EFFECT OF LOW-FLOW ON RIFFLE BEETLE SURVIVAL IN LABORATORY CONDITIONS

FINAL REPORT

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EXECUTIVE SUMMARY

The Comal Springs riffle beetle *Heterelmis comalensis* (Coleoptera: Elmidae) is a federally endangered aquatic beetle endemic to the Comal and San Marcos springs systems in central Texas. The ecology of *H. comalensis* under low-flow conditions is not well understood, and although the population in Comal Springs survived the 7-year drought of record in the 1950s via unknown mechanism(s), current knowledge of the species' physiology and water chemistry requirements indicates that riffle beetles such as *H. comalensis* may be poorly adapted to periods of drought/low-flow and the attendant increased temperature, decreased dissolved oxygen (DO), and water quality degradation.

A 2014 literature review found little available information on the effect of flow rates on aquatic invertebrate survival, particularly in subterranean or interstitial habitats such as those inhabited by *H. comalensis*. In this report we describe a series of novel experiments examining the effects of extended low-flow periods on riffle beetle survival and water quality under laboratory conditions. Experiments were conducted in the wet laboratory at the Freeman Aquatic Building at Texas State University using a custom-built Riffle Beetle Aquifer Simulation System (RBASS) aquarium unit constructed especially for these studies (shown in the cover photo). The RBASS was engineered to create "spring upwelling" mesocosms that provide water quality and light conditions simulating those found in riffle beetle habitats in the Comal Springs/River system. Riffle beetles are so small and logistically difficult to observe in the field that current understanding of their responses to environmental stressors is largely presumptive and derived from anecdotal evidence suggested by researchers familiar with the system. The introduction of the RBASS, which allows controlled experimentation in an upwelling environment with the application of several replicates and/or several different treatments simultaneously, is itself a major accomplishment of this study.

Initial study plans provided for the use of either *H. comalensis* or other surrogate species. The main study described here uses *Heterelmis glabra*, a riffle beetle closely related to the *H. comalensis*, as a surrogate. Several other riffle beetle species were used in the series of pilot studies that preceded the main study, including *H. comalensis*, *H. glabra*, and *Microcylloepus pusillus*. During the course of the project, two independent and unexplained mortality events with *H. comalensis* led to the use of *H. glabra* for the formal experiment to avoid risking further permitted take of an endangered species. The latter mortality event, which involved concurrent unexplained mortality of *H. comalensis* and *Heterelmis vulnerata* used in separate Habitat Conservation Plan (HCP) applied research projects (conducted by different principle investigators but within the same laboratory system), is concerning. Investigations into these events are underway, and research to compare conditions biologically at multiple laboratories has been proposed for 2015. The difficulties encountered in these efforts are illustrative of the general absence of life history and husbandry knowledge concerning these species.

Overall, the pilot studies provided a wealth of preliminary information on substrate use, handling stress, water temperature acclimation, surrogate suitability, movement, and various responses to environmental stimuli. In the formal experiment with *H. glabra*, flow conditions were found to have significant riffle beetle mortality effects under laboratory conditions. In that study, we observed higher riffle beetle mortality in no-flow conditions than in flow conditions. We also

found significant associated effects between flow and DO (mg/L) and flow and water temperature (°C).

Recommendations for Future Applied Research

For a variety of reasons, both *H. comalensis* HCP applied research projects conducted in 2014 (e.g., low-flow and plastron studies) used aquatic invertebrate surrogates (e.g., *M. pusillus*, *H. vulnerata*, and *H. glabra*). Such use of surrogate species broadens research horizons and remains an extremely attractive avenue toward further understanding the ecology of *H. comalensis*. However, events that occurred during the series of 2014 study trials described in this report (including unexplained *H. comalensis* mortality in a laboratory setting) raise concerns regarding the applicability of surrogate species for *H. comalensis*. Fortunately, a HCP applied research project to target the key questions related to the determination of appropriate surrogate species, as well as to investigate the importance of both horizontal and vertical connectivity to surface habitat for *H. comalensis*, has been approved for 2015.

In addition to the scheduled 2015 HCP applied research studies, we see high potential value in building upon the knowledge gained in 2014 in order to better refine our understanding of the effects of flow and water quality on adult and larval riffle beetle survival and adult movement.

Acknowledgments

The project team would like to acknowledge the U.S. Fish and Wildlife Service (USFWS) San Marcos Aquatic Resource Center (SMARC) scientists and staff for their guidance during this study. We especially appreciate SMARC's generosity in providing *H. glabra* on short notice, which was instrumental in the completion of the main experiment described in this report.

1.0 INTRODUCTION

The Comal Springs riffle beetle *Heterelmis comalensis* (Coleoptera: Elmidae) is a federally endangered aquatic beetle endemic to the Comal and San Marcos springs systems in central Texas. Current species range is restricted to the headwaters of the Comal and San Marcos springs, as well as to areas of seeps and upwellings from the Edwards Aquifer within Landa Lake in the Comal system (Bowles et al. 2003, BIO-WEST 2002, Gibson et al. 2008, Norris and Gibson 2013).

H. comalensis is a small, flightless riffle beetle that requires aquatic habitat throughout its life history (Bosse et al. 1988, Brown 1987). Like other elmid beetles, *H. comalensis* is understood to prefer spring habitats featuring high-quality water flowing over firm substrates with little-to-no silt cover and relatively uniform temperature, dissolved oxygen (DO), and pH levels (Brown 1987, USFWS 1997, Bosse et al. 1988, Crowe and Sharp 1997, Gibson et al. 2008, Bosse 1979). Riffle beetles such as *H. comalensis* are considered to be sensitive to deteriorating habitat conditions, probably due in large part to their physical respiratory mechanism: riffle beetles respire via a plastron of specialized hydrofuge setae with which they maintain a film of air over a large portion of their body surface (Brown 1987, Brown 1972). This mode of plastron respiration makes most elmid species vulnerable to changes in the specialized habitats conditions to which each is adapted (Brown 1987). In the case of *H. comalensis*, the event most likely to precipitate such a deterioration of habitat is reduction in system flow.

Reduced system flow often causes deterioration of habitat and water quality, *e.g.* increased temperature, decreased dissolved oxygen, and loss of vegetation (Flecker and Feifarek 1994). Although *H. comalensis* in Comal Springs survived the 7-year drought of record (DOR) in the 1950s by unknown mechanism(s), knowledge of the species' habitat requirements suggests that *H. comalensis* may be particularly vulnerable to detrimental effects during periods of extended severe low flow (Bowles et al. 2003, Gonzales 2008, Nice 2008). The present HCP flow regime projects periods of extended drying of the spring runs as well as drying of areas along the western shoreline and Spring Island in the Comal System, all of which are known to be key habitat for *H. comalensis* (Bowles et al. 2003, BIO-WEST 2002, Gibson et al. 2008, Norris and Gibson 2013). Although the HCP flow regime is not projected to be as severe on the minimum end as experienced in the DOR, the extended periods of <100 cfs projected in the HCP are well beyond what was observed historically (EARIP 2011).

One vital component of the HCP is applied research. The objective of HCP applied research is to fill in critical data gaps for the covered species and to answer key questions posed in the HCP in order to inform future management decisions and possible adaptive management solutions. To this end, in this document, we describe a series of experiments examining the effects of low-flow periods on riffle beetles under laboratory conditions.

We examine the effects of flow cessation on riffle beetle survival, physical parameters (*e.g.* temperature), and chemical properties (e.g., DO) in isolated upwellings no longer connected to spring water flows. We also describe a series of pilot studies that preceded the main experiment and covered subjects such as effects of substrate type and effects of handling stress on riffle beetle survival. Experiments were conducted at the wet lab at the Freeman Aquatic Building (FAB) at Texas State University.

Initial study plans provided for the use of either *H. comalensis* or surrogate species. The main study described here uses *H. glabra*, a riffle beetle closely related to *H. comalensis*, as a surrogate (Gonzales 2008), and several other riffle beetle species were used in the series of pilot studies (e.g., *H. comalensis*, *H. glabra*, and *Microcylloepus pusillus*). *H. glabra* was considered a likely candidate for a surrogate because it is found in habitats similar to *H. comalensis* and recent population genetics research indicates that *H. comalensis* evolved from an isolated population of *H. glabra* roughly 0.50 million years ago (Bosse et al. 1988, Gonzales 2008). *M. pusillus* was considered a candidate for a surrogate because it is also a related riffle beetle (Elminae subfamily) that has been observed to inhabit the general same areas and habitats of Comal Spring as *H. comalensis* (Bowles et al. 2003).

Prior to initiation of the study, an extensive literature review was conducted relating to riffle beetle food sources, riffle beetle habitat baseline and water quality conditions at average flows and low flows, and riffle beetle population characteristics at average and low flows. This literature review, summarized in Section 2, built upon the comprehensive literature review conducted as part of the HCP Ecological Modeling project that was submitted separately to the Edwards Aquifer Authority (EAA) in October 2013 (EAHCP 2013). Section 3 provides information on the study design, describes the methods used, and presents challenges observed during implementation of these studies. Results are provided in Section 4, followed by conclusions and recommendations in Section 5. Finally, Section 6 lists the references cited throughout the document.

2.0 LITERATURE REVIEW

Disappearance of surface flow in desert systems disconnects subsurface and surface habitats and has been observed to disrupt typical ecological processes in stream systems by changing species composition (Valett et al. 1992). Flow decreases also affect species composition of subsurface fauna in varying ways (Boulton and Stanley 1995). One such example is found in a 2008 study of the long-toed water beetle, *Postelichus immsi* (Coleoptera:Dryopidae), in an Arizona desert stream. When subjected to drying conditions, the beetles exhibited drought-escape adaptive behavior by moving away from drying areas and toward areas with higher flow at a rate faster than the drying rate (Lytle et al. 2008). However, while much work has been done on the impact of low-flow conditions and critical drought stages for surface taxa (e.g., Wright et al. 1994, Harrison 2000, Williams 1977), the same cannot be said for subsurface species: we were unable to locate any studies that determine a critical level of drought at which subterranean or hyporheic taxa are most at risk (Boulton 2003).

H. comalensis is endemic to the Comal and San Marcos springs systems in Central Texas, where it inhabits interstitial areas at spring upwellings of stream and lake beds (Cooke 2012). A member of the subfamily Elminae (the most aquatic of all riffle beetles), *H. comalensis* is flightless and requires aquatic habitat during every stage of its life cycle (Brown 1987, Bosse et al. 1988). The morphology of *H. comalensis* does not display adaptations typical of stygobionts (obligate subterranean organisms), such as reduced eyes and pigmentation. The Comal Springs population of *H. comalensis* survived the 7-year DOR in the 1950s (including a 143-day period of cessation of spring flow) via unknown mechanism(s), which may indicate an ability to survive inside the springs as well as in the interstitial habitat (Bowles et al. 2003). Elmids, like other aquatic invertebrates, are subject to detrimental effects when subject to conditions of excessive decreases in flow and water levels, as these are often accompanied by changes to normal physical and chemical properties of the water (Boulton 2003).

H. comalensis inhabits interstitial areas near spring upwellings of stream and lake beds, where they cling to substrates using specialized long tarsi and large tarsal claws (Brown 1987, Cooke 2012, Burke 1963). The distribution of *H. comalensis* is currently considered to be confined to the headwaters of the Comal and San Marcos springs and in areas of seeps and upwelling from the Edwards Aquifer within Landa Lake (Bowles et al. 2003, BIO-WEST 2002, and Gibson et al. 2008). The species typically inhabits spring and upwelling areas containing hard-packed rock and gravel substrates, swift flow, and little to no vegetation or silt (USFWS 1997, Bosse et al. 1988, Crowe and Sharp 1997). A 2002 survey of *H. comalensis* on the shoreline of Landa Lake found that all observed beetle habitat contained spring flow/seeps with flows ≤0.20 m/sec (BIO-WEST 2002). Microhabitat preferences are not well understood (Gibson et al. 2008).

Little is known about the life history of *H. comalensis*, but many elmid beetles tend to be long-lived, with accounts of some individuals surviving for several years (Brown 1987). Additionally, like other elmids adapted to spring habitats, *H. comalensis* may feature overlapping generations within populations, as pupae found in quarterly samples suggest non-seasonal emergence patterns (Bowels et al. 2003, Shepard 1990). Knowledge of feeding habits of *H. comalensis* is limited, but they are thought to be "detritivore-herbivores" that feed primarily on detritus and (probably fungal) biofilms (Brown 1976, Gibson et al. 2008), as are other riffle beetles (Seagle 1982, Elliott 2008), although microorganisms and decaying roots are also considered food sources for *H. comalensis* (USFWS 2007). The degree to which the diet of *H. comalensis* relies on leaf litter from surface habitats is currently unknown (USFWS 2007).

Some elmid species (e.g., *Macronychus glabratus* and *Stenelmis crenata*) are known to be able to survive several years in inhospitable conditions (e.g., no flow and very small enclosures) while in captivity, regardless of the fact that these species are understood to prefer highly oxygenated flowing water, as *H. comalensis* presumably does. Furthermore, *S. crenata*, which, like *H. comalensis*, uses plastron respiration (Thorpe and Crisp 1949), survived in very poor laboratory conditions (i.e., corked glass vial with no food or water changes) for between 394–398 days (Brown 1974). We are aware of only one study that presents information on *H. comalensis* movement in response to drought stimuli, or of their ability to survive underground within springs as flows decrease and physical and chemical properties of water are altered. In order to test the hypothesis that *H. comalensis* possess adaptations that allow them to inhabit the springs during periods of drought/low flow, the EAA sponsored a 2002 study that examined the response of *H. comalensis* and *M. pusillus* to alterations in flow regimes. The results of that study suggest that both *H. comalensis* and *M. pusillus* have a preference for flowing water and that they preferentially orient and move toward, not away from, a flow stimulus, indicating a possible adaptation for surviving drought conditions by moving underground along spring upwellings toward flowing water within the aquifer (BIO-WEST 2002).

Ultimately, the ecology of *H. comalensis* under low-flow conditions is not well understood. Existing research suggests that riffle beetle species are poorly adapted to long periods of dry conditions (up to several years in duration) and that extended drought has the potential to be highly detrimental to riffle beetle populations (Bowles et al. 2003, Gonzales 2008, Nice 2008). Current knowledge of the species' physiology and water chemistry requirements indicates that riffle beetles such as *H. comalensis* may be poorly adapted to periods of drought and the increased temperature, decreased DO, and degradation of water quality that accompanies low flow.

Adult *H. comalensis* utilize a plastron of specialized hydrofuge setae to obtain oxygen from the water and maintain a layer of air around their body, ultimately letting them remain submerged indefinitely in conditions with appropriate water pressure and nearly saturated DO levels. Larval *H. comalensis* do not rely on plastrons to respire; this fact is a possible explanation for the species' survival of the DOR in the Comal system. This reliance on plastron respiration is thought to be the root cause of riffle beetles' sensitivity to deterioration in water quality: riffle beetles require high-quality water containing minimal-to-no pollution, and dissolved salts, trace elements, pesticides, soaps and detergents and other compounds containing surfactants, heavy metals, fertilizer nutrients, petroleum hydrocarbons, pharmaceuticals and veterinary medicines, and semi-volatile compounds, such as industrial cleaning agents are all considered threats to their fragile respiratory mechanism (Brown 1987, USFWS 2007). In addition to the adverse effects pollution exerts on water quality suitability in riffle beetle habitats, water level decreases are often associated with declining water quality: affected parameters include increased temperature, decreased DO, (Flecker and Feifarek 1994), loss of submerged vegetative habitats necessary for food and shelter (Ormerod et al. 1987), and concentration of aquatic organisms in isolated pools, which alters the normal community structure of benthic invertebrates (Flecker and Feifarek 1994).

Our literature review found no reports of experiments in which flow rate was used to predict invertebrate survival. The study we describe in this report attempts to bridge this knowledge gap by using novel "spring upwelling" mesocosms to study the effect that flow rate exerts on riffle beetle survival in a laboratory setting. The vertical flow regimes in the laboratory mimic periods of low flow such that spring upwellings no longer connect the subterranean and surface habitats that *H. comalensis* likely inhabit. We also examine the ways in which physical (i.e., temperature) and chemical properties (e.g., DO, pH, conductivity) of spring water change as flow velocity decreases and how beetle survival is related to these properties.

3.0 PILOT STUDIES AND STUDY DESIGN, METHODS, AND IMPLEMENTATION

3.1 Study Chamber Design

Water used in this study originated from a series of water lines that connected the study chamber intake to a common spring water source located in a tank on the second floor of the FAB. The water supply was untreated Edwards Aquifer water from a partially capped artesian well located adjacent to the FAB.

3.1.1 Refugium Tanks

Two refugia tanks were constructed based on the designs of tanks used to house *H. comalensis* at the U.S. Fish and Wildlife Service (USFWS) San Marcos Aquatic Resource Center (SMARC). Polyvinyl chloride (PVC) piping with nine holes drilled along the length was installed at the top of the refugium tanks and connected to the wet lab's water source to allow constant, low-level flow to enter the refugia from the top at several points. Four PVC drains capped with fine mesh were installed in the bottom of the refugia tanks to facilitate constant outflow. Tanks were stocked with boiled limestone rocks, nylon mesh substrate, and air-dried leaves from trees growing along the banks of Comal Springs (Figure 1). Refugia were covered with a non-light-penetrating polyethylene tarp to block light, mimicking the darkness typical of subterranean and/or interstitial spring habitats.

Figure 1. Refugium tank, showing water flowing in through the top and draining out through the bottom, with rocks, mesh and leaf substrate.

3.1.2 Riffle Beetle Aquifer Simulation System

In order to conduct riffle beetle applied research for a wide variety of possible experiments, a self-contained hydraulic simulation system was designed and identified as the Riffle Beetle Aquifer Simulation System or "RBASS" (Figures 2–4). The primary objectives considered in the construction of the RBASS were to create a once-through flow hydraulic system that was both inert in composition and completely self-contained. The term "once-through flow" refers to the one-time use of source water that enters the system and exits without any reuse or recirculation. The term "self-contained" refers to the ability to host aquatic insects in multiple individual chambers and prevent escape. The RBASS can be divided into the following two main components: (1) the Hydraulic Distribution Arena (HDA) and, (2) the Experimental Flow Chambers (EFCs). The following narrative describes the construction and function of this system.

Figure 2. Photograph of RBASS system setup at the at Texas State University Freeman Aquatic Building laboratory showing six RBASS environmental flow Chambers, upwelling flow spigots, and black nylon cover.

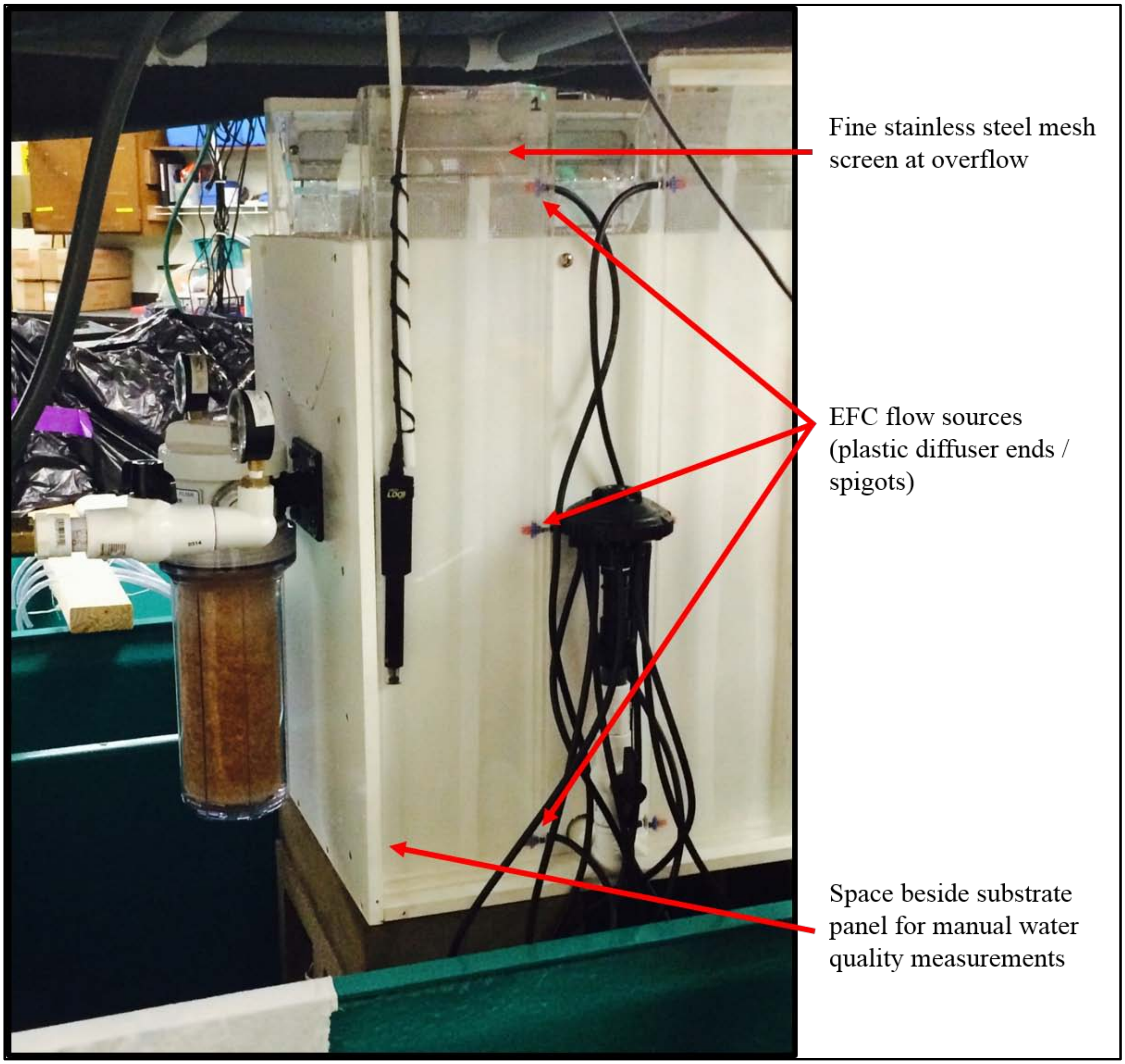

Figure 3. **Photograph of RBASS environmental flow chamber design showing flow spigots and mesh screen at chamber overflow point.**

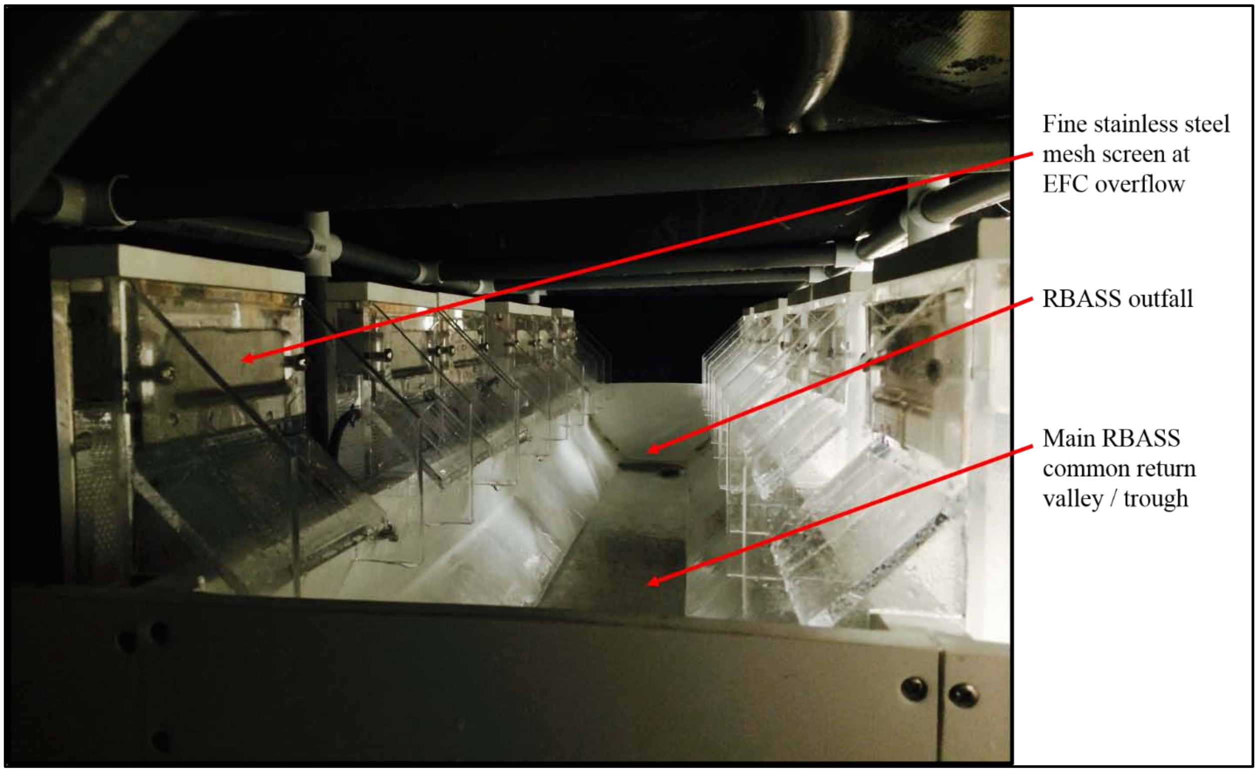

Figure 4. Photograph of RBASS trough/valley design showing interior sides of mesh screen at chamber overflow point.

Hydraulic Distribution Arena (HDA)

The purpose of the HDA is to distribute aquifer source water to numerous self-contained flow chambers and to act as a containment arena and visual flow return valley. This objective was accomplished by constructing a complex, variably controlled plumbing system housed within a highly visible support structure.

Flow

The hydraulic or once-through flow plumbing system starts with a 0.75-inch female hose fitting that can receive a standard garden hose end for source water. A PVC ball valve regulates flow into the system and is followed by a food-grade 50-micron polypropylene sediment depth filter. This filter is fitted with a diverter valve that enables the system to either utilize or bypass the filter unit. In addition to a clear housing for visual filter inspection, the system includes a pre- and post-filter water pressure gauge to identify pressure differentiation, which might indicate a clogged filter. The 50-micron filter system also provides secondary containment and visual detection of an aquatic beetle that might escape the flow chamber and travel upstream. After the filter system, 0.75-inch PVC pipe distributes the water to six PVC ball valves, each positioned between two flow chambers. From that point the water is distributed through a plastic manifold providing flow variations at three independent locations in each flow chamber. Water is expelled from the top of the flow chambers into a common return valley, which is drained through a 117-micron stainless steel wire mesh. The water level in the return valley can be regulated by the last PVC ball valve exiting the structure.

Construction
In addition to the aforementioned plumbing infrastructure, the HDA housing is constructed of white, 13-mm-thick expanded closed-cell PVC. The structure is held together with stainless steel screws and (where sealant is necessary) aquarium-grade silicone. The common return valley is removable from above to allow future plumbing access and to second as storage.

Experimental Flow Chambers (EFCs)
The EFCs is constructed of 0.24-inch clear acrylic measuring 24 inches high, 6 inches wide, and 0.75 inches deep (dimensions of open space between acrylic panel walls). Chamber seams are chemically fused (or "welded") for a watertight seal using Weld-On 4, an acrylic adhesive commonly used in aquarium construction. Water inlet ports are bored through the acrylic material along one side of the flow chamber near the bottom, middle, and top. These ports allow 0.25-inch tubing to be inserted and capped with a tapered, threaded plastic diffuser end/spigot. The plastic tapered end allows for a compression seal between the tubing and acrylic, while the diffuser not only evenly distributes flow, but also prevents aquatic beetles from escaping from the EFC into the source water. A 1.50-inch by 4.0-inch opening is cut into the acrylic approximately 1 inch from the top to allow water to exit the chamber before overflowing. At this opening, a 117-micron T304 stainless steel mesh is secured by a removable compression plate to allow for cleaning and any necessary future replacement. Outside of the ECF, an acrylic shelf is affixed below and on the sides of this opening to divert outflow water into the HDA return valley. Lastly, an acrylic insert topped with closed-cell PVC was used as a sealed removable top.

A total of 12 EFCs were constructed to allow multiple replicate samples and flow variations. During experimentation, a total of six EFCs could be placed on each side of the HDA.

Ambient Light Enclosure
In order to mimic aphotic aquifer conditions, a cover was constructed to prevent ambient light exposure to the RBASS (Figure 4). This cover was constructed from a black, non-light-penetrating, vinyl-reinforced nylon, which is supported by a 0.75-inch PVC sub-frame. Four independent panels are sewn into each side of the cover to allow access to the RBASS at selected locations. Each panel is sewn together at the top and secured laterally by Velcro seams and extended to the facility floor.

3.2 *Microcylloepus pusillus* Substrate Pilot Study 1

3.2.1 Study Rationale
Microcylloepus pusillus was chosen as a surrogate for *H. comalensis* in initial trials because *M. pusillus* occurs sympatrically with *H. comalensis* at several spring outflows within the Comal system, and is often found on the same cloth lures as *H. comalensis* during regular field monitoring and collecting events (BIO-WEST 2014, unpublished data). The use of a surrogate was warranted for initial tests in order to minimize take of *H. comalensis* from the Comal system. The first trial was designed to simultaneously test the hypotheses of (1) no difference in survival of *M. pusillus* between the RBASS unit and the refugium and (2) no difference in survival or detectability of *M. pusillus* between two different types of substrate within the RBASS unit. Substrate choices were made based on the availability of substrate material, the transparency of the material, and commonly used substrates for scientific studies in aquaria

systems for other local spring species, such as the San Marcos, Texas blind, and Barton Springs salamanders.

3.2.2 Study Design

Twenty-three *M. pusillus* were collected from cotton cloth lures at Comal Springs and placed in one of the refugium tanks for a 48-hour acclimation period. Following the acclimation period, eight beetles were transferred to an RBASS EFC filled with glass marbles of uneven sizes and nine were transferred to an RBASS EFC filled with crystal beads of uniform size (Figure 5). After 24 hours, visual surveys were conducted to determine the detectability of beetles within the RBASS EFCs containing different types of substrate, and then EFCs were emptied in order to assess whether the beetles could withstand the physical forces of substrate removal. Survival rates were recorded for both EFCs and for the refugium.

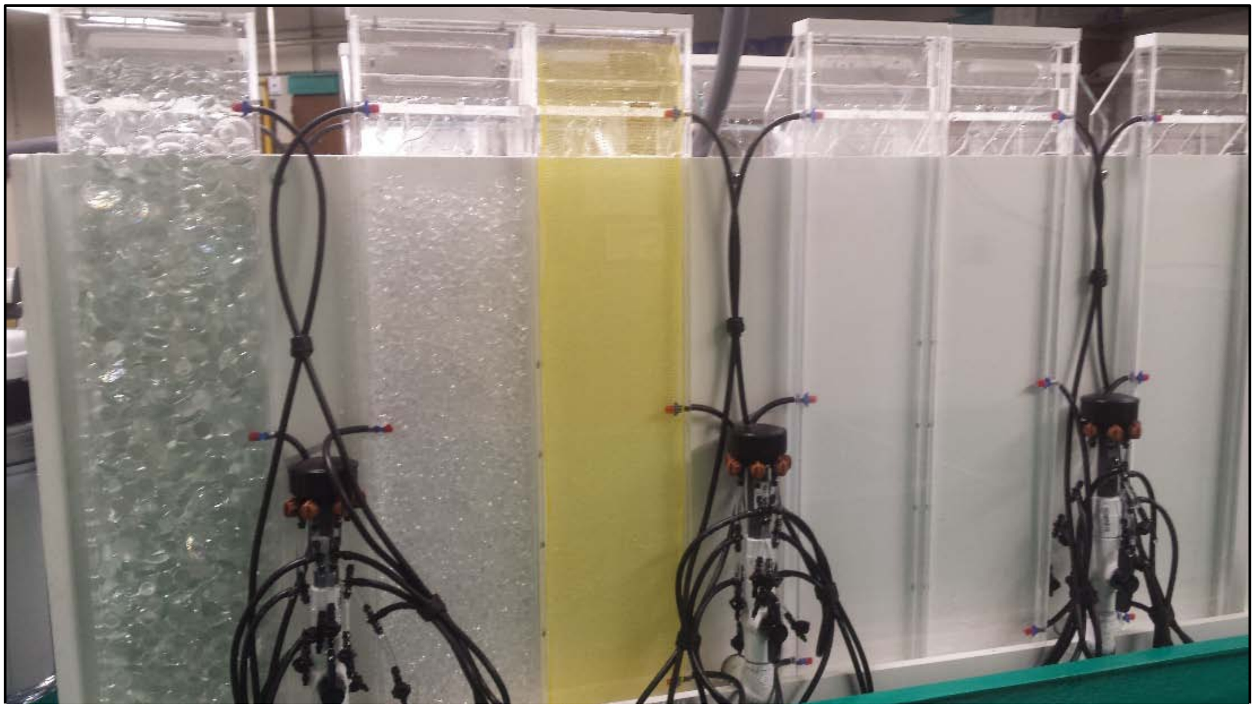

Figure 5. Photograph of RBASS system showing environmental flow chambers with different substrates tested in the Microcylloepus pusillus Substrate Pilot Studies.

3.2.3 Study Outcome

In the RBASS EFC with marbles of uneven sizes, 88% (n=7) *M. pusillus* were readily observed in the tank during visual inspection. In the RBASS EFC with uniformly sized crystal beads, 57% (n=6) *M. pusillus* were observed in the tank during visual inspection. Detection increased to 100% for both EFCs when substrate was removed and sorted. There was no observed difference in beetle survival between either the substrate treatments or the refugium, and there was no observed mortality in any of the three treatments.

Visual detection of beetles was higher in the uneven marble substrate; however, this substrate was problematic to remove from the RBASS EFCs. The glass differently sized marbles and uniformly sized crystal beads were both abandoned as substrates due to low visual detection rates and/or due to the difficulty of removing substrates from the EFCs. Although all of the beetles survived, the risk associated with substrate removal and manipulation, especially for the uneven marble substrate, was high due to the force required to remove the substrate from the EFCs.

3.3 *Microcylloepus pusillus* Substrate Pilot Study 2

3.3.1 Study Rationale

Microcylloepus pusillus was again used as a surrogate. The first trial revealed that visual detection of beetles was higher in the uneven marble substrate; however, this substrate was problematic to remove from the RBASS EFCs. Due to the complications and risks associated with using the large marbles and beads tested in the previous study, we proposed to test different sizes of mesh material for appropriateness as a substrate during riffle beetle studies. The second trial was designed to test the hypothesis of no difference in detectability of *M. pusillus* between two different types and configurations of mesh substrates within the RBASS unit. Substrate choices were made based on the transparency of the material and commonly used substrates for scientific studies in riffle beetle aquaria at the SMARC (R. Gibson, personal communication, 2014).

3.3.2 Study Design

Additional *M. pusillus* were collected from Comal Springs and added to the refugium tanks. Ten beetles were transferred to an RBASS EFC with two layers of stiff, large-diameter yellow mesh and ten were transferred to an RBASS EFC with five layers of fine, flexible, white mesh (Figure 5). After 24 hours, visual surveys were conducted to determine the detectability of beetles within the RBASS EFCs with the different types of substrate.

3.3.3 Study Outcome

In the RBASS EFC with two layers of stiff, large-diameter yellow mesh, 60% (n=6) *M. pusillus* were readily observed in the tank during visual inspection. In the RBASS EFC with five layers of fine, flexible, white mesh, 100% (n=10) *M. pusillus* were observed in the tank during visual inspection. Beetle distribution followed a clumped pattern orienting near the spigots in each EFC (Figure 6). There was no difference in beetle survival between either substrate; there was zero mortality across both treatments.

3.4 *Microcylloepus pusillus* Substrate Pilot Study 3

3.4.1 Study Rationale

Microcylloepus pusillus was again used as a surrogate. The second trial revealed that visual detection of beetles was higher on the white mesh substrate, so this substrate was used exclusively for the third trial. The third trial was designed to test the hypothesis of no difference in survival of *M. pusillus* between flow and no-flow treatments.

Figure 6. **Photograph of RBASS EFCs showing the clumped spatial distribution of *M. pusillus* in areas orienting toward the source of flow. Small black dots are live beetles, while large blue dots are sharpie marks denoting beetle locations on previous days.**

3.4.2 Study Design

Six RBASS EFCs were configured with four layers of fine white mesh substrate. Ten beetles were transferred from the refugium into each of the six EFCs for a 24-hour acclimation period. Following the acclimation period, flow to two EFCs was manually turned off; one beetle was undetectable in one of the no-flow EFCs at this time, thus the study was conducted with n=40 beetles exposed to flow treatment and n=19 beetles exposed to no-flow treatment. Visual surveys were conducted every 48 hours over ten days (five observation periods – numbered 1 to 5 in Table 1) to assess beetle survival between flow and no-flow treatments. Clean cotton cloth lures were added to one flow and one no-flow EFC after the third observation period.

3.4.3 Study Outcome

By the end of the 10-day trial, the flow treatment had lower survival overall than the no-flow treatment. Eighteen percent (n=7) of *M. pusillus* in the flow treatment were active and 63% (n=12) of *M. pusillus* in the no-flow treatment were active. Beetles were not observed on the cotton cloth lures at any time.

Table 1. ***Microcylloepus pusillus*** **activity in flow and no-flow treatments over 10-day observation period.**

48-HOUR OBSERVATION PERIOD	ACTIVE:INACTIVE (FLOW)	ACTIVE:INACTIVE (NO-FLOW)
1	39:1	17:2
2	36:4	16:3
3	30:10	16:3
4 [a]	10:30	12:7
5 [a]	7:33	12:7

[a] cotton cloth lures added.

3.5 Heterelmis comalensis Food vs. Flow Pilot Study

3.5.1 Study Rationale

Results of previous surrogate trials suggested that the most effective substrate of those tested for use in the RBASS EFCs was fine white mesh and that differences in flow and no-flow treatments on beetle activity were detectable. Additionally, the observed absence of beetles on the clean cloth lures used in the preceding study contrasted with our many field observations of wild riffle beetles congregating on similar cloth lures left *in situ* in the Comal system. This prompted the decision to pre-inoculate cotton cloth lures via prolonged submersion in the refugia tank in order to culture organic matter and/or food organisms for the beetles.

This study was designed to test two hypotheses relating to *H. comalensis* responses to varying food and flow regimes; the null hypotheses being that there is no difference in survival of *H. comalensis* between flow and no-flow treatments and between food and no-food treatments.

3.5.2 Study Design

On 24 July 2014, BIO-WEST formally received an amendment to federal endangered species permit number TE037155-0, which allows collection of *H. comalensis* from the wild and use of the same for experimentation at the FAB. On 25 July 2014, 67 *H. comalensis* were collected from Comal Springs using collection techniques developed by researchers at the SMARC to collect wild *H. comalensis* for stocking the refugia at that facility. Beetles were brought immediately to the FAB and placed in the refugia for a 48-hour acclimation period. Survival within the refugium was tallied on 28 July 2014 and the beetles were then left alone for an additional 24 hours.

The RBASS unit was set up to implement a 2x2 factorial design in which two levels of food availability (presence or absence of a cloth substrate inoculated with organic material) were cross-classified with two levels of flow rate (presence or absence of upwelling flow in RBASS EFCs). On 29 July 2014, five *H. comalensis* were placed in each of 12 RBASS EFCs with a substrate of four equal-sized panels of fine white mesh layered together in each EFC. Six EFCs had a strip of an already inoculated cloth lure attached to the substrate. With the water supply to the RBASS unit turned on, each EFC received 200–250 mL water per 30 seconds (approximately 7 mL/s). Beetles were acclimated in the RBASS EFCs for 48 hours, after which point flow to six EFCs (three with food, three without food) was turned off.

Qualitative beetle spatial location data was recorded by marking observed beetle positions within RBASS EFCs both in a logbook chart and on the clear acrylic EFC surface itself (Figure 6). However, limitations of this method include: (1) the impossibility of identifying individual beetles (and thus their movement) and (2) difficulty in ascertaining whether some beetles were dead or simply inactive when making observations through the EFC surface (this determination is best performed once beetles have been removed from EFCs and can be examined closely). Because of these limitations, we found only limited opportunity for analysis of beetle movement patterns, and have restricted the use of this data to qualitative discussion and observations only.

3.5.3 Study Outcome

High mortality (82%; n=49) was observed during the first observation period on 2 August 2014 (nine days after initial collection). All *H. comalensis* were removed from the RBASS, and live individuals were moved to the refugium in an effort to minimize additional mortality; however, no live *H. comalensis* were observed in the refugium on 13 August 2014.

3.6 *Heterelmis comalensis* Handling Stress Pilot Study

3.6.1 Study Rationale

The sudden and unexpected mortality observed in the laboratory population of *H. comalensis* suggested the need for revised handling and testing protocols, with a goal of minimizing stress effects on the animals. Revised methods included no-contact protocols in which beetles were collected and manipulated with fine paintbrushes, pipets, and/or other tools rather than by hand, in the event that skin salts, oils or other handling factors were having an unknown effect on plastron retention or some other aspect of survival.

In addition to handling stress, it was thought that acclimation to water temperature may be necessary to reduce stress in riffle beetles. Although SMARC does not perform water temperature acclimation for riffle beetles brought into their refugia, the average water temperature in Comal Springs that support riffle beetles is ≈23.4 °C, whereas the water temperatures in the FAB refugia tank is on average 22.667± 0.053°C, more similar to water temperature conditions at SMARC. Because there is no literature to support sensitivity of *H. comalensis* to temperature fluctuations, another addition to the protocol was to maximize temperature stabilization during field collecting and to include a serial acclimation period once beetles were at the FAB wet lab.

3.6.2 Study Design

On 21 August 2014, 24 *H. comalensis* were collected from the Comal Springs system. Beetles were collected from cotton lures that had been set *in situ* in Spring Run 3 for 27 days and colonized by local *H. comalensis*. Colonized lures were removed in their entirety, placed in bottles filled *in situ* with local spring water, housed in a two-liter cooler full of local spring water, and transported to FAB within 1 hour in order to minimize temperature fluctuations in the field.

The goal of this trial was to test the hypothesis of no difference in *H. comalensis* survival between those exposed to a serial acclimation period and those placed directly into the refugium. Twelve *H. comalensis* were placed immediately into Refugium 1 by gently pouring the collected beetles, lure, and local spring water directly into spring water and refugium water directly from the collection bottle into the refugium tank. Survival was assessed after 7 days.

3.6.3 Study Outcome

After the 7-day observation period, there was no difference in observed mortality between *H. comalensis* exposed to the serial acclimation method and *H. comalensis* placed directly into the refugia; both refugia tanks contained 10 live and 2 dead individuals. The survival rate of 20 out of 24 (83%) beetles after 7 days is comparable to the success rate observed at SMARC when bringing in *H. comalensis* from the wild (R. Gibson, personal communication, 2014).

3.7 *Heterelmis comalensis* Substrate Pilot Study

3.7.1 Study Rationale

Because previous testing did not support acclimation methods as a cause of increased mortality, and because observations during the high-mortality event suggested that *H. comalensis* may be getting caught in the mesh substrate, another study was conducted to compare responses of *H. comalensis* to varying substrates.

3.7.2 Study Design

Four RBASS EFCs were configured with different substrate treatments, including: (1) a large diameter (1.5cm) plastic grate, (2) 2000-micron nylon mesh, (3) the fine mesh tested in the previous study, and (4) no substrate. Using beetles collected for the previous trial, four *H. comalensis* were placed in each EFC containing a substrate treatment and exposed to similar water quality conditions and flow rates, approximately 2 mL/sec. Four *H. comalensis* were left in the refugia tank as a control. Riffle beetle survival was assessed via visual inspection daily over a period of 10 days.

3.7.3 Study Outcome

For the first several days of the trial, beetles appeared to be responding as expected with differing results unveiling per EFC/substrate type. Beetles were observed to have difficulty in clinging to the large plastic grate substrate, and fell to the bottom when the substrate was disturbed. At a point midway through the experiment, beetle health in all EFCs appeared to deteriorate, regardless of EFC treatment combination; after which point beetles in every EFC showed very little movement. At the end of 10 days, no *H. comalensis* activity was observed in any of the four treatment EFCs. When riffle beetles were removed from the RBASS unit, mortality was observed in 100% (n=4) of beetles in the EFCs containing mesh substrates and in 75% (n=3) of beetles in EFCs containing large plastic grates. Although no mortality was observed in the EFC containing no substrate, the surviving beetles in that treatment appeared inactive and exhibited behaviors that appeared to indicate inevitable mortality in the near future (e.g., listlessness and extremely depressed response to handling stimulus). The refugium tank was then examined; all beetles in the refugium had died during this time period as well.

This second event of near-complete mortality of *H. comalensis* in a laboratory setting raised immediate concerns. Up until this point, the filter apparatus on the RBASS had not been in operation, as the decision was made that it might filter out biologically important components of aquifer water (Birdwell and Engle 2009, Birdwell and Engle 2010, Engle et al. 2010). When this potential water quality concern arose, however, BIO-WEST engaged the filter and flushed the system. The result was extensive rust build-up on the filter and rusted chunks in the unscreened inflow water line (Figure 7). The water quality conditions at the FAB are currently under investigation as to whether this may have been the cause of the unexplained mortality.

Although BIO-WEST's federal endangered species permit (#TE037155-0) allows collection and experimental use of wild *H. comalensis*, it remains necessary to judiciously budget take. In order to avoid risking further permitted take of endangered species, no *H. comalensis* were used for the completion of 2014 activities.

Figure 7. Photographs showing large and small iron oxide particulates found in RBASS unit water system.

3.8 *Heterelmis glabra* Surrogate Flow Study

3.8.1 Study Rationale

Based on the unexpected mortality of *H. comalensis* in the previous study, the study team was left with two choices. The first was to extend the contract and conduct formal experimentation at SMARC. The second was to carry out the final 2014 study with *H. glabra* at the FAB. The former option was deemed unfeasible because of space limitation at SMARC and complexity of extended applied research contracts across calendar years. Therefore, the decision was made to use *H. glabra* at the FAB.

The riffle beetles used in this experiment were 70 *H. glabra* that were given to the project team by researchers at SMARC. SMARC personnel collected these *H. glabra* from Finegan Springs in the Devil's River State Natural Area, Val Verde County, Texas, on either of two sampling events (either June or July of 2014). The beetles had been kept in aquaria at SMARC after collection. As this formal experiment had now morphed into using a surrogate species not from Comal Springs, the decision was made to abandon the food source component of the experiment. Therefore, the experiment focused on flow vs. no flow, and so the design was downsized to using six EFCs.

3.8.2 Study Design

A 28-day experiment was conducted in which *H. glabra* survival was tested at two levels of flow (with vs. without). Treatments were randomly assigned to 6 EFCs (3 EFCs with flow and 3 EFCs without flow). All EFCs were equipped with two layers of 2,000-micron nylon mesh substrate. Visual counts were conducted and observed beetle spatial location was plotted within each EFC every other day. Water quality parameters were recorded every other day at two depths in each EFC (upper and lower). Water quality measurements included flow (L/s), water temperature (ºC), and DO (mg/L), and were taken on the side of each EFC opposite the flow spigots. Additional water quality measurements were logged by thermistors (HOBO® TidbiT v2 Water Temperature Data Loggers) every 10 minutes. Temperature and DO were recorded with a handheld portable meter (HACH® HQ40d multi-parameter meter) using a luminescent optical DO probe (HACH® IntelliCAL™ LDO101 probe). Flow was measured by collecting the volume of water that flowed out the overflow of each experimental EFC during a 30-second interval and recorded to the nearest milliliter.

Qualitative beetle spatial location data was recorded by marking observed beetle positions within RBASS EFCs both in a logbook chart and on the clear acrylic EFC surface itself (Figure 6). However, limitations of this method include: (1) the impossibility of identifying individual beetles (and thus their movement) and (2) difficulty in ascertaining whether some beetles were dead or simply inactive when making observations through the EFC surface (this determination is best performed once beetles have been removed from EFCs and can be examined closely). Because of these limitations, we found only limited opportunity for analysis of beetle movement patterns, and have restricted the use of this data to qualitative discussion and observations only.

3.8.3 Statistical Analysis

Survival data (proportion of survivors pooled across replicates within each treatment due to small sample size) from the 28-day trial were assessed for differential survival between flow and no-flow treatments by a standard binomial test of two proportions (with continuity correction for small sample size) as implemented in R version 3.0.3 (R Development Core Team 2008). A two-factor analysis of variance (ANOVA) was used to determine if significant differences in water quality parameters existed between flow and no-flow treatments, or between upper and lower EFC sections, over the 28-day experiment. Other descriptive statistics were generated with Minitab 17 Statistical Software (2013).

4.0 Results

4.1 *Heterelmis glabra* Surrogate Flow Study

4.1.1 Survival

The proportion of individuals that survived in each treatment differed significantly (chi-square = 4.6147, df=1, p=0.03, α=0.05). The average proportion of individuals surviving the flow treatment was 0.39 vs. 0.08 for the no-flow treatment (Figure 8).

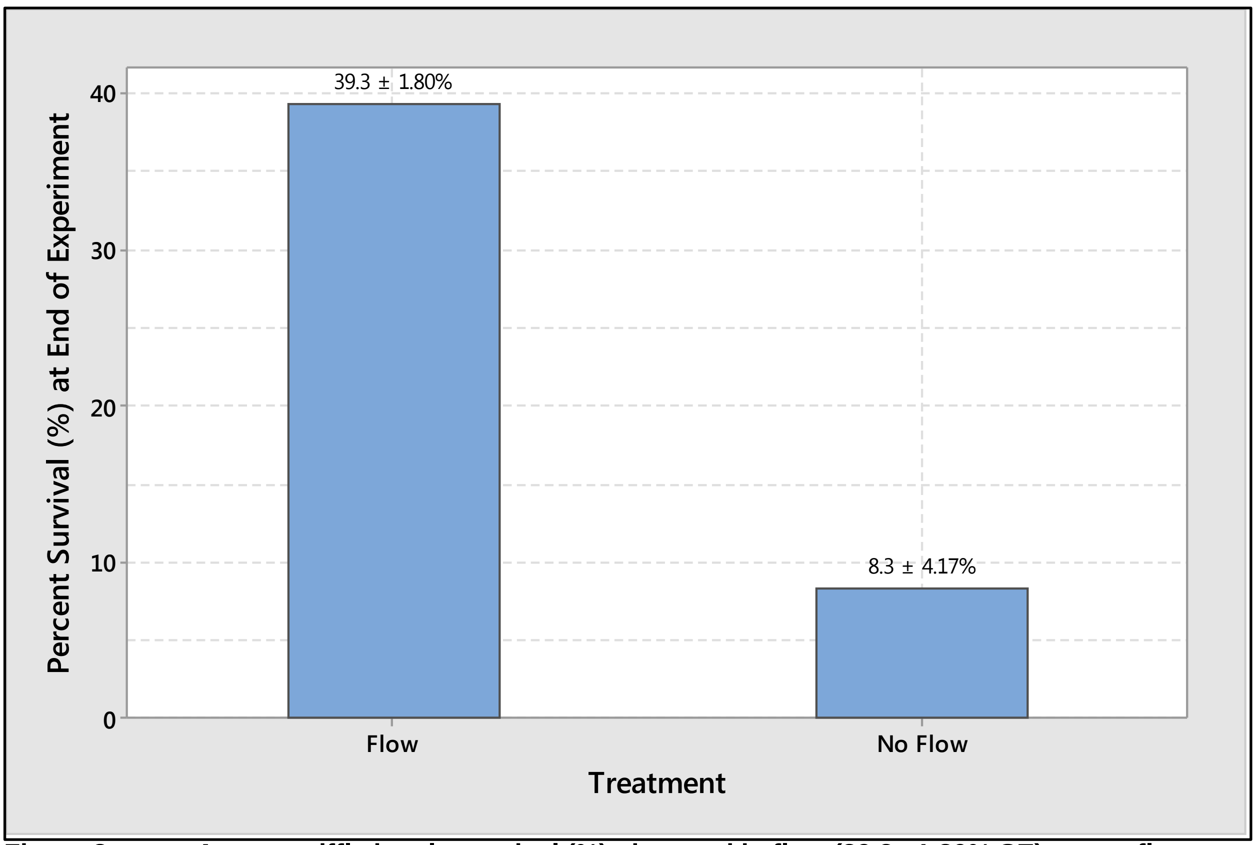

Figure 8. Average riffle beetle survival (%) observed in flow (39.3 ±1.80% SE) vs. no-flow (8.3±4.17% SE) treatments at end of 28-day experiment.

4.1.2 *Water Quality Conditions*

4.1.2.1 Dissolved Oxygen

A two factor ANOVA showed that no significant differences existed between upper and lower EFC sections (p=0.202). However, significant differences were found to exist between flow (DO mean=6.900 mg/L ±0.014) and no flow (DO mean=2.400 mg/L ±0.22) treatments (p<0.001) (Table 2).

Table 2. Water quality averages with standard error (SE) between experimental treatments measured over the course of the 28-day study.

TREATMENT	DISSOLVED OXYGEN (DO) (MG/L) ± SE	TEMPERATURE (°C) ± SE
Flow	6.900 ± 0.014	22.250 ± 0.034
No flow	2.400 ± 0.220	21.667 ± 0.049

A plot of DO (mg/L) data over time displayed marked roughly inverse exponential decline in the no-flow treatment, while DO in the flow treatment was steady (Figure 9).

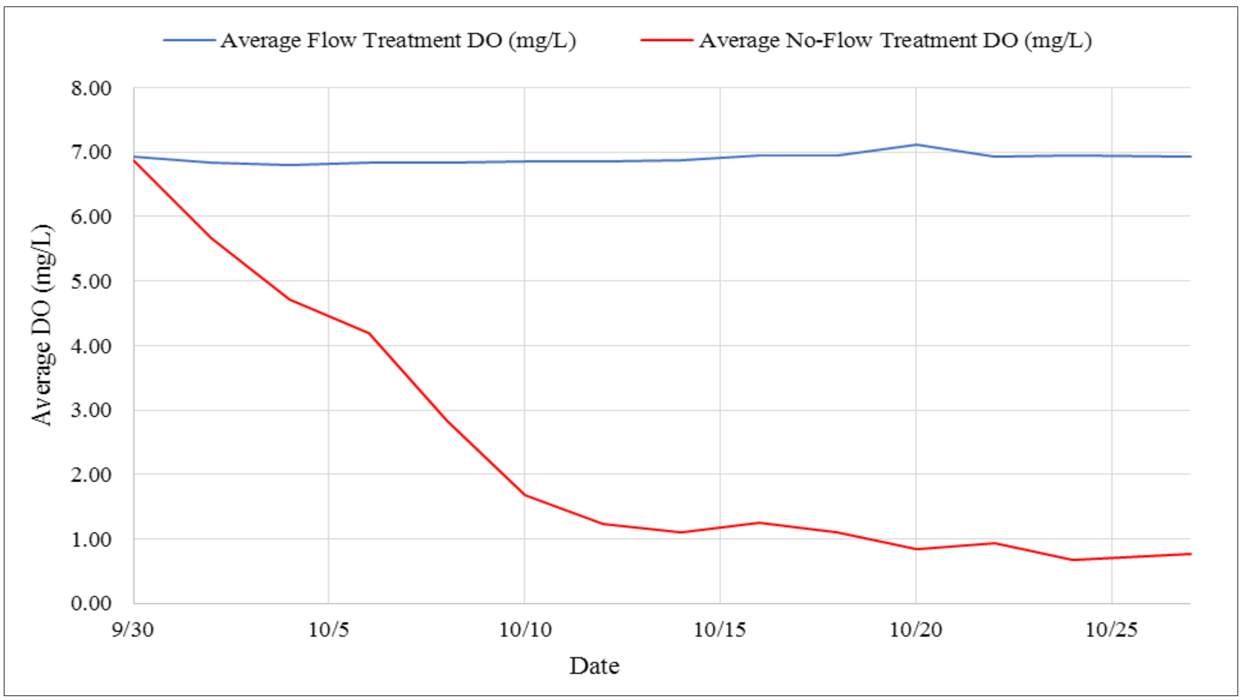

Figure 9. Time series plot of environmental flow chamber DO (mg/L) over course of experiment.

4.1.2.2 Temperature

A two-factor ANOVA showed that no significant differences existed between upper and lower EFC sections (p=0.241). However, significant differences were found to exist between flow (temperature mean=22.250°C ±0.034) and no flow (temperature mean=21.667°C ±0.0.49) treatments (p<0.001) (Table 2).

A plot of temperature data (°C) over the course of the experiment suggests that similar trends in temperature of flow and no-flow EFCs, although no-flow EFCs were significantly cooler than flow EFCs and displayed greater variation in measured temperature values (Figure 10).

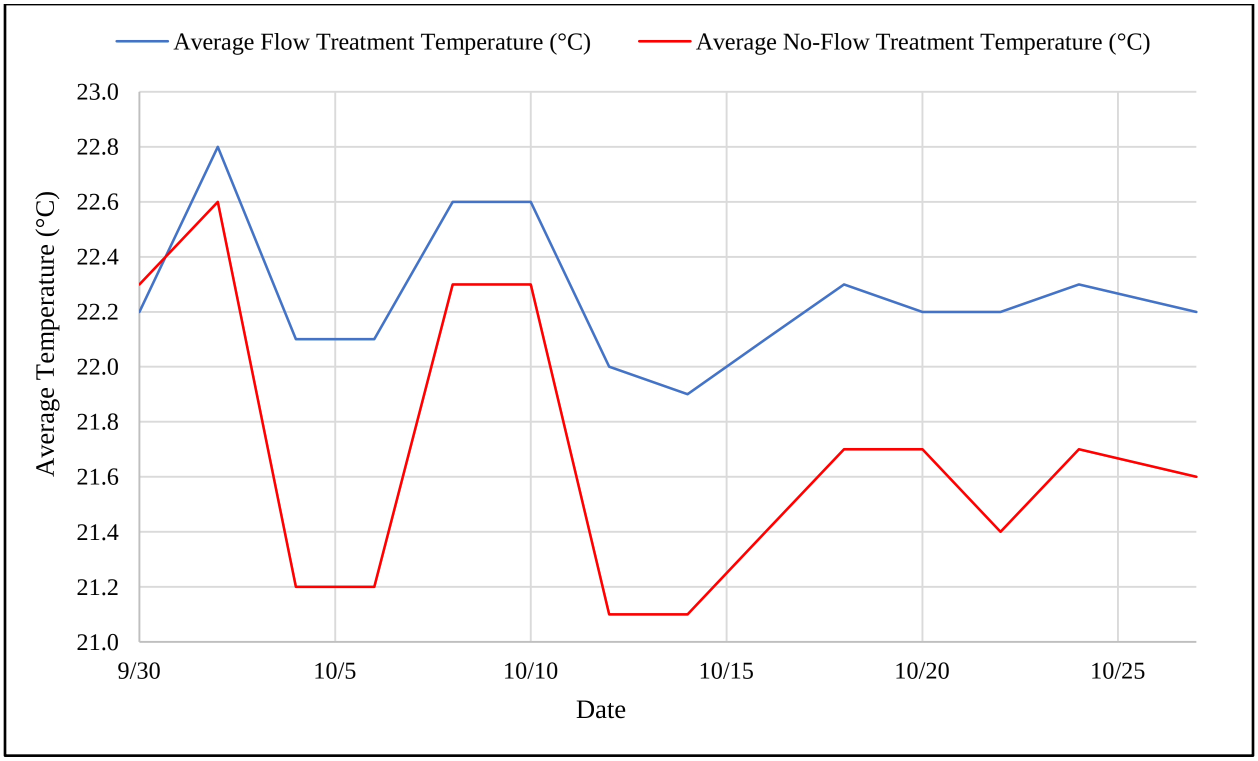

Figure 10. Time series plot of environmental flow chamber temperature (°C) over course of experiment.

Trends of temperature data (°C) in the RBASS and refugia also suggest and effect of flow rate, with thermistor data from the relatively high-flow Refugia 1 showing slightly higher temperatures than the lower-flow RBASS outfall throughout the duration of the experiment (Figure 11). These differences are slight (<1C°), and may be due to temperature differences between the FAB wet laboratory's common spring water source and ambient air; this may account for observed differences in average temperatures between flow (22.250 ±0.034) and no-flow treatments (21.667 ±0.049) in the RBASS EFCs (Table 2, Figure 10).

4.1.2.3 Flow

A time series plot of flow (mL/second) data displayed similar trends in EFCs in each flow treatment group (Figure 12).

The water source for the FAB wet laboratory is spring water from a common tank on the floor above, which is gravity-fed through pipes attached to the wet laboratory ceiling. The way in which the RBASS systems were set up to use this water source allows for flow rates to be decreased, but at this time does not allow maintenance of flow rates above the default flow rates provided by the gravity-fed system. We recorded qualitative observations of decreased flow/head pressure in the RBASS during periods of heavy water use upstream, which may account for the change in flows rates observed in flow treatment EFCs (Figure 12).

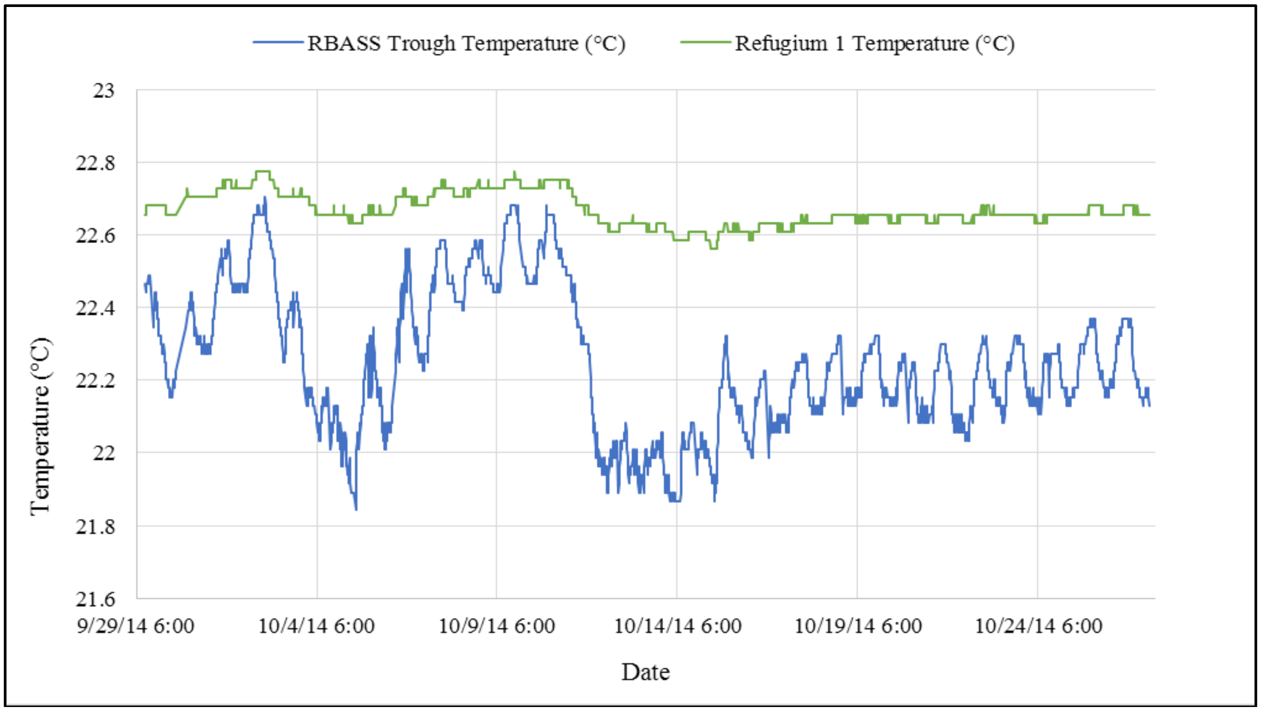

Figure 11. **Time series plot of RBASS outfall vs. refugia temperature (°C) 10-minute thermistor data over course of experiment.**

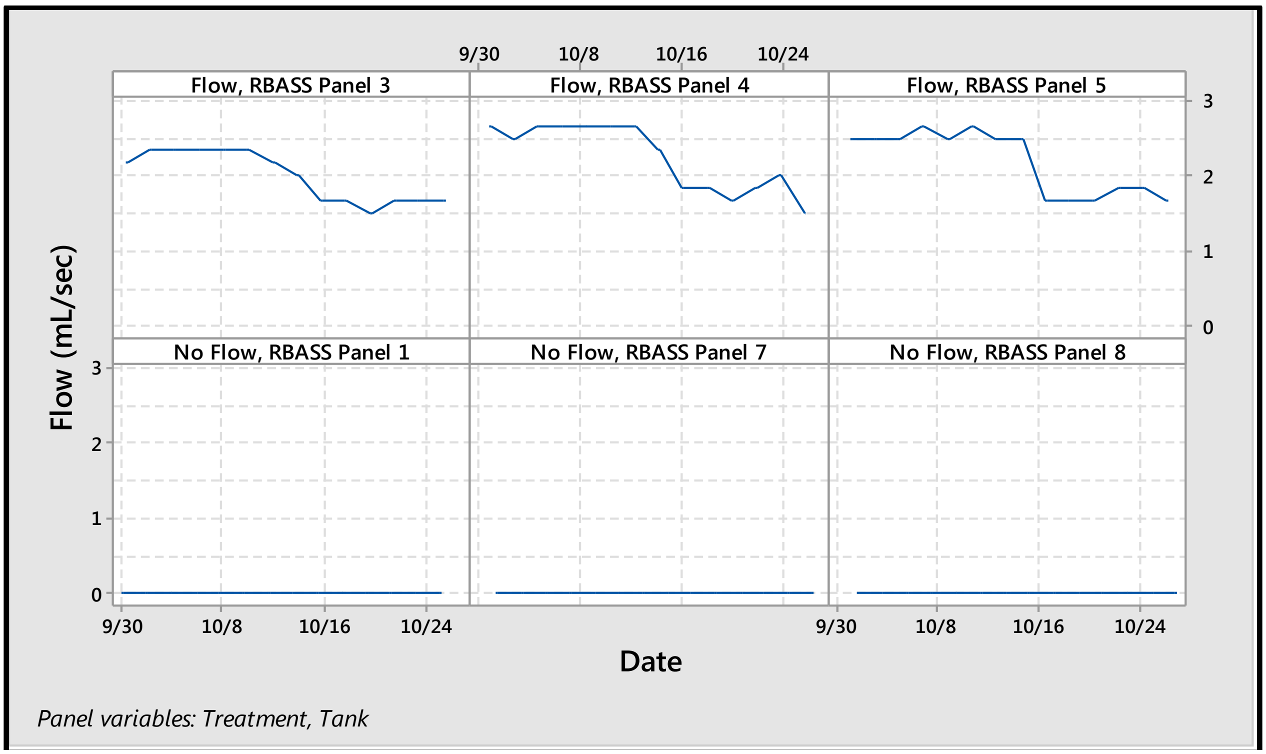

Figure 12. **Time series plot of environmental flow chamber ("panel") flow (mL/sec) over course of experiment.**

4.1.3 Qualitative Analysis of Movement Patterns

Previous laboratory research (Edwards Aquifer Authority 2007, BIO-WEST 2002) and field observational studies (Brown 1987) indicated that riffle beetles move preferentially nearer to current. We observed this incidentally in only one of 8 laboratory studies, all of which were testing hypotheses unrelated to beetle movement/orientation. However, *M. pusillus* were observed to position themselves toward the side of the EFC nearest the flow spigots in an early substrate pilot study (Figure 6). We did not observe significant difference in DO (mg/L) between the top and bottom halves of each EFC (Table 2).

We also noted that live beetles in the *H. glabra* surrogate flow study were frequently observed to be situated above the waterline on the fine mesh spillover screen. The screen was frequently damp/wet (Figure 13).

Figure 13. Photograph of beetles (dark dots) and waterline on fine mesh spillover screen at top RBASS environmental flow chamber. Beetles in this image are situated below the waterline.

5.0 SUMMARY AND CONCLUSIONS

Spring-associated invertebrate species are generally adapted to high quality water conditions that include stable temperatures, pH, and dissolved oxygen (Bosse 1979, Bosse et al. 1988, Brown 1987, Crowe and Sharp 1997, Gibson et al. 2008, USFWS 1997). The life history of *H. comalensis* is poorly understood, due in part to a lack of data specific to their survival and recruitment under varying conditions and a to lack of general baseline data, including presumptions about their nutritional requirements and the presence of adaptive responses to decreased quality or quantity of flow. Several researchers have suggested that *H. comalensis* may be sensitive to changing environmental parameters resulting from reduced springflows (Bowles et al. 2003, Gonzales 2008, Nice 2008); however, this would be difficult to illustrate *in situ*, as detectability of these organisms may vary under changing conditions. For example, it is probable that *H. comalensis* responds to low flows and reduced water quality, such as occurred during the DOR, by disproportionately utilizing the subsurface and retreating to the water table. This type of adaptive habitat utilization in response to changing surface environments makes the beetles nearly impossible for researchers to detect in the field during these conditions.

Because so few empirical studies of the species exist, the development and successful deployment of the RBASS unit, which lends itself to a wide range of potential future research applications, is a valuable step forward in efforts to improve understanding of the movement and response mechanisms of *H. comalensis.* Riffle beetles are so small and logistically difficult to observe in the field that current understanding of their responses to environmental stressors is largely presumptive and derived from anecdotal evidence suggested by researchers familiar with the system. The introduction of the RBASS, which allows controlled experimentation in an upwelling environment with the application of several replicates and/or several different treatments simultaneously, is itself a major accomplishment of this study.

Several field collection methods have been utilized to observe and collect *H. comalensis* from the Comal Springs system. BIO-WEST (2002) reports using manual collection methods utilizing fingers and/or soft forceps and dip-nets. More recently, Gibson et al. (2008), BIO-WEST (2013) and Gibson (pers. comm. 2014) reported success with cotton cloth lures allowed to “culture” *in situ* for a period of 4 weeks, after which time the lures grow fungal colonies believed to attract *H. comalensis* and *M. pusillus.* There is a lack of available behavioral and survival data following each of these methods of collection. This study utilized the least apparently disruptive method, with a no-contact approach focused on collecting the lures, rather than the individual beetles, by simply placing the lures in a water tight bottle with native water, storing it in a double-lined insulated thermos also filled with just-collected native water, and transported immediately back to the laboratory. This may be the least invasive approach to riffle beetle handling and collection in the field, and potentially refines the way endangered invertebrates could be brought into a laboratory for refugia or experiments.

Our data suggest that serial acclimation to laboratory water conditions is not necessary for *H. comalensis* transported from Comal Springs to the FAB. Similar facilities, such as the SMARC, also do not utilize serial acclimation procedures for this species. The implication for future transport of *H. comalensis* to one of these two facilities is that it provides support for resource managers to bypass this step, thus conserving resource costs and reducing necessary transfer and acclimation time.

Our evaluation of different substrates for use in the RBASS EFCs resulted in the dismissal of hard pieces of individual substrate in favor of mesh substrates that allowed for efficient visual interpretation, easy removal, and more accurate assessments of movement or survival. These types of substrates appear suitable for riffle beetle studies up to 1 month in length, and may support the addition of a food component to future studies.

The RBASS can be used to mimic rapid declines in DO during low/no flow events without the addition of supplemental nitrogen (or other means) to the system to deplete DO levels. Because DO in the system is anticipated to naturally decline over a short period of time without flow, riffle beetle responses—such as modified levels of activity—can be observed at different levels of progressive DO reduction. In addition, the bounds of the flow treatment with no food added have been defined by this study through the observation that almost all *H. glabra* died within 27 days of no-flow treatment. The preliminary nature of this study precluded independent analysis of the influences of DO, temperature or flow. Future studies investigating the effect of duration of no-flow conditions on beetle survival are recommended in order to determine limits surrounding *Heterelmis* spp. survival in the RBASS under these conditions and inform future experimental designs. Such studies could be conducted with the EFCs submerged in a controlled-temperature water bath in order to remove the potential influences of temperature fluctuations.

The main study described in this report did not incorporate a food presence treatment, so it is unknown whether the addition of potential food sources would affect survival of *H. glabra, H. comalensis* or *M. pusillus* in the laboratory. The consideration of food as a factor in laboratory experiments using multiple individuals in the same experimental chamber is problematic for several reasons. Microbial communities issuing from the Edwards Aquifer are known to be prolific near the fresh/saline water interface (Gray and Engel 2012, Randall 2006), and some obligate subterranean beetles are known to obtain their nutrients from allochthonous organic material dissolved in water underground (Paoletti et al. 2011). Researchers observed organic buildup on the overflow screens of the RBASS system over several weeks, which may have resulted either from organic matter introduced through the groundwater or from the metabolic activity of the beetles themselves. Additionally, when multiple individuals are maintained in the same experimental chamber, mortality may result in the immediate (e.g., necrophagy) or delayed (e.g., mycophagy) availability of a food source for surviving beetles. Because so little has been firmly established about riffle beetle dietary requirements, the possibility of these and other such alternative food sources cannot be ruled out and should to be at least a consideration of future experiments.

Although these studies employed *M. pusillus* and *H. glabra* as surrogates for *H. comalensis*, their suitability as surrogates is not firmly confirmed, as there is still a relative lack of understanding of any key differences between *H. comalensis* and these other species regarding morphology, ecology, and genetics. Both *M. pusillus* and *H. glabra* exhibited longer-term survival in the RBASS than *H. comalensis*, suggesting perhaps that these species are more adaptable to rapid environmental changes, or are longer lived than *H. comalensis*, or that there are other factors that have not been considered that may indicate the suitability of either of these species. Studies with additional surrogate species should be considered for future research. Additionally, only adult beetles of all species were considered for this study. Larval stage beetles may be differently suited to the study, and may respond differently than adult beetles. Differential survival of larval-stage beetles under stressful conditions is one hypothesis addressing *H. comalensis* persistence after the DOR.

Unexpectedly high mortality in laboratory populations of *H. comalensis* during our study coincided with a concurrent mortality event for *H. vulnerata* that were kept by other researchers in the same laboratory system. During the late August event, both *H. comalensis* in two separate BIO-WEST project team tanks (refugia and experimental chambers) and *H. vulnerata* in Texas State University holding tanks died over a period of several days. Another species, *H. glabra* survived in Texas State University holding tanks during this same time period. The simultaneous unexplained high mortality of *H. comalensis* and *H. vulnerata* is concerning, and current investigations are underway along with 2015 research proposed to compare conditions biologically at both the SMARC and FAB.

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