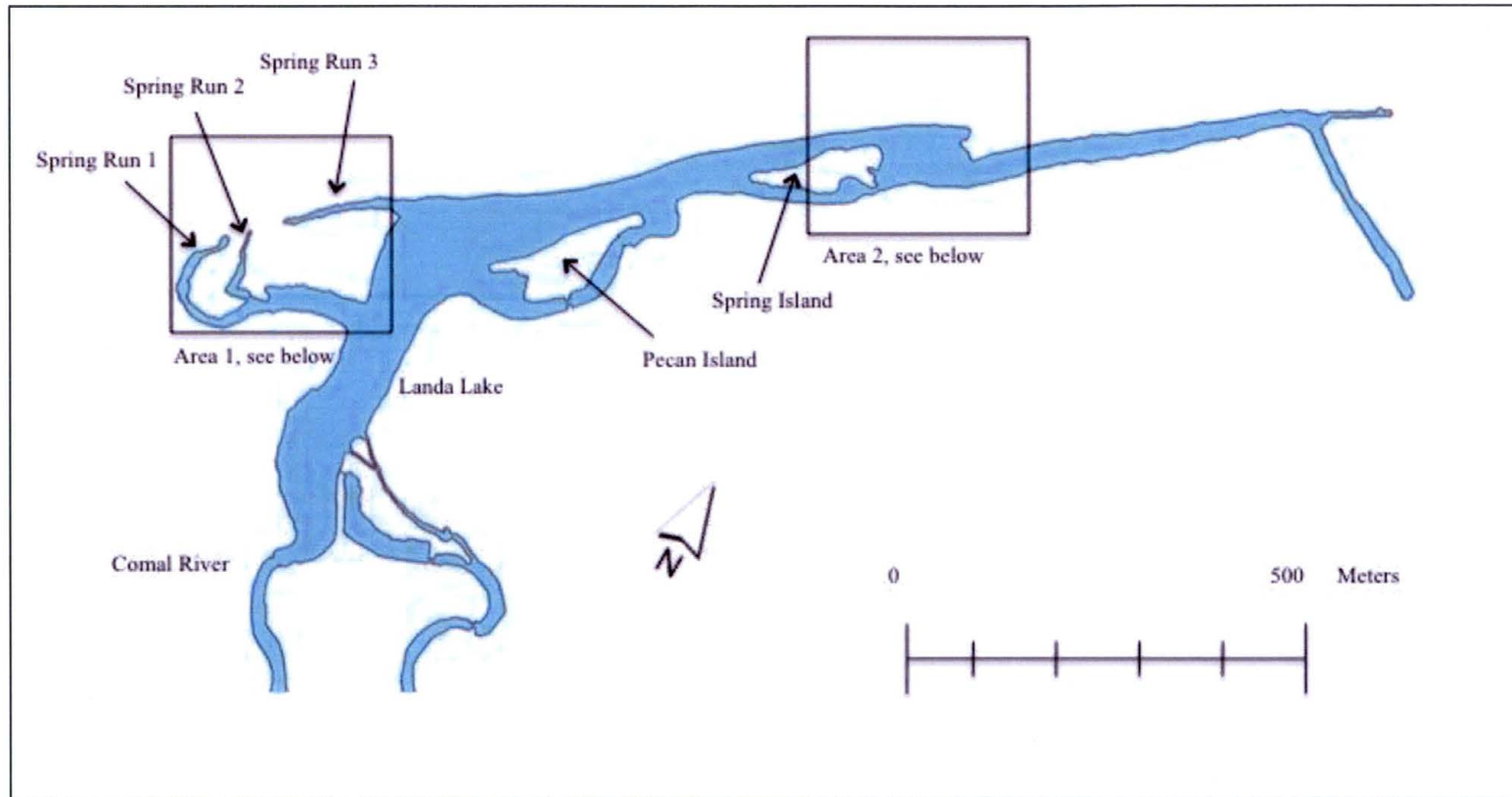


Figure 3. Location of spring runs and Landa Lake in New Braunfels, Texas. Area enlargements are shown on page 50.

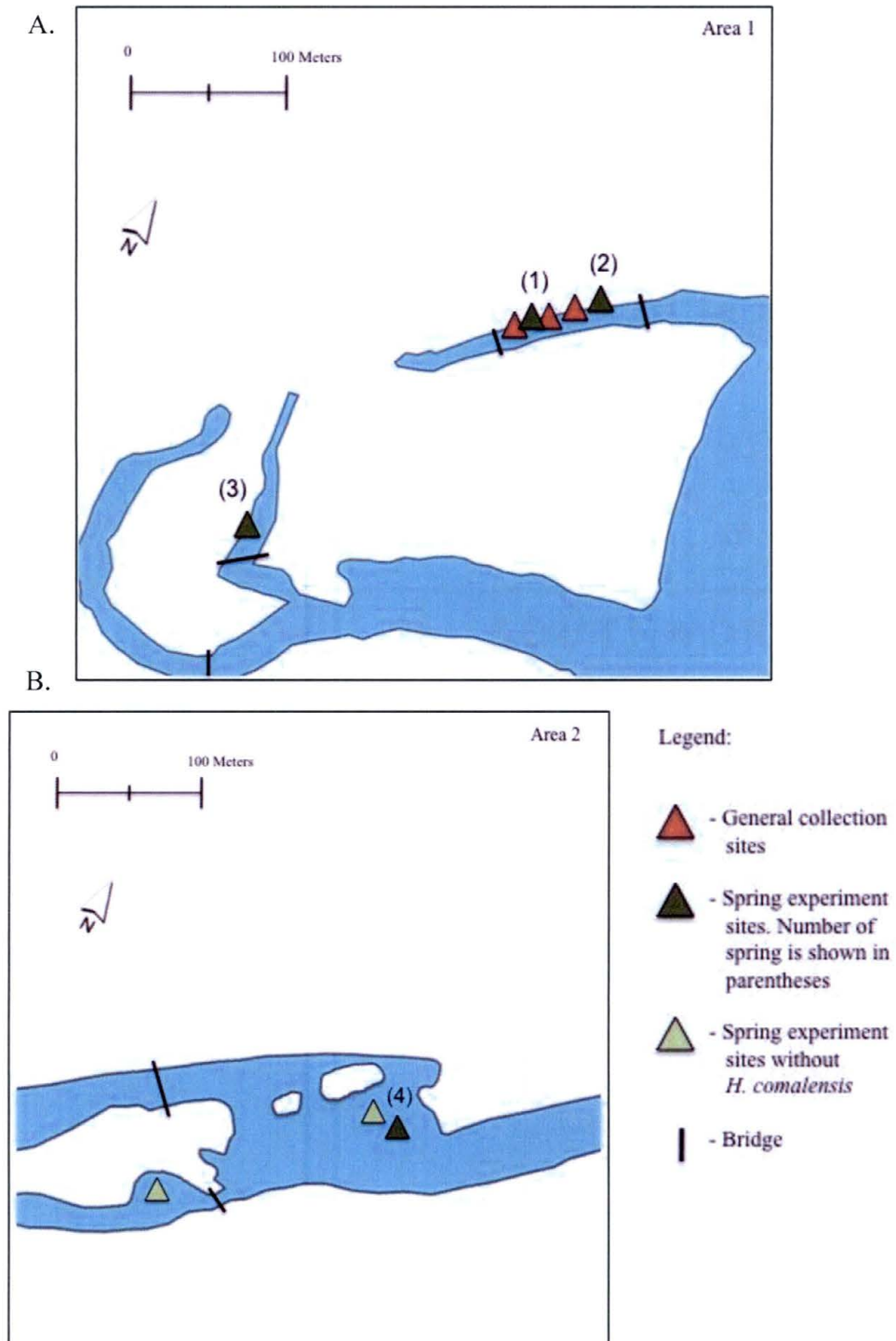


Figure 4. Sampling locations of *H. comalensis* and spring experiments locations at spring runs 2 and 3 (A) and Landa Lake (B). Numbers in parenthesis indicate springs used for analysis of spring experiment (see table 1).

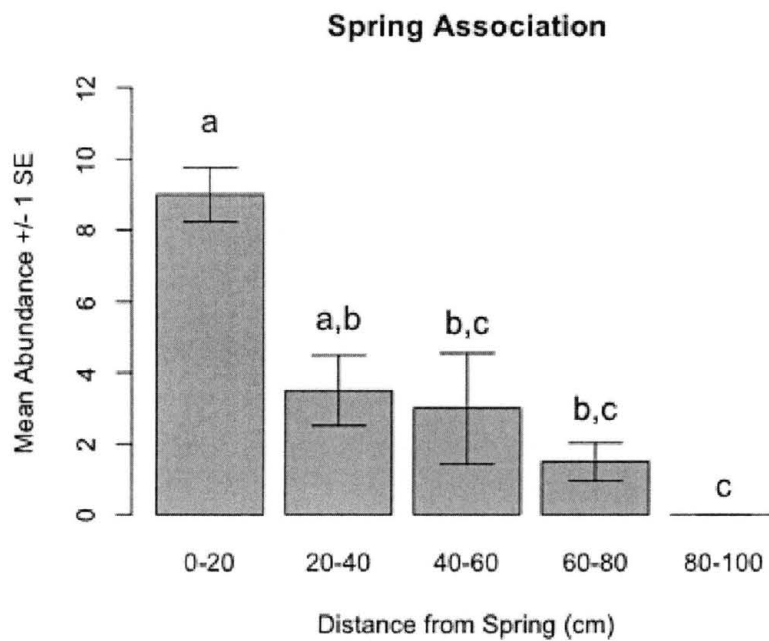


Figure 5. Association of *H. comalensis* with spring discharge in four springs. Shown is the mean abundance and standard error for different distances from the spring outlet. (Friedman test, p -value=0.01089, n = 68). Different letters indicate statistically significant differences between distance categories as determined by multiple comparisons.

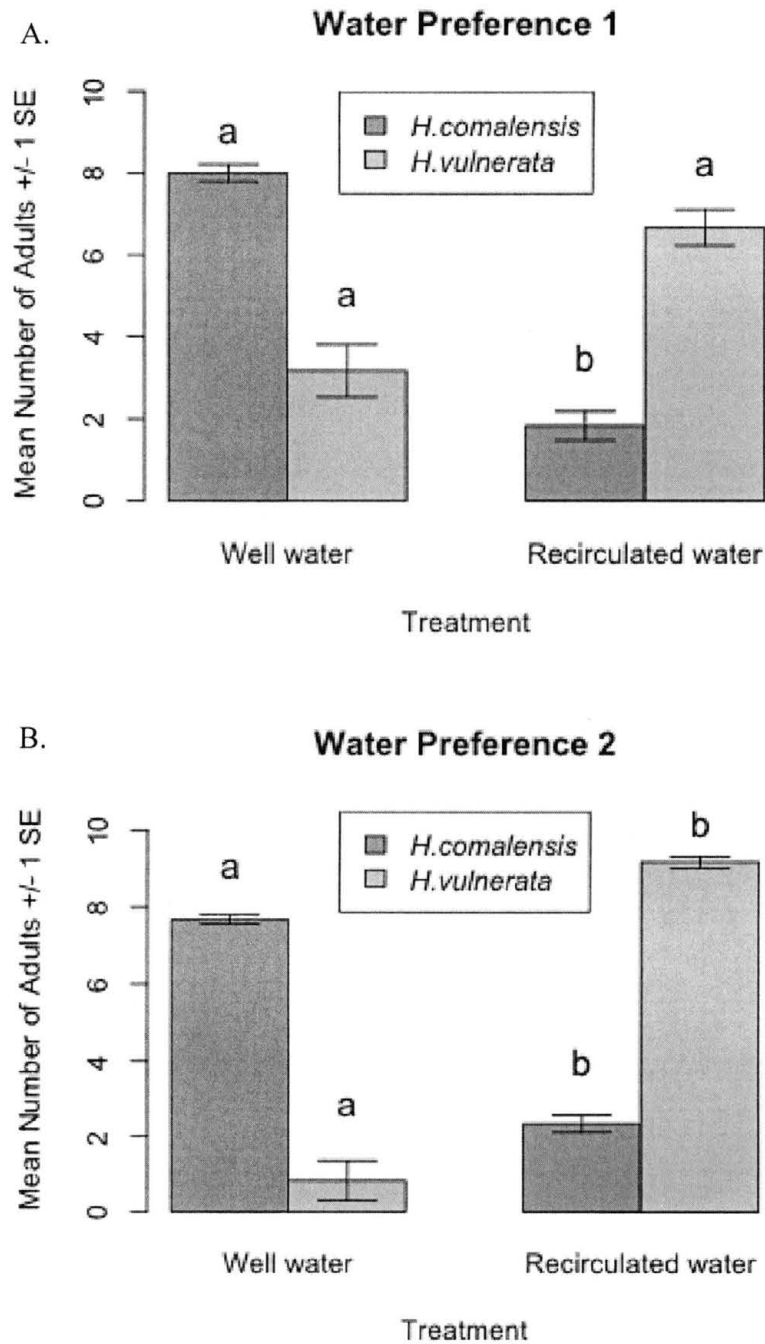


Figure 6. Water preference for *H. comalensis* and *H. vulnerata*. A) Dataset 1 (*H. comalensis*: Quade test, $p=0.0056$, $n=59$; *H. vulnerata*: Quade test, $p=0.1537$, $n=59$). B) Dataset 2 (*H. comalensis*: Quade test, $p=0.0043$, $n=60$; *H. vulnerata*: Quade test, $p=0.0043$, $n=60$). Different letters indicate significant difference between sections as determined by multiple comparisons.

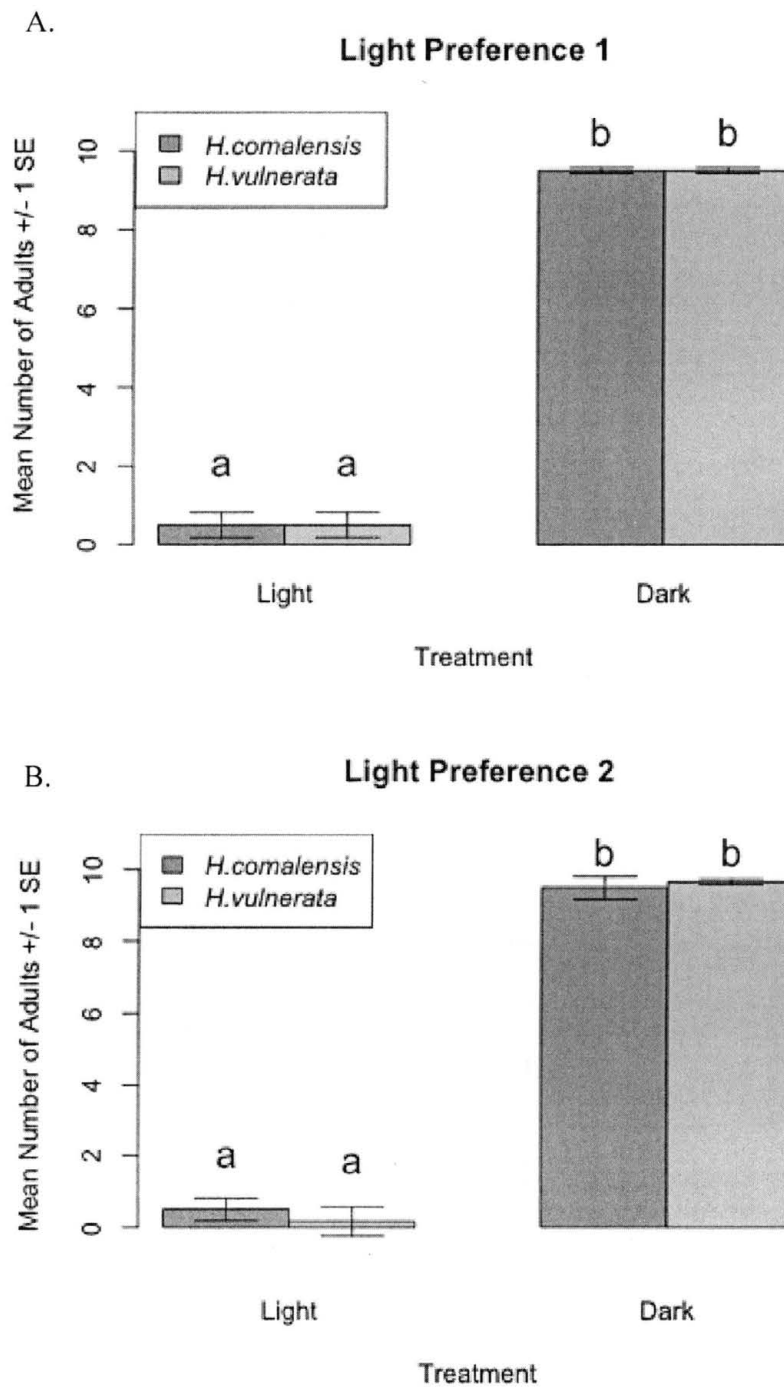


Figure 7. Light preference for *H. comalensis* and *H. vulnerata*. A) Dataset 1 (*H. comalensis*: Quade test, $p=0.0034$, $n=60$; *H. vulnerata*: Quade test, $p=0.0034$, $n=60$). B) Dataset 2 (*H. comalensis*: Quade test, $p=0.0034$, $n=60$; *H. vulnerata*: Quade test, $p=0.0029$, $n=59$). Different letters indicate significant difference between sections as determined by multiple comparisons.

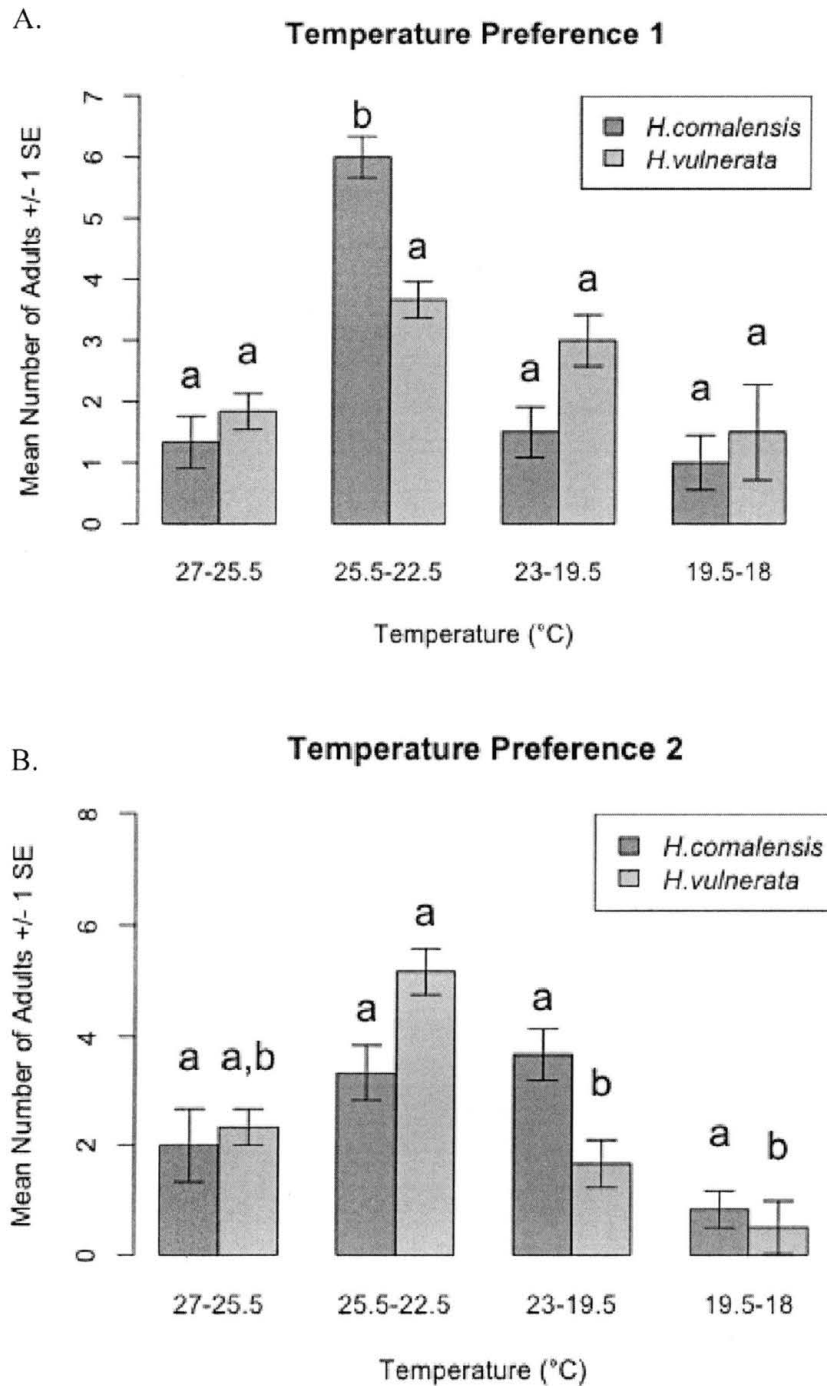


Figure 8. Temperature preference for *H. comalensis* and *H. vulnerata*. A) Dataset 1 (*H. comalensis*: Quade test, $p=0.0061$, $n=59$; *H. vulnerata*: Quade test, $p=0.3247$, $n=60$). B) Dataset 2 (*H. comalensis*: Quade test, $p=0.1435$, $n=59$; *H. vulnerata*: Quade test, $p=0.0009$, $n=58$). Different letters indicate significant difference between sections as determined by multiple comparisons.

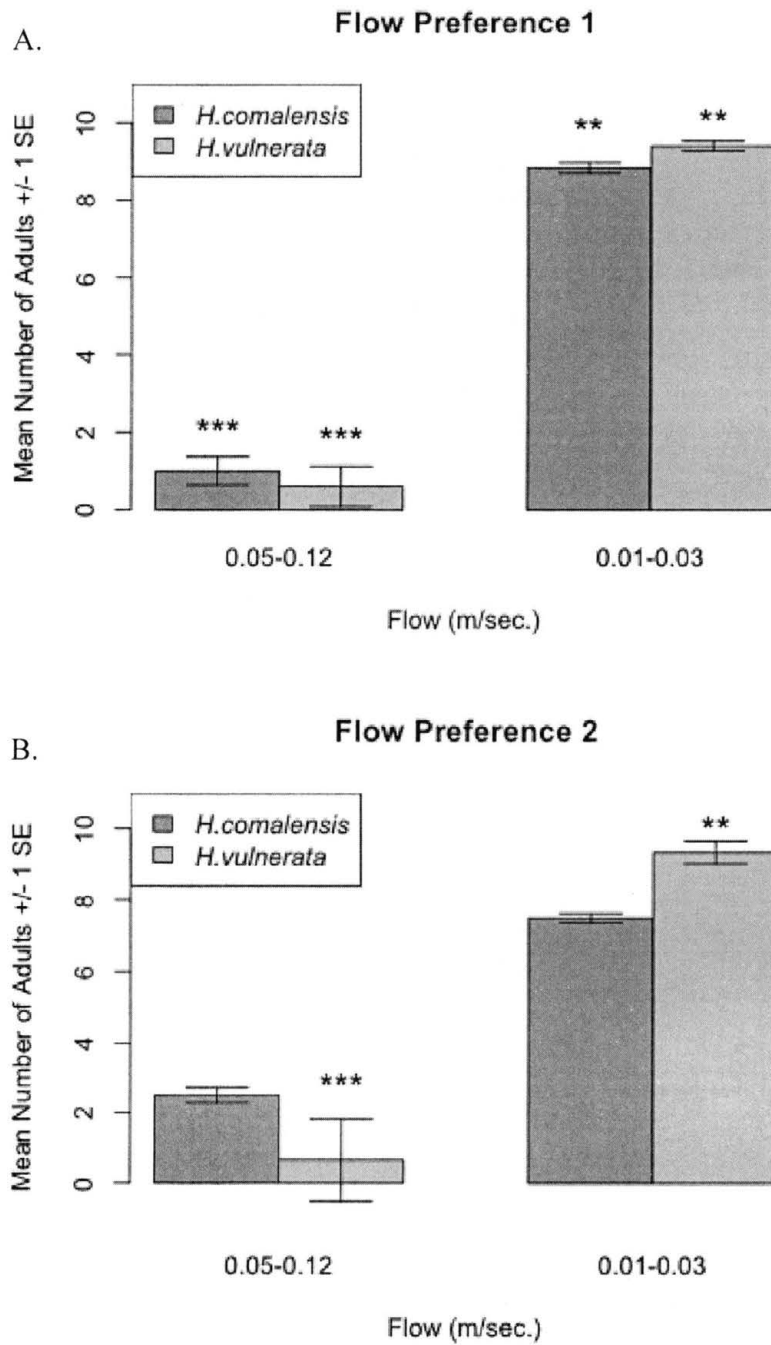


Figure 9. Flow preference for *H. comalensis* and *H. vulnerata*. A) Dataset 1 (*H. comalensis*: G-test, $p=0.0039$, $n=59$; *H. vulnerata*: G-test, $p=0.0004$, $n=50$). B) Dataset 2 (*H. comalensis*: G-test, $p=1$, $n=60$; *H. vulnerata*: G-test, $p=0.0002$, $n=60$). Differences are indicated by asterisks (***) = greatest difference from expected, ** = second greatest difference from expected, * = smallest difference from expected, but still different).

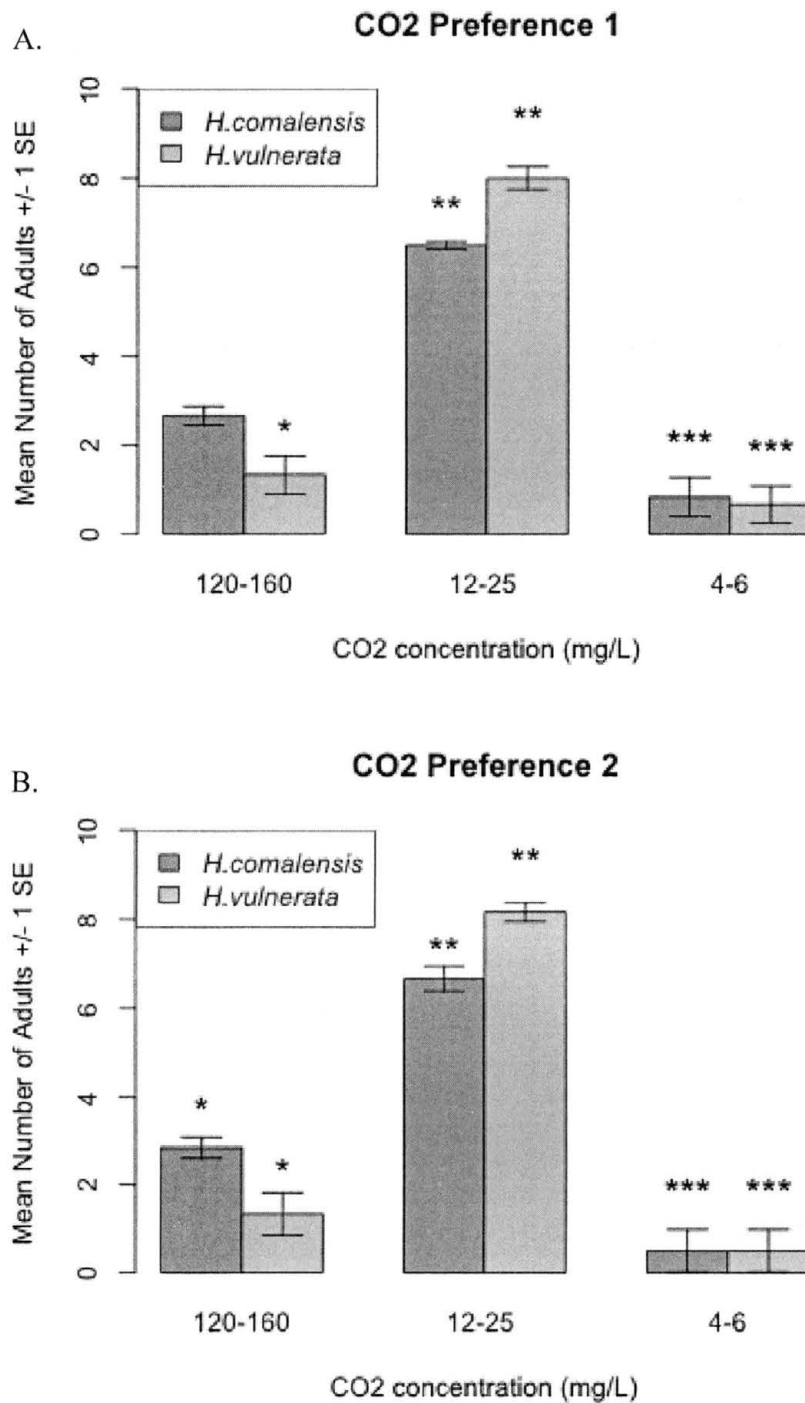


Figure 10. CO₂ preference for *H. comalensis* and *H. vulnerata*. A) Dataset 1 (*H. comalensis*: G-test, $p=0.0031$, $n=60$; *H. vulnerata*: G-test, $p<0.0001$, $n=60$). B) Dataset 2 (*H. comalensis*: G-test, $p=0.0002$, $n=60$; *H. vulnerata*: G-test, $p<0.0001$, $n=60$). Differences are indicated by asterisks (***) = greatest difference from expected, (**) = second greatest difference from expected, (*) = smallest difference from expected, but still different).

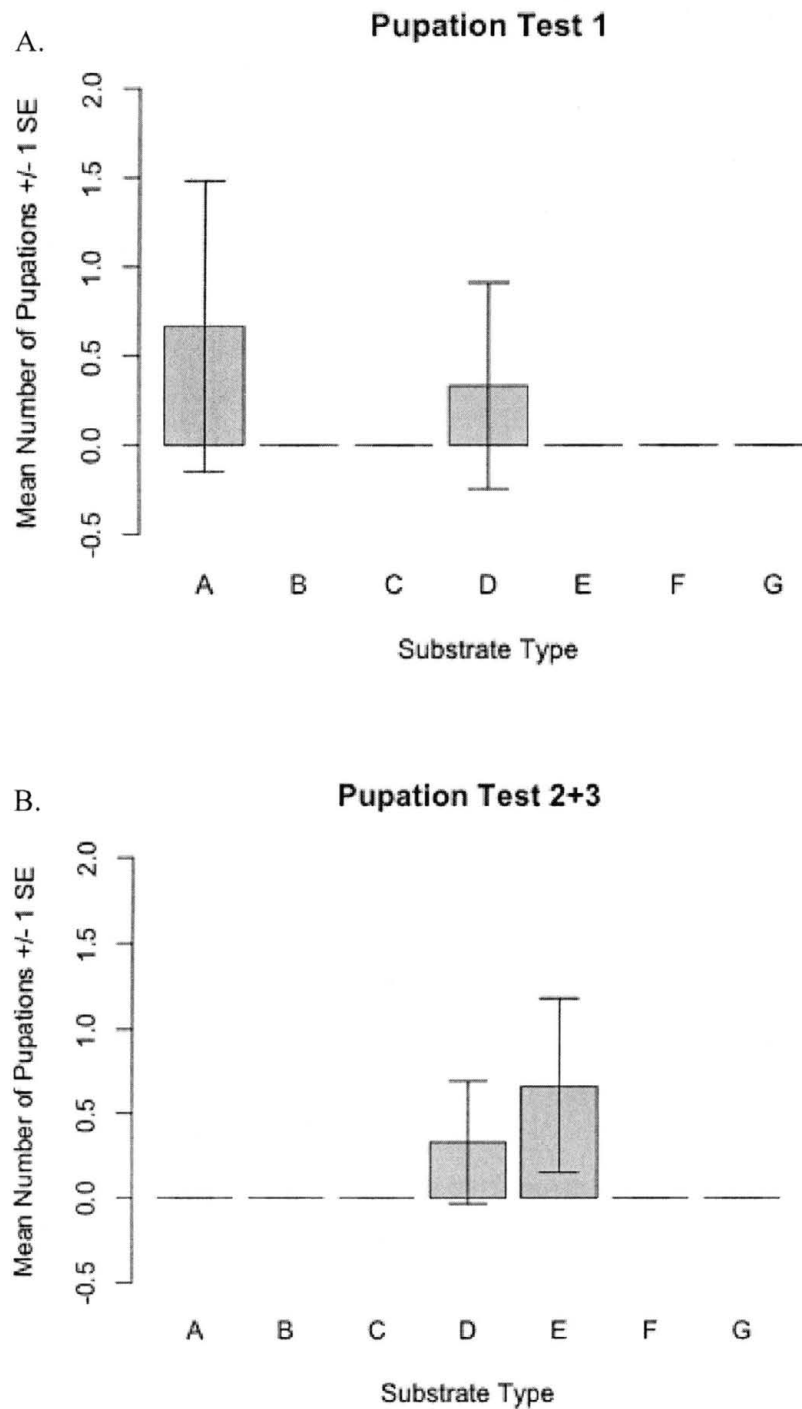


Figure 11. Pupation results for different substrate type tested. A) Test 1 (Friedmans test, $p=0.42$, $n=3$). B) Test 2+3 (Friedmans test, $p=0.1247$, $n=6$). Substrate types: A=Sand, B=Rocks, C=Sand+Rocks, D=Sand and Leaves, E=Rocks+Leaves, F=Sand+Rocks+Leaves, G=Cotton strips.

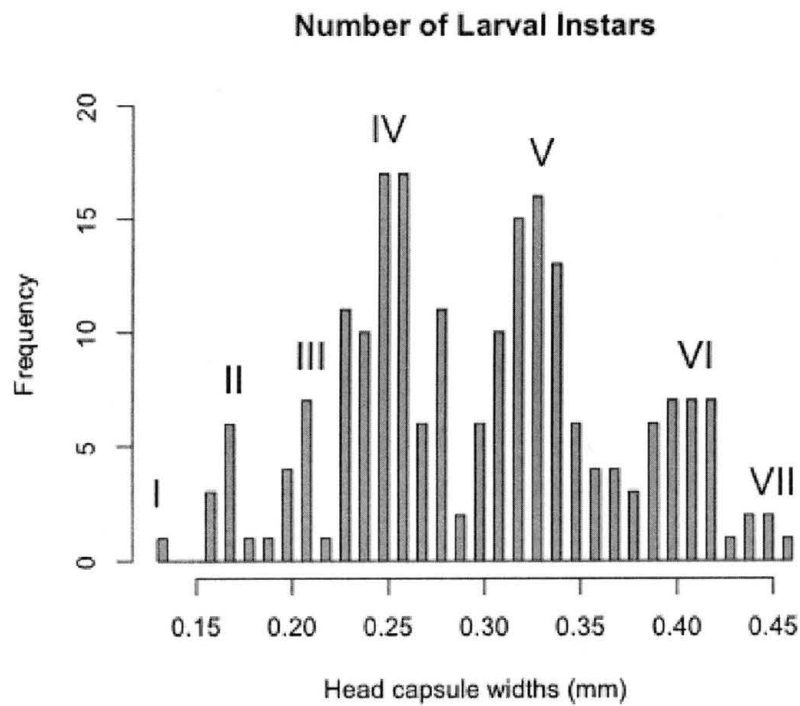


Figure 12. Frequency distribution of head capsule measurements of *H. comalensis* larvae. N=208. Measured were preserved larvae produced at the NFHTC. Larval instars are indicated by roman numerals, as represented by peaks in the distribution.

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