Comal Springs Riffle Beetle Habitat Connectivity Study

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Executive summary

The Comal Springs riffle beetle *Heterelmis comalensis* (Coleoptera: Elmidae) is a federally-endangered aquatic beetle endemic to the Edwards Aquifer—specifically, areas of seeps and spring upwellings in the Comal and San Marcos systems. These beetles and their associated habitat are covered under a 2013 United States Fish and Wildlife Service (USFWS) Incidental Take Permit (ITP) maintained by the Edwards Aquifer Habitat Conservation Plan (EAHCP). The objective of the EAHCP applied research is to fill in critical data gaps for the covered species and to answer key questions posed in the EAHCP in order to inform future management decisions and possible adaptive management solutions. To this end, in this document we describe an applied research project performed in 2015 that examined water quality and survival of Comal Springs riffle beetles to establish a laboratory location for 2015 experimentation, a subsequent evaluation of potential surrogate beetle species, and a series of laboratory and field investigations examining key components of upwelling and lateral habitat connectivity.

Although both the USFWS San Marcos Aquatic Resource Center (SMARC) and Texas State University Freeman Aquatic Building (FAB) laboratories exhibited fairly similar water quality conditions with similar lack of temporal variability during our preliminary investigation, there were profound differences in the survival of riffle beetle adults with extensive mortality at FAB. As such, all subsequent 2015 riffle beetle laboratory studies associated with this project were conducted at SMARC. In response to the unknown causes for this mortality concern, Dr. Nowlin and Texas State University (both outside of this project) have been working closely with the Edwards Aquifer Authority to determine the cause for the adult mortality and how to manage the issue.

The surrogate evaluations suggest that *Microcylloepus pusillus* is likely not the best candidate to serve as a surrogate species for the Comal Springs riffle beetle in physiological stress studies associated with variation in environmental conditions, especially water temperature. This conclusion was further reinforced with stable isotope results that documented that the diet of *M. pusillus* was much more reliant upon periphyton and therefore feeds on an entirely different food chain than the Comal Springs riffle beetle.

Results from the upwelling habitat connectivity study conducted at SMARC using the Riffle Beetle Aquifer Simulation System revealed greater survival in treatments connected to organic matter via water flow than in treatments that were disconnected to organic matter over the course of the study. Additionally, qualitative observations of beetle movement over the course of the upwelling experiment suggest that beetles in Experimental Flow Chambers with flowing water connected via flow through organic material tended to be more active and often frequented the organic material provided near the surface of the upwelling. The laboratory observation regarding connectivity and organic matter was independently supported by field studies using stable isotopes to examine the Comal food chain.

Results from the field investigations indicate that inputs of terrestrial-derived materials are likely to be particularly important for the diet (and thus conservation) of the Comal Springs riffle beetle. In fact, model results indicate that Comal Springs riffle beetle adults and larvae derive 80% and 73% of their diet from terrestrial-derived materials (wood and leaves combined). The

results of this study suggest that a lateral connection to terrestrial matter sources may be particularly important for two of the invertebrate species listed under the EAHCP: Comal Springs riffle beetle and the Comal Springs dryopid beetle (*Stygoparnus comalensis*). Lower spring discharge and declining flows can lead to disconnection of the aquatic environment from the bank and to lower water surface areas of aquatic habitats, potentially leading to decreased inputs of terrestrial material that may support populations of these wood-associated taxa. In addition, decreased flow rates and water velocities could also lead to lower rates of downstream transport of terrestrial materials from more canopy-covered upstream areas (e.g., the more canopy-covered Spring Runs) to more open areas with lower rates of terrestrial inputs (e.g., Spring Island area).

Results from the stable isotope work indicate that the third EAHCP covered and federally listed Comal invertebrate, Peck's cave amphipod (*Stygobromus pecki*), another spring-associated consumer may have a more plastic feeding strategy than the Comal Springs riffle beetle and Comal Springs dryopid beetle and may be able to switch to an alternate basal resource food chain as environmental conditions vary spatially.

Relative to this investigation, it was clear that there are potentially a large number of additional studies that should be considered. Experiments for consideration could include examining the performance of riffle beetles to environmental stimuli (water temperature, organic matter connection or disconnection, levels of siltation, etc.) over extended periods of time (months) in order to establish "preferred" conditions for maintenance of both wild and refuge populations. It may also be extremely insightful to monitor physiological responses (instead of behavioral responses) to increasing temperature, such as respiration rates, immune function, and the concentration of various biomolecules.

Finally, based upon the two preliminary studies we conducted, it appears that passive sampling pits or wells and Bou-Rouch samples are not likely to be efficient methods to estimate beetles. However, the efficiency of these and other methods should be considered for more thorough investigation in a systematic study that explicitly examines and compares various collection methods in the field. In addition to these comparisons, other data should be considered for collection in the lab, including the dispersal or movement ability of beetles, the ability to mark and re-capture beetles in small-scale settings, and the ability of beetles to hold onto surfaces at different flow or suction rates.

Acknowledgments

The project team would like to acknowledge the USFWS SMARC scientists and staff for the generous use of their facilities and expertise.

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). *Heterelmis 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 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).

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). However, though research on low-flow conditions and critical drought stages exist 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).

In this report we describe some initial testing of research facilities, an evaluation of surrogate beetle species, and a series of novel experiments examining key components of upwelling and lateral habitat connectivity of *H. comalensis*. Due to potential issues associated with differences in the survivability of *H. comalensis* at the Freeman Aquatic Building (FAB) at Texas State University and the United States Fish and Wildlife Service (USFWS) San Marcos Aquatic Resource Center (SMARC) observed in 2014, intensive water quality monitoring at both sites were conducted in 2015 (Section 1). Subsequently, we examined the suitability of several riffle beetle species as surrogates for *H. comalensis* for future studies (Section 2). However, the main focus of this applied research effort was testing the effects of water level decrease and surface habitat disconnection on *H. comalensis* under laboratory (Section 3) and field conditions (Section 4).

To test upwelling connectivity, we conducted laboratory experiments to assess the importance of organic matter availability on the spatial habitat preference and survival and/or ecological death of *H. comalensis* subjected to constant upwelling flow that was either connected or disconnected from organic material. This effect is of interest in light of the possibility that worsening drought and low-flow conditions may increase the disconnection of subsurface habitats from the surface edge habitats hypothesized to be important to *H. comalensis* in terms of protective habitat, food availability, or a combination of the two. Experiments were conducted in a wet laboratory at SMARC using a custom-built Riffle Beetle Aquifer Simulation System (RBASS) aquarium unit. 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, and is valuable in that it allows controlled experimentation in an upwelling environment with the application of several replicates and/or several different treatments simultaneously.

The field portion of the study examined whether the abundance and distribution of *H. comalensis* is associated with spring openings that contain organic matter (i.e., root material, leaves), whether they utilize the organic matter and/or biofilms on the organic matter as a food source, and whether riffle beetles have the ability to respond to changing water levels over the course of the drawdown season. Conclusions and recommendations are presented in Section 5 with literature cited and reviewed presented in Section 6.

2.0 LITERATURE REVIEW

Prior to initiation of the study, an extensive literature review was conducted relating to 1) population distributions and habitat associations of *H. comalensis*; 2) dispersal ability, habitat connectivity, and life history of *H. comalensis*; 3) surface-subsurface interactions for flowing water invertebrates; 4) food habits and trophic ecology of *H. comalensis*; and 5) use of surrogate species for habitat connectivity studies. Since periods of extended drought greatly influence the amount and persistence of spring flows at Comal Springs, determining the likelihood of low flow periods in the future is critical for habitat management of the listed species dependent on these habitats for their continued presence in the system. The probabilities of a shorter-term (3-5 years) and longer-term (7-10 years; equivalent to the drought of record in the 1950's) drought periods are estimated to be approximately 0.2% and 0.1%, respectively (EARIP 2011). The Edwards Aquifer Recovery and Implementation Plan (EAHCP) set the long-term mean and minimum daily discharge objectives for Comal Springs at 225 ft^3/s (cubic feet/second) and 30 ft^3/s, respectively.

Historical data and modeling results indicate some of the potential loss of habitat and habitat degradation associated with reduction in spring flows. It has been observed that Spring Runs 1 and 2 generally cease to flow when total Comal Springs flow is ~130 ft^3/s, and Spring Run 3 generally ceases to flow when Comal Springs total flow is about 50 ft^3/s (LBG Guyton 2004). Modeling results suggest that discharge will be less than 120 ft^3/s for a total of 127 months and less than 45 ft^3/s for a total of seven months during a repeat of the drought of record (in the 1950's) with Phase 1 of the EAHCP implemented (EARIP 2011). Modeling efforts also indicate that a repeat of the drought of record (with Phase 1 of the EAHCP fully implemented) will lead to the total flows in the Comal Springs system to be < 30 ft^3/s for a two month period (EARIP 2011). If flows drop below 30 ft^3/s, it is expected that the main spring runs in the system (Spring Runs 1 through 6) will be dry for a considerable period of time, and that the remaining aquatic habitat for the Comal Springs riffle beetle within the system will be limited to portions of Landa Lake and the Spring Island area. Cumulatively, this information indicates that it is possible for several, if not most, of the spring runs in the Comal system to cease flowing for extended periods of time (i.e., months to years) and for a significant reduction of aquatic habitat to occur, should the Comal system again experience conditions similar to those of the drought of record.

Obviously, there is substantial uncertainty inherent with predictions about the duration and extent of low flow conditions at Comal Springs, but the effects of these predicted scenarios and droughts of lesser durations will likely affect the quality and quantity of habitat for listed species. In particular, the Comal springs riffle beetle has a fairly limited spatial distribution within the system, so changes in flow could lead to areas suitable for riffle beetle habitat in the system becoming reduced in area and fragmented, potentially leading to the spatial separation of beetles from potential higher quality food resources they utilize. However, little is known about consequences of decreased flows and the potential effects on habitat connectivity for the Comal Springs riffle beetle. The purpose of this literature review is to provide information on the life history and surface-subsurface interactions and how these factors may affect the population and distribution of the Comal Springs riffle beetle. We also reviewed the literature for research that discusses the use of aquatic invertebrate surrogates.

Population Distributions and Habitat Associations of the Comal Springs Riffle Beetle

Riffle beetles (Order Coleoptera, Family Elmidae) typically live in swifter flowing areas in high water-quality streams and rivers. Adult riffle beetles generally crawl along benthic surfaces and respire through a plastron on the ventral side of their abdomen (Brown 1987, White and Roughley 2008, Elliott 2008a). *Heterelmis comalensis* is typically collected on cotton cloth lures in areas within a 80 cm from spring sources in the Comal system (Gibson et al. 2008, Cooke 2012). In hard-packed gravel habitats commonly found adjacent to spring openings, *H. comalensis* is found at the depths of 2-10 cm (Bosse 1979). The precise mechanisms for the apparent spatial restriction of *H. comalensis* within the Comal system are unknown, but may involve spatial variation in water quality parameters and habitat associations. Data indicate that riffle beetles prefer spring water characterized by high CO_2, low DO, and slightly lower pH in comparison to surface-water-dominated streams (Cooke 2012). Other species of more widespread elmids (i.e., *Stenelmis* spp.) also exhibit habitat preferences for stable gravel-cobble substrates and coarse woody debris, rather than unstable sand and mud substrates (Phillips 1995).

It is currently thought that a reduction in spring flow that leads to loss (desiccation) of habitat or reduces water quality of occupied riffle beetle habitat will likely impact their fitness and survival. Undoubtedly, water quantity will be the primary issue in the Spring Runs and along the western shoreline during substantial low-flow events, as springs within these areas will cease to flow and the habitat associated with the presence of the Comal Springs riffle beetle (i.e., areas around spring orifices) will be dry. However, as flows decline at Comal Springs and the remaining aquatic habitat is reduced to portions of Landa Lake along the western shoreline downstream of Spring Island (EARIP 2011), it is likely that the water temperature will increase and dissolved oxygen (DO) concentrations will drop. A recent study conducted for the EAHCP found that Comal Springs riffle beetles could tolerate rapid changes in temperature and DO concentrations (i.e., beetles could withstand up to 45°C before loss of function and tolerate 0 mg DO/L for several minutes without suffering obvious ill effects), but that their tolerance ranges to short-term temperature changes were substantially narrower than a closely-related elmid species (*Heterelmis glabra*; Nowlin et al. 2014). However, the sensitivity of riffle beetles to longer-term and more slowly-occurring changes in temperature and DO remain to be determined.

Dispersal Ability, Habitat Connectivity, and Life History of the Comal Springs Riffle Beetle

Although many adult aquatic coleopterans emerge from aquatic habitats for dispersal flights to other aquatic locations, many riffle beetle species typically cannot disperse great distances via flight (White and Roughley 2008). Indeed, some elmid species are thought to be flightless as adults (Elliott 2008a) and other species can fly relatively short distances after emergence but their flight muscles degenerate after they re-enter the water (Hinton 1976). The complete loss of adult flight (degenerated wings) or the rapid loss of wings after adult emergence suggests that retaining flight capability may not be compatible with utilization of plastron respiration because of the maintenance of substantial sub-elytral air space required for functional wings (Thorpe and Crisp 1947). Thus, smaller bodied adult elmids like *H. comalensis* typically do not have the ability to disperse great distances and move relatively slowly via crawling in their aquatic habitats or through drifting downstream.

On benthic surfaces, smaller-bodied invertebrates like elmids are more resistant to dislodging during high flow events than larger-bodied invertebrates (Turcotte and Harper 1982), but the use of benthic surface habitats can vary substantially among species and within life stages of a single species. In general, elmid larvae are less sensitive to lower water velocities and hydraulic stagnation than adult stages (Walters and Post 2011) because larvae are less dependent on flowing water conditions (i.e., less rheophilous) than adults because they utilize gills for respiration (Elliott 2008a). Later larval elmid instars can develop tracheal air sacs that provide a Cartesian driver system for controlling the specific gravity of the body and allow drift towards preferred pupation sites along stream banks (Brown 1987). Drifting of individuals occurs mostly at night, most likely in response to gaining access to food resources or to escape sub-optimal environmental conditions (Elliott 2008b; Brown 1987; Reisen 1977). Higher mortality rates of newly hatched or overwintering larvae occurs during drift events, as they are then at high risk of being washed away by the current and/or dispersed to sub-optimal habitats (Reisen 1977; Elliott 2008a).

Variation in environmental conditions can alter the timing and magnitude of emergence of adult elmids, including *Microcylloepus pusillus* and *Heterelmis* spp. (Reisen 1977). In temperate systems with a high level of seasonal variation in environmental conditions (e.g., Quebec, North America), individual elmids can persist in the larval stage for 2-3 years and the adult stage lasts for approximately a year (Lesage and Harper 1976). In temperate systems, both adult and larval elmids exhibit a great deal of seasonal variation in life stage timing, and air/water temperatures greatly influences the duration of the pupation period (Lesage and Harper 1976). In contrast, *H. comalensis* and *M. pusillus* in the Comal Springs system exhibit non-seasonally influenced emergence patterns and have overlapping, asynchronous generations (Bowles et al. 2003). This lack of seasonality in emergence and life history patterns in *H. comalensis* is largely thought to be a consequence of environmental conditions at spring-influenced systems like Comal and San Marcos springs because they exhibit little seasonal variation. In other systems with limited variation in environmental conditions (e.g., the tropics), emergence of *Heterelmis* adults occurs during periods of low intensity, low current velocities, and high food availability, and oviposition occurs when temperature is highest and water level is lowest (Passos et al. 2003). It is important to note that although the USFWS can successfully house both adult and larval *H. comalensis* to the extent that adults mate and oviposit in aquaria, they have so far had only minimal success in getting adults to emerge after pupation. Currently, there is a clear need to better understand potential mechanisms and conditions leading to successful adult emergence of *H. comalensis*.

Surface-Subsurface Interactions for Flowing Water Invertebrates

The subsurface hyporheos, or hyporheic zone, of flowing water systems may act as a refuge for benthic invertebrates during periods of low flow or even in apparently dry stream beds (Williams and Hynes 1974). It provides refuge from drying and enables invertebrates to recolonize the surface once the disturbance has passed (Dole-Olivier et al. 1997). Drought-induced changes to in-stream environment such as hydraulic stagnation, increased/decreased water temperatures, increased fine sediment deposition, and altered macrophyte composition can affect the macroinvertebrate community by reducing habitat quantity and quality as well as access to preferred food resources. During low flow periods, the contraction of wetted stream width can cause in-stream organismal densities to increase and lead increased resource competition in the hyporheic zone (Dewson et.al 2007). Complete cessation of flow in the hyporheic zone can lead to loss of suitable habitat for invertebrates seeking refuge in the hyporheos through the eventual complete desiccation of hyporheic sediments (Boulton & Stanley 1995), anoxia in the hyporheos (Smock et al. 1994), and the lack of interstitial habitat due to clogging of interstices by fine sediments (Bo et al. 2006).

In addition to its limited geographic distribution, specificity in preferred habitat types, lack of mobility, and potential sensitivity to habitat degradation, the genetic variation of *H. comalensis* populations in the West Shoreline, Spring Island, and San Marcos springs populations suggests limited gene flow among these populations. Therefore, if springs at Comal or San Marcos springs cease to flow for extended periods of time, genetic variation across the remaining variable populations could be lost (Gonzales 2008). Although *H. comalensis* was described as a species after Comal Springs stopped flowing during the drought of record (Bosse et al. 1988), is has been assumed that *H. comalensis* populations were present in the Comal system prior to the drought of record and were able to persist for the 144-day no-flow period in 1956. It remains unknown precisely how beetles persisted in the Comal system during the drought of record, or how the drought of record affected riffle beetle populations, and if riffle beetles have the ability to rapidly recover from a large-magnitude drought events. It has been hypothesized that *H. comalensis* persisted through the drought of record through life cycle aestivation or by retreating into spring heads, the aquifer, or down into the hyporheos (Bowles et.al 2003).

Previous research has determined that for perennial species of riffle beetles (i.e., species that live for multiple years), pool habitats connected via some flowing surface water can serve as refugia for both larvae and adults during drought periods (Burk and Kennedy 2013). Elmid use of these refuge areas is thought to be associated with the relatively higher water quality and constancy of flow in these habitat patches during drought conditions (Burk 2012). In addition, during extreme drought events, elmids can take refuge in shaded disconnected pools where environmental temperature and evaporative losses are moderated by riparian shading (Burk and Kennedy 2013). However, elmids may also utilize subsurface (hyporheic) environments when flows are low; elmid body shape is such that they can tolerate small spaces (elmid adults are typically of small body size, while larvae have slender, flexible bodies), and elmids have been found to survive in the hyporheos for relatively long periods of time during periods of low flow (Boulton and Foster 1998; Marchant, 1988).

Food Habits and Trophic Ecology of Comal Springs Riffle Beetle

Potential food resources for *H. comalensis* have not been clearly identified in the Comal and San Marcos systems. Most literature sources state that riffle beetles are generally biofilm scrapers that can utilize detrital materials (Brown 1987). Currently, the standard capture method for *H. comalensis* in Comal is through the use of cloth lures (R. Gibson, USFWS; *pers. comm.*). Presumably, Comal Springs riffle beetles are attracted to the lures to gain access to the biofilms that grow there (Gibson et al. 2008). A more widely-distributed elmid species, *Heterelmis vulnerata,* is often associated with coarse woody debris with biofilm coverage and loose bark and/or interstitial spaces. The biofilm and interstitial spaces are thought to be used as concealment from the predators and biofilms may serve as algal and fungal food sources for the beetles (Phillips 1995). Seagle (1982) found that the gut contents of larvae and adults of three different riffle beetle species (*Stenelmis crenata, Stenelmis mera,* and *Optisoservus trivittatus*) were dominated by detritus-like materials, including wood xylem and unidentified organic matter and mineral particles, while algal material was consumed to a much lesser extent. Thus, it has been suggested that elmids should be reclassified as detritivores-herbivores rather than as strictly herbivores, with the exception of known xylophagus genera (i.e., *Lara*) (Seagle 1982). Cannibalistic foraging has been observed in some elmids (i.e., *M. pusillus*), but this behavior was attributed to nutritional deprivation and is probably not a common foraging strategy (Brown and Shoemake 1969). Currently, the precise food sources and trophic ecology of *H. comalensis* remains unknown. In addition, it is unknown whether a reduction in spring flow may lead to the disconnection of *H. comalensis* from potential or preferred food sources, such as terrestrial organic matter and detritus which may be most concentrated along the bank.

Use of Surrogate Species for Habitat Connectivity Studies

Given the sensitive nature and protected status of the *H. comalensis*, there is high potential value in determining whether other similar insect species may be used as test surrogates, especially as such could allow for improved understanding of tolerance to changes in habitat and quantity and quality while preventing the unnecessary take of protected organisms. Suitable surrogate organisms should share certain traits in common with the organism in question, such as life history, physiology, developmental patterns, food preferences, trophic ecology, habitat associations, sensitivity to environmental conditions, and dispersal ability.

Several species of elmids have been used as surrogate species to *H. comalensis* for a number of studies. The elmid *M. pusillus,* while in another genus, occurs in some of the same habitats as *H. comalensis* in the Comal system; the mechanisms facilitating the occurrence of *H. comalensis* and *M. pusillus* in similar habitats in Comal Springs are not well known, but their coexistence may be related to patterns in availability of preferred substrates, and competition with each other and with other species may influence their population distribution (Bowles et al. 2003). Both *M. pusillus* and *H. comalensis* occur in the same habitats at Comal Springs (spring runs 1, 2, and 3), indicating a possible preference for similar substrate composition (i.e., substrates dominated by gravel-sized particles), although the absence of *H. comalensis* and presence of *M. pusillus* at Spring Run 4, where substrate composition is dominated by silt, sand, and smaller gravels, suggests that *H. comalensis* may be more sensitive to changes in substrate size than *M. pusillus* (Bowles et al. 2003). In addition, adult *M. pusillus* are winged and are assumed to be capable of flight, whereas *H. comalensis* adults are generally thought to be incapable of flight. This suggests that *M. pusillus* may have greater potential dispersal ability, a wider population distribution in

Comal Springs, as well as a greater tolerance to changes in habitat than *H. comalensis* (Brown and Shoemaker 1969; Bowles et al. 2003). However, these differences are not well-elucidated and have yet to be examined in depth.

Heterelmis glabra and *H. vulnerata* may also serve as potential surrogates for *H. comalensis*. Data suggest that while *H. comalensis* and *H. glabra* are closely related, populations have been historically isolated from one another with little-to-no recent gene flow (Gonzales 2008). More recently, research associated with the EAHCP found that tolerance ranges to rapid and short-term temperature changes for *H. comalensis* were substantially narrower than for *H. glabra* (Nowlin et al. 2014), suggesting that *H. comalensis* is more sensitive to changes in habitat and water quality than *H. glabra*. Finally, *H. vulnerata* is typically found in surface-water-dominated sites and not with spring-dominated systems, suggesting that it has clearly different water quality and habitat requirements that *H. comalensis*. Cumulatively, this information indicates that there is clearly a need to determine whether effects of low flow on *H. comalensis* can be correctly assessed by using *M. pusillus*, *H. glabra,* and *H. vulnerata* as surrogate species.

3.0 METHODS AND MATERIALS

3.1 Potential Water Quality Differences between SMARC and FAB

Due to potential issues associated with differences in the survivability of *H. comalensis* and other riffle beetle species at FAB at Texas State University, we conducted high-frequency water quality monitoring at FAB and SMARC over a two-week period to determine whether either facility exhibited rapid changes in conditions that might affect the captive beetle populations. At each facility, an auto-logging datasonde (YSI multiparameter probe at SMARC and an In-Situ Inc. TROLL multiparameter probe at FAB) was placed on March 1, 2015 in the incoming water flow to a living stream in a plastic bucket (so that there were no influences of aeration) that logged data on temperature (°C), dissolved oxygen (DO; mg/L), conductivity (µS/cm), and pH at 10 minute intervals. Each sonde was calibrated to known standards on site. We did not cross-calibrate the sondes with each other because we were not interested in direct comparison of conditions across sites, but rather in the relative change in conditions within each site and how any potential changes would affect survival of beetles.

Concurrently with the installation of the sondes at each facility, we collected fifty individuals of both *H. comalensis* and *M. pusillus* on March 16, 2015 from Comal Springs by hand picking and transported individuals in high-quality coolers filled with site water to both facilities. Twenty-five individuals of each species were held in separate flow-through chambers on living streams at FAB (n = 25) and SMARC (n = 25). We additionally collected *H. glabra* (n = 50) from Finnegan Springs at the Devils River on March 21, 2015 by hand-picking from cloth lures. *Heterelmis glabra* individuals were transported to FAB and SMARC in high-quality coolers containing Finnegan Spring water and introduced into the flow-through chambers at FAB (n = 25) on March 21, 2015 and at SMARC (n = 25) on March 23, 2015. The plastic flow-through chambers that housed riffle beetle populations of each species at both the facilities contained pre-cleaned limestone river cobbles and well-conditioned sycamore (*Plantanus occidentalis*) leaves, a presumed food source. Data from the sondes were downloaded on a weekly or bi-weekly basis and examined to determine if there were pronounced or rapid fluctuations in the conditions of the supply water quality (e.g., rapid drops in DO or abrupt changes in conductivity) at both the facilities. Survival of beetles at both facilities was checked every day and beetle mortality was recorded. The beetle survival at both the facilities was observed and recorded everyday until March 31, 2015 (Section 4.1).

On April 1, 2015, an additional flow-through chamber containing adults of *M. pusillus* (n = 22) was transferred from SMARC to FAB and connected to the water source in FAB wet lab. This transfer was done to verify the occurrence of 100% beetle mortality at FAB in a short period of time (Section 4.1) when all the conditions in the flow-through chambers were exactly similar at both the facilities and the only difference was the water source.

3.1.1 Data Analysis

Differences in riffle beetle survival and water quality at the two facilities were assessed by examining the number of beetles that were alive at each location during the study duration and by examining the variability in the various water quality parameters at each location over the study period. Variability in water quality parameters was assessed by determining the minimum, maximum, and standard deviation of values during the temporal span of the study.

3.2 Surrogate Testing for Effects of Water Temperature

Based on the results obtained by survival testing at SMARC and FAB (Section 4.1), all subsequent experiments examining riffle beetle responses to different environmental temperatures were performed in temperature-controlled living stream systems at SMARC. Adult beetles of each species (i.e., *H. comalensis*, *M. pusillus* and *H. glabra*) were collected in the wild using the previously described methods, transported to SMARC, and maintained in flow-through chambers with a constant addition of Edwards Aquifer well water for at least two weeks before being used in experiments.

Populations of adults were housed in flow-through chambers held within living stream systems and gradually acclimated to temperatures set at approximately spring outflow temperatures (23^{o}C) prior to the start of experiments. *Heterelmis glabra* was selected as a potential surrogate because it is the most closely related species to *H. comalensis* and is also considered to be spring-associated in terms of its habitat use. We also utilized *M. pusillus* in experiments because it is often found in the same habitats as *H. comalensis* and *H. glabra*, but its overall distribution within these habitats is thought to be more cosmopolitan and not as spring-restricted as the two *Heterelmis* species.

In this study, we focused on potential differences in survival among the three species during relatively long-term exposure to gradually-increasing temperatures. We elected to use this approach in our comparison for two main reasons. First, we decided to use temperature as the variable of interest because previous experiments with *H. comalensis* and *H. glabra* indicated that these species appeared to be sensitive to temperature when they increased to ~30^{o}C (Nowlin et al. 2014). Second, we elected to use a long-term and gradual increase design so that we could somewhat mimic the types of conditions that these beetles would experience in natural systems.

Previous studies (Nowlin et al. 2014) only had the opportunity to examine responses of *H. comalensis* to relatively short-term temperature increases (changes over a few hours); however, in spring systems, temperature increases are likely to be persistent and gradual as spring flows decline in the summer months. Thus, we elected to examine how gradual step-wise increases affected survival of beetles so that longer-term effects would have time to manifest themselves in animals.

For this portion of the study, the experimental setup included 10-L plastic tubs which lay within a living stream with continuous supply of Edwards Aquifer water (Figure 1). The plastic tub had a layer of pea-sized aquarium gravel in the bottom and a magnetic drive pump (~700 g/h or 44 L/min; Pentair Aquatic ecosystem, Model MD7). A temperature control unit (Frigid Units, Inc.) was placed in an initial reservoir and could be set to the desired temperature via digital interface. A temperature and optical DO probe was placed in the reservoir and DO and temperature were recorded continuously over the period of the experiment on a laptop computer with Loggers Pro software. The outlet of the magnetic pump was connected to flexible clear vinyl tubing which led to a manifold with multiple splitters in the line – each line led to an airtight high density polyethylene (HDPE) chamber that had inflow and outflow lines and would serve to house beetles during experiments. Openings to the inflow and outflow tubes to the chambers were covered with a fine mesh (100µm aperture) to prevent beetle escape. Individual beetles of each species were housed in individual HDPE chambers that were suspended in a living stream using a plastic screen mesh. Three plastic screens were suspended in the living stream and each plastic screen with 5 cups housed 5 individuals of the same species (one individual in each cup). Replacement of water into each beetle holding chamber was estimated at 120 mL/min, allowing for complete replacement of the chamber volume every 30 seconds.

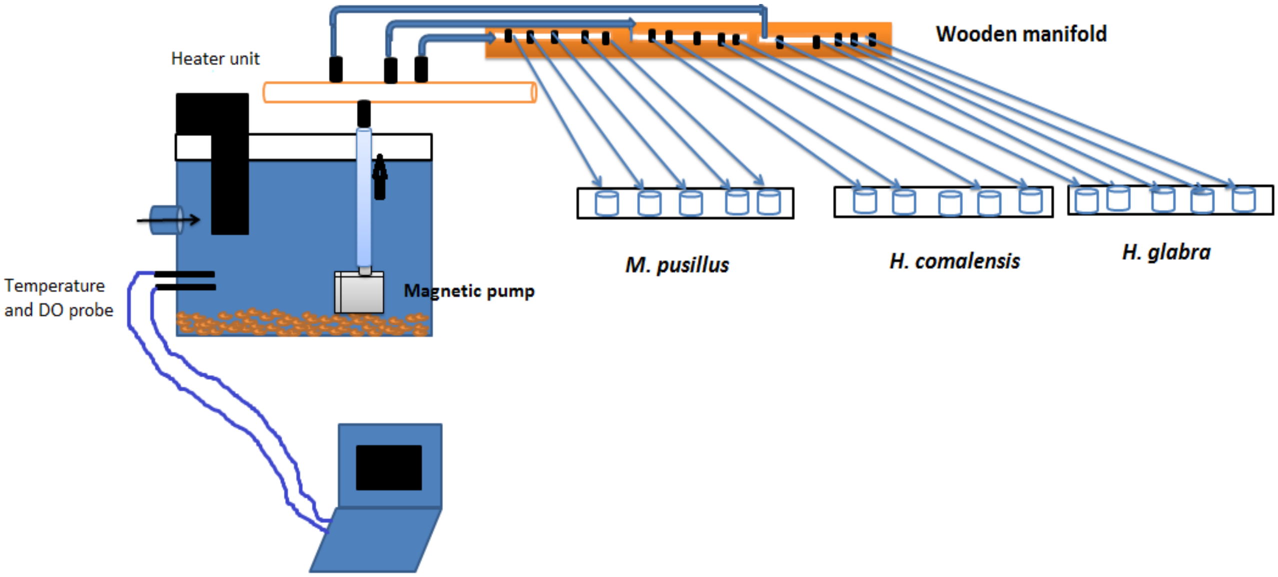


Figure 1. Schematic of the system used for the surrogate experiment. Incoming water temperature was ~23°C, and changes to water temperature were accomplished by setting the desired temperature on the heater unit.

The long-term experiments at different temperature treatments with *H. comalensis*, *H. glabra* and *M. pusillus* were conducted at SMARC from June 15 to July 20, 2015 (35 days). Five individuals of each species were subjected to gradually increasing target temperature treatments of 23°C, 26°C, 28°C, 29°C, 30°C, and 32°C or until all beetles died or exhibited a loss of response to a stimulus. The DO concentrations during experiments was always ≥5.9 mg/L. Beetles were initially kept at 23°C for one week (7 d) and then the temperature was slowly raised to 26°C over a period of ~2.5 days to allow for acclimation to experimental temperatures. Water temperatures in the reservoir varied from the programmed temperature by ± 1°C over the course of a given week, but mean temperatures were maintained at the desired temperature. After the period of acclimation to the new temperature, beetles in all the chambers were kept at each treatment temperature for 5 days and checked daily at 10:00 AM for signs of mortality or a loss of response (LOR) to an external stimulus (gentle agitation of the chamber; see Nowlin et al. 2014). If a beetle was observed to exhibit an LOR, it was immediately removed from its chamber and placed into an individual container at initial acclimation conditions (23°C, >4 mg/L) and repeatedly observed every few hours to note whether it recovered or died.

3.2.1 Data Analysis

In order to examine potential differences among the different riffle beetle species over the course of the progressively increasing temperatures, we examined the temperature at which beetles would die. Temperatures at which individuals became unresponsive/died were compared among species using one-way ANOVA. The temperature of death of each individual of each species was considered an independent observation. We assessed assumptions of normality and homogeneity of variances prior to analysis. Significance was inferred at $p \leq 0.05$.

3.3 Upwelling Habitat Connectivity

Based on the results obtained by survival testing at SMARC and FAB (Section 4.1), all subsequent 2015 laboratory experiments examining riffle beetle responses were conducted at SMARC. All water used in experimental apparatuses in the course of this laboratory investigation originated from a series of groundwater wells operated by SMARC

3.3.1 Refugium Tanks

Specific to the upwelling experiment, two refugia tanks were constructed based on the designs of tanks used to house *H. comalensis* at 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 and oven-dried leaves from trees growing along the banks of Comal Springs (Figure 2). 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 2. Refugium tank, showing water flowing in through the top and draining out through the bottom, with rock and leaf substrates.

3.3.2 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" (Figure 3). 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 summarizes the construction and function of this system.

Figure 3. Photograph of RBASS system setup at SMARC wet laboratory showing six RBASS environmental flow chambers, upwelling flow spigots, black nylon cover, and inlet water line. The refugia tank can be seen at the far end of the green fiberglass living stream.

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 are 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. For this study, six randomly chosen EFCs were used to support two treatments.

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.

Heat Exchange Process

Water destined for the RBASS and refugia tanks was sent through a chiller and hose assembly in order to regulate water temperature by protecting against periods of possible temperature variation caused by thermal convection between warm daytime soil and underground pipes (Figure 4). The chiller was maintained at approximately 68.3°F and contained 150 feet of 5/8’’ garden hose for the RBASS inlet and a length of 50 feet of ½’’ garden hose for the refugia inlet.

Figure 4. Photograph of RBASS cover and adjacent chiller and heat exchange system.

Surface Organic Layer

To supply organic matter for the experiment originating from known *H. comalensis* habitat, Anacua leaves from Spring Run 3 at Comal Springs were collected and transported to SMARC. Leaves were then dried in SMARC's drying oven and subsequently weighed to determine equal amounts for placement in each surface organic layer (SOL) (Figure 5). Once placed within the 2 –ply mesh SOL, the bundles were allowed to condition together in a bucket supplied by the RBASS inlet water for one week prior to use in the study.

Figure 5. Photograph of Anacua leaves used in the surface organic layer (SOL) packs.

3.3.3 Study design

On September 10, 2015, 60 *H. comalensis* were collected from Comal Springs using “hand-picking” collection techniques developed by researchers at SMARC to collect wild *H. comalensis* for stocking the refugia at that facility. Beetles were brought immediately to SMARC and placed in the refugia for a weeklong acclimation period.

The RBASS unit was set up to implement a design to test the effect of the organic matter connection or disconnection on beetle survival in upwelling flow in RBASS EFCs over a 30-day period. On 21 September 2015, nine *H. comalensis* were placed in each of 6 RBASS EFCs equipped with a substrate of two equal-sized vertical layers of 2,000-micron nylon mesh layered together. Following the acclimation period, SOL packets were added to the top of each EFC and secured by the addition of an extra layer of mesh substrate (Figure 6). After one additional acclimation week, outlet lines in the middle of the three disconnection-treatment tanks were opened at a rate which allowed the formation of a gap between the water line and the SOL layer over a period of three days. Beetles were then kept in the tanks until October 21, 2015.

Treatments were randomly assigned to 6 EFCs (3 EFCs with organic matter connection and 3 EFCs without organic matter connection). Visual counts were conducted and observed beetle spatial location was plotted within each EFC every other day. Water quality parameters were recorded once per week at two depths in each EFC (upper and lower). Water quality measurements included flow (L/s), water temperature (ºC), and dissolved oxygen (DO) (mg/L), and were taken on the side of each EFC opposite the flow spigots. Additional water quality measurements were logged by thermisters (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 recording the time required to fill a 100 mL beaker from an EFC inlet line identical to those providing water to experiment EFCs.

3.3.4 Data Analysis

Survival data (proportion of survivors pooled across replicates within each treatment due to small sample size) from the trial were assessed for greater survival of beetles in connection treatments than disconnection treatments by a one tailed test for the equality of two proportions (prop. test) with continuity correction for small sample size as implemented in R version 3.0.3 (R Development Core Team 2008).

Figure 6. Photograph of EFC with surface organic layer (SOL), EFC inlet (bottom diffuser cap), EFC outlet (middle diffuser cap), and grid overlay.

3.4 Lateral Habitat Connection and Diet Study

This field portion of the study examined whether the abundance and distribution of *H. comalensis* are associated with spring openings that contain organic matter (i.e., woody material, leaves), whether they utilize the organic matter and/or biofilms on the organic matter as a food source, and whether riffle beetles have the ability to respond to changing water levels over the course of the drawdown season. Thus, this portion of the study had three objectives: (1) to determine if beetles can be sampled with the use of passive sampling pits located within areas that are considered to be "preferred" or "associated" habitat; (2) to determine if beetles populations at spring sites have the ability to change patterns in their dispersion within spring run habitats both laterally and vertically as water levels decline over the course of a summer and fall; and (3) to determine if beetles are actively using terrestrial organic matter and/or the biofilms associated with organic matter as a food source.

3.4.1 Passive Sampling Pit Study

In order to meet the first two objectives, we utilized passive sampling "pits" or "wells" that were installed into benthic substrates around spring openings that are frequently associated with the presence of *H. comalensis*. We elected to use sampling pits because they would passively sample the beetles in their habitat and not act as a "lure" that would cause them to aggregate in specific area. Currently, the technique used to collect and to enumerate beetles relies upon the use of poly-cotton cloth lures, which beetles (adults and larvae) are attracted to and the resulting densities on the cloths may be higher than in the surrounding environment. The use of sampling pits is commonly done to passively collect hyporheic and sub-surface invertebrate fauna (e.g., Clinton et al. 1996), but passive sampling pits have not been previously used to sample for *H. comalensis*. Thus, we first performed a pilot study to determine if pits would effectively collect beetles. Sampling pits were constructed of 3/4" (1.905 cm) diameter PVC that was cut into 25 cm lengths and were perforated (5 mm diameter openings) to assess vertical distribution of beetles along the 5 cm depth intervals (Figure 7). PVC spacers were inserted into the pit which separated the 5 cm vertical intervals within sampling pits. Half of the sampling pits contained a small piece of poly-cotton cloth lure (used for the collection of *H. comalensis*) and the other half did not contain the cloth lure. Wells were capped at both ends.

Figure 7. Picture of the passive sampling pits utilized to examine the vertical and lateral distribution of riffle beetles in Comal SR3.

For the pilot study, sampling pits were installed at three sites in Comal Spring Run 3 (SR3; Figure 8) by hand digging up to a depth of 50 cm on April 8, 2015. Site 1 was a running seep surrounded with gravel and cobble located under a tree near the first bridge at SR3. The second site located downstream to the first site, was a seep covered with gravel and cobbles and was shaded by a tree. Site 3 was located downstream of Site 2 and was again within a spring outflow which was shaded by a tree. Site 3 had higher composition of silt in comparison to the other two sites. For the study, we installed a total of six pits, with 2 pits (one with cloth lures and one without) at each site. After installation, sampling pits were removed from sites after 3 weeks on April 30, 2015 and the organisms present in the pits were recorded.

Figure 8. **Spring site along Spring Run 3 in the Comal system where *H. comalensis* commonly occurs. Sampling pits and Bou-Rouch collections were performed along this reach.**

3.4.1.1 Data Analysis

For this portion of the project, we were initially interested if we could capture riffle beetles using passive pit samplers. If we received positive results, then we would have expanded the study, but because we did not capture any riffle beetles in the sampling pits (Section 4.4), we did not statistically analyze the data for this initial experiment.

3.4.2 Bou-Rouch Collection Study

In addition to the installation of passive sampling pits, we also utilized another passive sampling method to examine the lateral and vertical distribution of riffle beetles in the Comal system: a Bou-Rouch sampler. We extracted eight interstitial water (and presumably organisms) samples from three sites in Spring Run 3 using Bou-Rouch method (Bou and Rouch, 1967) on April 30, 2015. Bou-Rouch sampling involves the process of sampling hyporheic water from a temporarily-installed well and a hand-pump (Figure 9). Bou-Rouch sampling is a fairly common practice to passively-sample hyporheic invertebrates (Hunt and Stanley 2000), but its effectiveness for sampling *H. comalensis* has not been explored.

Figure 9. Picture of a Bou-Rouch pump used for collections along Comal SR3.

For this portion of the study, we collected eight Bou-Rouch samples from four locations near spring openings in areas where *H. comalensis* have been previously collected using cloth lures. At each of the four locations, we collected two Bou-Rouch samples: one sample in which we installed the temporary well and pumped water for a fixed period of time and one sample in which we first bubbled CO_2 into the well for a known period before pumping water out of the well. Previous studies have expressed concern that some organisms have the ability to grasp onto larger substrates and prevent uptake during pumping (Fraser and Williams 1997), thus we elected to bubble CO_2 into ½ of the well samples in an attempt to anesthetize organisms prior to sampling.

The Bou-Rouch samples were collected by hammering a steel pipe (2.5 cm internal diameter, with 0.5 cm pores in the pipe) to a depth of 25-50 cm below the streambed. Interstitial water and fine sediment were extracted using a hand held pump attached to Bou-Rouch pipe (Bou & Rouch, 1967) and filling a 5 gallon plastic bucket (approximately 19 liters) and then filtered the contents through a 100-µm aperture mesh net. For half of the samples, we first bubbled CO_2 into the internal space of the Bou-Rouch pipe and below ground to anaesthetize organisms in the vicinity of the sampler before extracting the interstitial water. CO_2 was bubbled into the well at a relatively low pressure for a 3 minute period from a commercial CO_2 canister with a flexible vinyl tube connected to an air stone; while directing CO_2 into wells, bubbles were clearly visible emanating from around the well at a radius extending up to approximately 10 cm from around the well. All hyporheic samples collected in the mesh net were preserved using 95% ethanol after we looked through the sample to remove any species of concern (e.g., *S. pecki*). Invertebrates in each sample were sorted and identified in the lab.

3.4.2.1 Data Analysis

For the Bou-Rouch study, we examined if there were differences in the number of captured invertebrates in standard Bou-Rouch collections and CO_2-amended Bou-Rouch collections using Students t-test. We assessed assumptions of normality and homogeneity of variances prior to analysis. Significance was inferred at $p \leq 0.05$.

3.4.3 Stable Isotope Study

In order to examine the potential diet of *H. comalensis* and to assess whether lateral connectivity is important for supplying resources to the riffle beetle, we conducted a study in which we assessed the diet of the Comal Springs riffle beetle and other invertebrate consumers in the Comal Springs food web using stable isotopes of carbon (δ13C) and nitrogen (δ15N). The published literature suggests that riffle beetles in general are biofilm scrapers, but there is a diversity of biofilm scraping strategies that can be utilized by stream organisms. Thus, to determine if riffle beetles are utilizing organic matter or biofilms associated with organic matter, we collected *H. comalensis* and other organisms from sites where they are present for analysis of stable isotopes. Stable isotopes are a commonly-used method in diet studies, and the ratio of stable isotopes of carbon (13C:12C or δ13C) can be used to determine which food items a consumer it utilizing (Fry 2006; Boecklen et al. 2011; Layman et al. 2012).

In late June 2015, we collected macroinvertebrates (including adult and larval *H. comalensis*) by hand picking along Spring Run 3 and along Spring Island. Invertebrates were generally sorted in the field while alive and active and kept in vials containing water from the site for 1-2 hours to allow them to void their gut contents. Organisms were then brought back to the lab, sorted and then dried at 60^{o}C for 48 h in plastic weigh boats.

In addition to organisms, we also collected several types of basal resources that would potentially support consumers in the Comal food web. We collected in-stream detritus of two types that are present in the system: well-conditioned leaves (typically sycamore and pecan, *Carya illinoinensis,* leaves) and well-rotted wood. We chose to collect wood because *H. comalensis* (and the Comal Springs dryopid beetle, *Stygoparnus comalensis*) is frequently found on pieces of porous rotted woody material (R. Gibson and W.H. Nowlin, *pers. obs.*). Because wood was relatively rotted, we did not attempt to identify wood to species. Both leaf and wood samples were collected in at least triplicate from each location (SR3 and Spring Island). Wood and leaf material was stored in plastic bags and brought back to the lab, where it was dried at 60^{o}C for 48 h in plastic weigh boats. We additionally collected at least 6 relatively flat rocks from each location for algal biofilms. Rocks were placed in plastic bags within a cooler and brought back to the lab to remove biofilms. Biofilms were removed by scrubbing the upper rock surface with a nylon bristled brush and washing the material with DI water into a clean HDPE beaker. A portion of this slurry was then filtered onto ashed and pre-weighed 25-mm diameter

Whatman GF/F filters; for each rock, we collected two filters. One filter (to be used for δ15N analyses) was immediately placed into an oven and at dried at 60°C for 48 h. The other filter was placed into a fuming HCl chamber for at least 8 hours to remove inorganic precipitated carbonates from the algal biofilms, which affect bulk δ13C values (Pound et al. 2011); this filter would be used for δ13C values only. After fuming, filters were dried at 60°C for 48 h.

After all samples were sorted, treated and dried, we prepared samples for analysis at the University of California – Davis Stable Isotope Facility. All organisms, leaves, and wood samples were homogenized and ground to a fine powder using a mill grinder or a mortar and pestle that was thoroughly cleaned between samples. In a few cases, individual organism were of great enough mass for individual analysis, but in most cases we were forced to create composite samples of 2-10 individuals so that there was enough mass to run ^{13}C and ^{15}N analyses. For all organisms, leaves, and wood samples, we analyzed at least 3 and up to 6 independent samples for stable isotopes of C and N after packing homogenized material into tin capsules. We folded and packed algal filters into tin capsules for analysis.

Stable isotopes of C and N were analyzed using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Samples were combusted at 1000°C in a reactor packed with chromium oxide and silvered copper oxide. Following combustion, oxides were removed in a reduction reactor (reduced copper at 650°C). The helium carrier then flowed through a water trap (magnesium perchlorate) and the resulting N_2 and CO_2 were separated on a Carbosieve GC column (65°C, 65 mL/min) before entering the IRMS. Glass fiber filters were analyzed for ^{13}C and ^{15}N isotopes using an Elementar Vario EL Cube or Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Samples were combusted at 1000°C in a reactor packed with copper oxide and lead chromate. Following combustion, oxides were removed in a reduction reactor (reduced copper at 650°C). The helium carrier then flowed through a water trap (magnesium perchlorate). N_2 and CO_2 were separated using a molecular sieve adsorption trap before entering the IRMS. For each isotope run, samples were interspersed with several replicates of at least two different laboratory standards. The laboratory standards, which are selected to be compositionally similar to the samples being analyzed, were previously calibrated against NIST Standard Reference Materials (IAEA-N1, IAEA-N2, IAEA-N3, USGS-40, and USGS-41). Each sample's preliminary isotope ratio is measured relative to reference gases analyzed with each sample. These preliminary values are finalized by correcting the values for the entire batch based on the known values of the included laboratory standards. The long-term standard deviation during analysis is ± 0.2‰ for ^{13}C and ± 0.3‰ for ^{15}N. Final delta values are expressed relative to international standards Vienna PeeDee Belemnite for C and Air for N.

3.4.3.1 Data Analysis

Stable isotope data for the various basal food resources (leaves, rotted wood, and algae) and for consumer species (including *H. comalensis*) were initially compared between sites (Spring Run 3 and Spring Island) to determine if there were any longitudinal changes in stable isotope values for $\delta^{13}C$ and $\delta^{15}N$ as water moved from Spring Run 3 to the Spring Island area. Food resources of the same type and the same species were compared between sites using separate one-way ANOVAs for $\delta^{13}C$ and $\delta^{15}N$ values. We also initially compared the various potential basal food web resources (leaves, rotted wood, and algae) within a given site using separate one-way ANOVAs for $\delta^{13}C$ and $\delta^{15}N$ values. We assessed assumptions of normality and homogeneity of variances prior to analysis and significance was inferred at $p \leq 0.05$.

In order to determine the likely basal food resources for consumers in the food web, we utilized a Bayesian inference approach to food source determination using the stable isotope composition of the various food sources and consumers. Bayesian approaches to isotopic mixing models are becoming more widely used (Layman et al. 2012) because, unlike traditional linear mixing models, they allow us to incorporate sources of variability within the model, permit the analysis of multiple dietary sources for consumers, and generate potential dietary solutions as true probability distributions (Parnell et al. 2010). Although, there is some debate about the relative importance and kinds of assumptions that must be made with the use of isotopic mixing models, many ecologists are utilizing Bayesian approaches because of their utility when compared to traditional models.

In order to determine the relative importance of different basal food resources to different consumers in the Comal Springs foodweb, we followed the "best practices" guidelines as described in Phillips et al. (2014). We ran all isotope mixing models in the program Stable Isotope Analysis in R (SIAR) Version 4 (Inger et al. 2014). We first identified the potential basal food resources as leaves, rotted wood, and epilithic algae (periphyton) and used these as our resources in models (see Results below). Trophic enrichment factors (TEFs) for the models were taken from a meta-analysis of the literature (Caut et al. 2009) and used the mean (± 1SD) trophic enrichment factors of 1.33 ‰ ± 0.454 and 2.75 ‰ ± 1.637 for ^{13}C and ^{15}N, respectively. These values are the Δ13C for all "freshwater organisms" and the "overall" Δ15N for all organisms reported by Caut et al. (2009). We ran isotope mixing models to determine the proportional contribution of each basal food resource to each consumer species in the food web. We initially ran separate models for the Spring Run 3 and Spring Island food webs, but here we only report on the results of the mixing model that was run on the overall Comal Springs system, which includes consumers from both reaches. However, some consumer species appeared to exhibit differences in the basal food resources between the two sites (Section 4.4) and these differences were identified when we ran the initial ANOVAs examining spatial differences in $\delta^{13}C$ and $\delta^{15}N$ values in the same consumer species.

4.0 Results

4.1 Potential Water Quality Differences between SMARC and FAB

Water quality conditions (e.g., temperature, DO, and conductivity) within each site did not exhibit any large variation or display any values that would indicate that conditions were inappropriate for holding riffle beetles. At SMARC, we logged conditions from March 2, 2015 until March 8, 2015 when the sonde discontinued gathering data (Figure 10). However, during this time period there was minimal loss of riffle beetles at SMARC (in contrast to FAB; see below), so we discontinued collecting water quality data from SMARC. During the period of data collection at SMARC the mean temperature was 20.62°C and only varied from 20.01 – 21.28°C (Table 1). In general, temperature increased but only by approximately 1°C over the time period. Dissolved oxygen and conductivity also exhibited minimal changes over the same time period, with mean DO and conductivity being 5.05 mg/L (range = 4.59 – 6.01 mg/L) and 554 µS/cm (range = 539 – 564 µS/cm), respectively (Figure 10; Table 1).

Table 1. Mean, median, minimum, maximum, and the coefficient of variation (CV; as a percent) of the water quality parameters measured at SMARC and FAB during the period of observation at each location.

	SMARC			FAB		
	Temperature (oC)	Conductivity (µS/cm)	DO (mg/L)	Temperature (oC)	Conductivity (µS/cm)	DO (mg/L)
Mean, Median	20.62, 20.51	553.94, 562.00	5.05, 5.08	22.21, 22.25	574.45, 574.83	6.82, 6.83
Min, Max	20.01, 21.28	539.00, 564.00	4.59, 6.01	21.86, 22.45	559.00, 583.34	6.56, 7.06
CV	1.98	1.98	5.47	0.71	1.07	1.07

At FAB, water quality conditions were logged from March 2, 2015 until April 14, 2015, well after the conclusion of the survival trials (Figure 11; see survival trial results below). FAB wet lab water was also of relatively high quality (as determined by the measured parameters) and did not exhibit any wide temporal changes over the recoding period. FAB mean temperature was 22.21°C and varied from 21.86 – 22.45°C (Table 1). Over the time period, temperature of FAB water appeared to oscillate slightly, but this change was <1°C over the time period. Dissolved oxygen and conductivity also exhibited minimal changes over the same time period, with mean DO and conductivity being 6.82 mg/L (range = 6.56 – 7.06 mg/L) and 574 µS/cm (range = 559 – 583 µS/cm), respectively (Figure 11; Table 1). Over the time period, the conductivity at FAB increased slightly (Figure 11), but this increase was minimal (~30 µS/cm) and could be entirely due to drift associated with the extended deployment of the sonde.

Although both SMARC and FAB exhibited fairly similar water quality conditions with similar lack of temporal variability, there were profound and striking differences in the survival of riffle beetle adults at each location. Of the 25 individual *H. comalensis*, *H. glabra*, and *M. pusillus* that were initially placed at SMARC on March 16, 2015 (for *H. comalensis* and *M. pusillus*) and March 23, 2015 (for *H. glabra*), only 1 *M. pusillus* died and all other individuals were alive after several weeks. In contrast, mortality of individuals of all three species was observed within 3 days of introduction to FAB. By March 31, 2015, all individuals of all three species at FAB had died.

Examination of the water quality data for FAB during this period does not reveal any rapid fluctuations or exhibit any values that would be immediately distressing for riffle beetles (i.e., drop of DO near 0 mg/L, sudden increase in temperature to >30°C; Figure 11). Thus, at that point, we elected to continue and perform all subsequent laboratory studies associated with this project at SMARC.

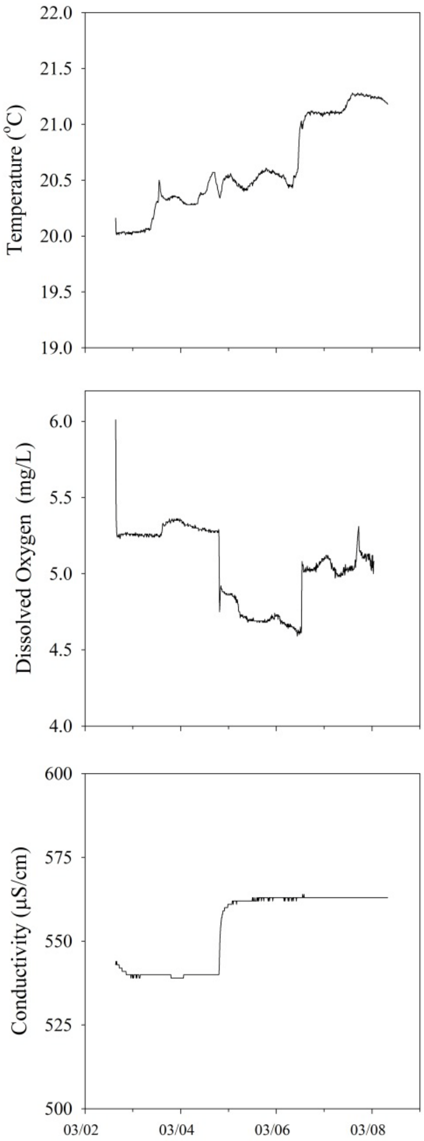


Figure 10. **Temporal changes in the water quality parameters measured at SMARC during the experimental period. The data sonde terminated measurements on March 8, 2015; however, a majority of the beetles were still alive at SMARC facility more than 6 months after the period of observation ended.**

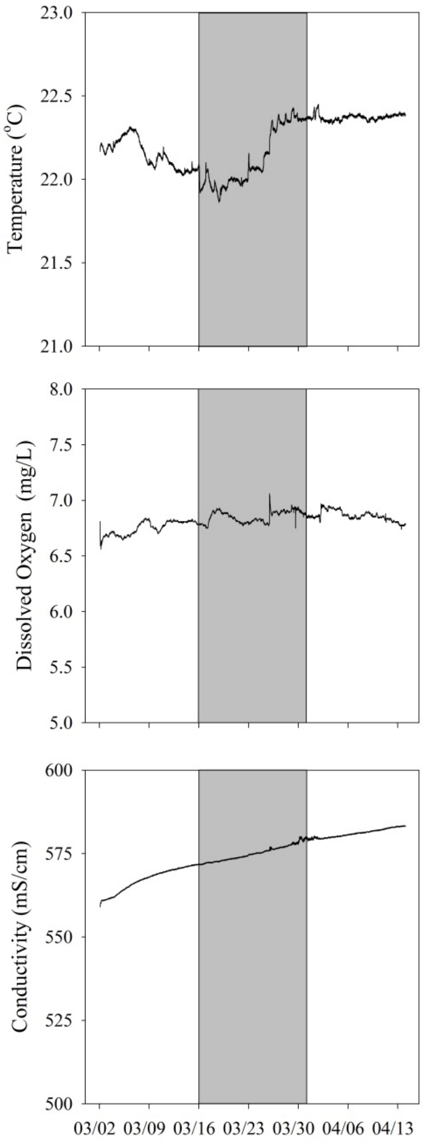


Figure 11. Temporal changes in the water quality parameters measured at FAB during the experimental period. The sonde measured water quality parameters throughout the period of beetle observation. The period of beetle observation is indicated by the shaded time period in each panel. All beetles were dead by the end of March.

4.2 Surrogate Testing for Effects of Water Temperature

During the experiment in which individuals of each species were exposed to gradually increasing water temperatures, the temperatures achieved in the experimental set-ups was relatively close to the desired set-points. In the beginning of the experiment and during the initial 7-day acclimation period, all individuals of all of species survived until the first increase in temperature (Figure 12). However, once the first temperature adjustment began and the temperature was increased to 26^{o}C, individuals of both *H. comalensis* and *H. glabra* died. As the temperature further increased over the 35-day experiment, both *H. comalensis* and *H. glabra* individuals died until all individuals were dead by 29^{o}C. In contrast, *M. pusillus* exhibited a different mortality pattern as temperatures increased (Figure 12). All *M. pusillus* individuals survived until 28^{o}C and then individuals began to quickly die after temperatures were increased beyond that threshold (all individuals were dead by 30^{o}C). These differences in temperature-dependent survival translated into significantly different temperatures among species for individual deaths ($F_{2,12} = 4.84$; $p = 0.029$), with *M. pusillus* exhibiting significantly higher impairment temperatures than *H. glabra* (Tukey HSD; $p = 0.023$). However, results of the post-hoc analyses indicated that *H. comalensis* was not significantly different than *M. pusillus* (Tukey HSD; $p = 0.301$) or *H. glabra* (Tukey HSD; $p = 0.301$).

These relatively long-term temperature-dependent survival thresholds are consistent with previous work that examined upper temperature limits in *H. comalensis* and *H. glabra* (Nowlin et al. 2014). In relatively short-term temperature experiments (temperature change over a few hours) on these species, Nowlin et al. (2014) found that both *H. glabra* and *H. comalensis* exhibited evidence of stress (increased movement around the experimental chamber) when temperatures crossed a threshold of 28 – 31^{o}C. The same study conducted longer-term temperature experiments (change over a few days) with *H. glabra*, and found that LOR thresholds were 34 - 36^{o}C. Nowlin et al. (2014) concluded that *H. comalensis* and *H. glabra* likely experience physiological stress starting at temperatures ~28^{o}C and that the longer beetles spend at these temperatures, the effect is likely to be cumulative. Indeed, the "thresholds" we found for all three species in the present study are also in line with thresholds reported for other plastron utilizing beetle species (i.e., Harpster 1941, 1944).

In the present study, we found that *M. pusillus* exhibited a slightly higher mortality threshold temperature (onset of mortality at 29^{o}C) than the two *Heterelmis* species (mortality onset occurred at 26-27^{o}C). These results make sense in light of the types of habitats these species are associated with in natural systems. It has been hypothesized that organisms living in thermally stable environments, such as subterranean systems, the deep oceans, and spring-influenced ecosystems should be stenothermal (having a narrow thermal tolerance range) (Mermillod-Blondin et al. 2013). Both *H. glabra* used in this research and *H. comalensis* are associated with spring outflows, but *M. pusillus* is found in a variety of habitats often with less thermally-stable environments. Our results are consistent with this hypothesis and indicate that *M. pusillus* is less sensitive to longer-term temperature changes than *H. comalensis* and *H. glabra*. These results also suggest that *M. pusillus* is likely not the best candidate to serve as a surrogate species in physiological stress studies associated with variation in environmental conditions, especially water temperature.

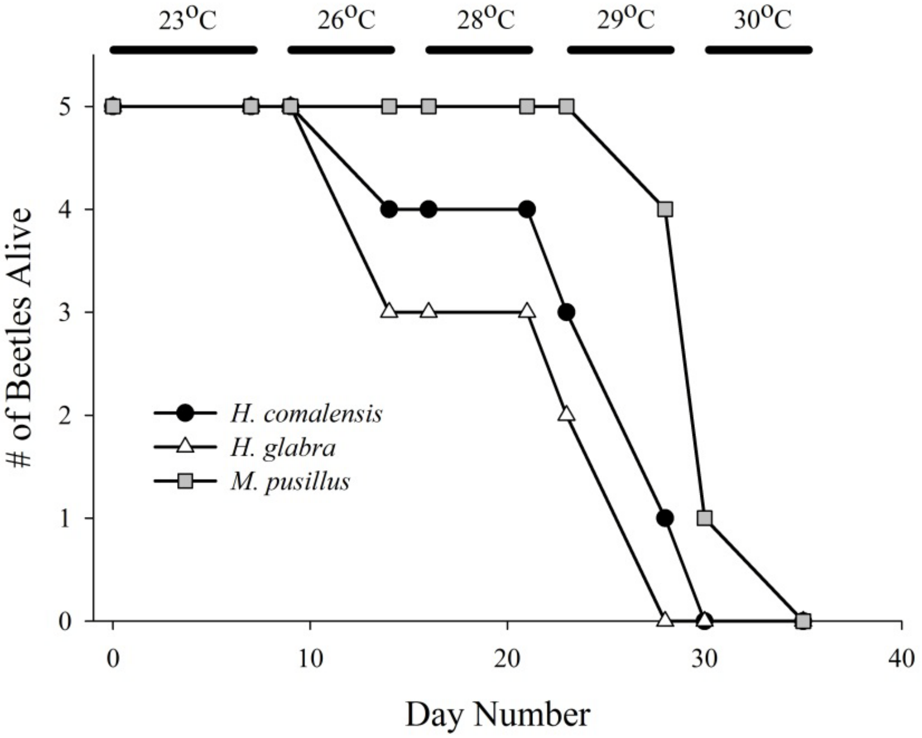


Figure 12. Time series of the results of the longer-term gradual temperature increase surrogate testing study. The number of beetles alive (n = 5 initially) of each species is plotted as a function of time. The time period of consistent temperature maintenance is indicated by the dark lines across the top of the graph and the intervening short-duration periods of increasing to the next temperature are indicated by the gaps in the lines.

4.3 Upwelling Habitat Connectivity

Discharge was represented as continuous water movement through each EFC at a constant rate that did not cause riffle beetle agitation. Several preliminary trials were conducted to determine levels at which beetles would become disoriented or dislodged from the mesh substrate and fall to the bottom of the EFC. Several trials were also conducted to make sure that the discharge rates would stay constant between treatments after water levels were dropped with flow exiting the EFC through the middle outlet versus the top of the EFC as for the connected treatments.

Overall, discharge within each EFC remained constant and similar to each other over the course of the experiment ranging from approximately 0.000084 to 0.000095 cfs. Discharge was measured in the EFCs as the time (approximately 37 to 42 seconds) it took to fill a 100 mL beaker. Measurements were based on an average of 5 reps conducted weekly over the course of the experiment in conjunction with the standard water quality parameter measurements.

Table 2 shows the standard water quality measurements (DO and temperature) that were taken weekly over the course of the experiment. As shown in Table 2, measurements across all treatments remained very consistent with DO ranging from 4.19 to 4.85 mg/L and water temperature from 21.4 to 22.2 °C over the course of the experiment. Figure 13 shows the continuous (10-minute intervals) thermister data from the RBASS inlet and outlet locations which further supports the water temperature conditions experienced within the EFCs.

Table 2. Standard parameter water quality results per treatment during the experiment.

Date	Time Range	EFC No. and Panel Section								
		EFC 1 Disconnected	EFC 2 Connected		5 Disconnected	8 Connected		9 Disconnected	12 Connected	
		lower	lower	upper	lower	lower	upper	lower	lower	upper
		Dissolved Oxygen (mg/L)								
10/6/2015	14:30 to 16:25	4.36	4.3	4.24	4.32	4.3	4.22	4.28	4.38	4.25
10/13/2015	13:47 to 14:04	4.81	4.75	4.69	4.85	4.72	4.66	4.71	4.78	4.67
10/20/2015	15:59 to 16:41	4.36	4.23	4.23	4.33	4.26	4.19	4.24	4.27	4.21
		Water temperature (°C)								
10/6/2015	14:30 to 16:25	21.6	21.6	21.6	21.9	21.6	21.7	21.5	21.8	21.8
10/13/2015	13:47 to 14:04	22.0	21.8	21.9	21.9	21.7	21.8	21.4	22.0	22.2
10/20/2015	15:59 to 16:41	21.7	21.5	21.5	21.6	21.6	21.7	21.5	21.8	21.8

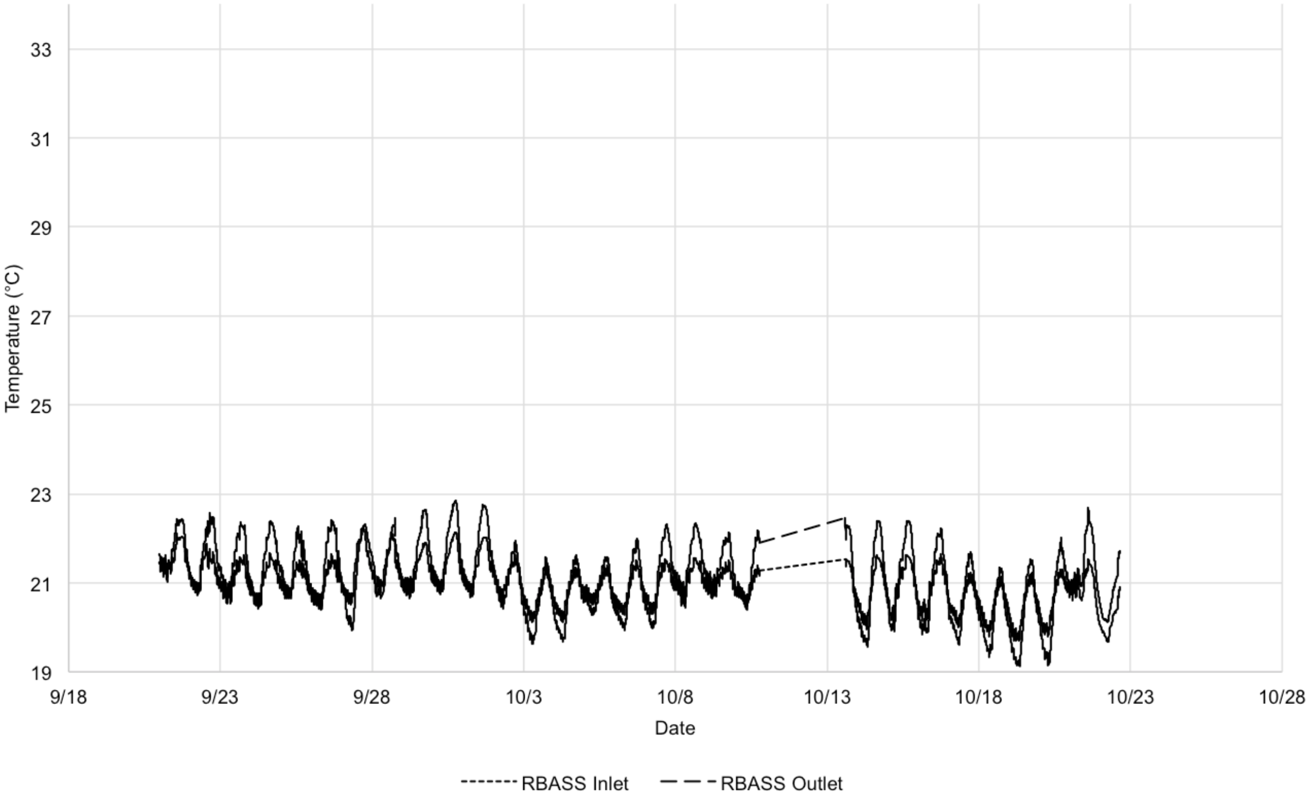


Figure 13. **Time series plot of RBASS inlet and outlet temperature (°C) over course of experiment.**

Following the termination of the study on October 21, 2015, the experiment tear down procedure was performed as follows. The beetle position within each SOL was noted. Each SOL was removed and cut with scissors and placed in a labeled cup. Each SOL was carefully taken apart and leaves examined for beetles, leaf by leaf. The EFC mesh (substrate) panel was then removed and placed on a labeled white tray. Beetle locations and activity of each beetle (i.e. "active" = crawling around; "inactive' = motionless) was logged to the extent practicable. Once retrieved all beetles were placed in cups with refugia water (Figure 14). Any beetles remaining in the EFC were removed via a long-handled acrylic fan-bristle brush (Figure 14). The number of beetles was counted and recorded, locations logged and all recovered beetles were placed in recovery bags inside the refugia tank. Recovery bags consisted of aquarium filter bags labeled for each EFC tank which were subsequently held in the refugia for 24 hours, before final determination of survival. Upon completion of the study, all live beetles were donated to SMARC.

Figure 14. Comal Springs riffle beetles during experiment tear down.

Table 3 presents the survival results from the experiment. Twenty-six of 27 beetles survived in EFCs that maintained flowing water connection through the SOL, whereas a total of 7 beetles died in the EFCs that were disconnected via water flow from the SOLs. Results of the one tailed test of proportions supported the conclusion that survival was higher for individuals in connected treatments (p = 0.028). Even when the data is subjected to a less powerful 2 tailed test it is still significant at the 90% level (p=0.055).

Table 3. Results of upwelling connectivity study in terms of survival per treatment.

EFC No.	Treatment	Comal Springs riffle beetles		
		Stocked	Survived	Percent survival
1	Disconnection	9	9	100
2	No disconnection	9	8	89
5	Disconnection	9	5	56
8	No disconnection	9	9	100
9	Disconnection	9	6	67
12	No disconnection	9	9	100
	Total	54	46	85

SUMMARY		
Treatment	Average Survival	STDEV
Disconnection	74.07	23.13
No disconnection	96.30	6.42

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 every other day. When practical, visual identification was conducted under the cover to minimize potential disturbance to the beetles from light exposure (Figure 15). Even so, 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 interpretation of this data to a brief qualitative discussion presentation of all movement data (Figure 16, Appendix A).

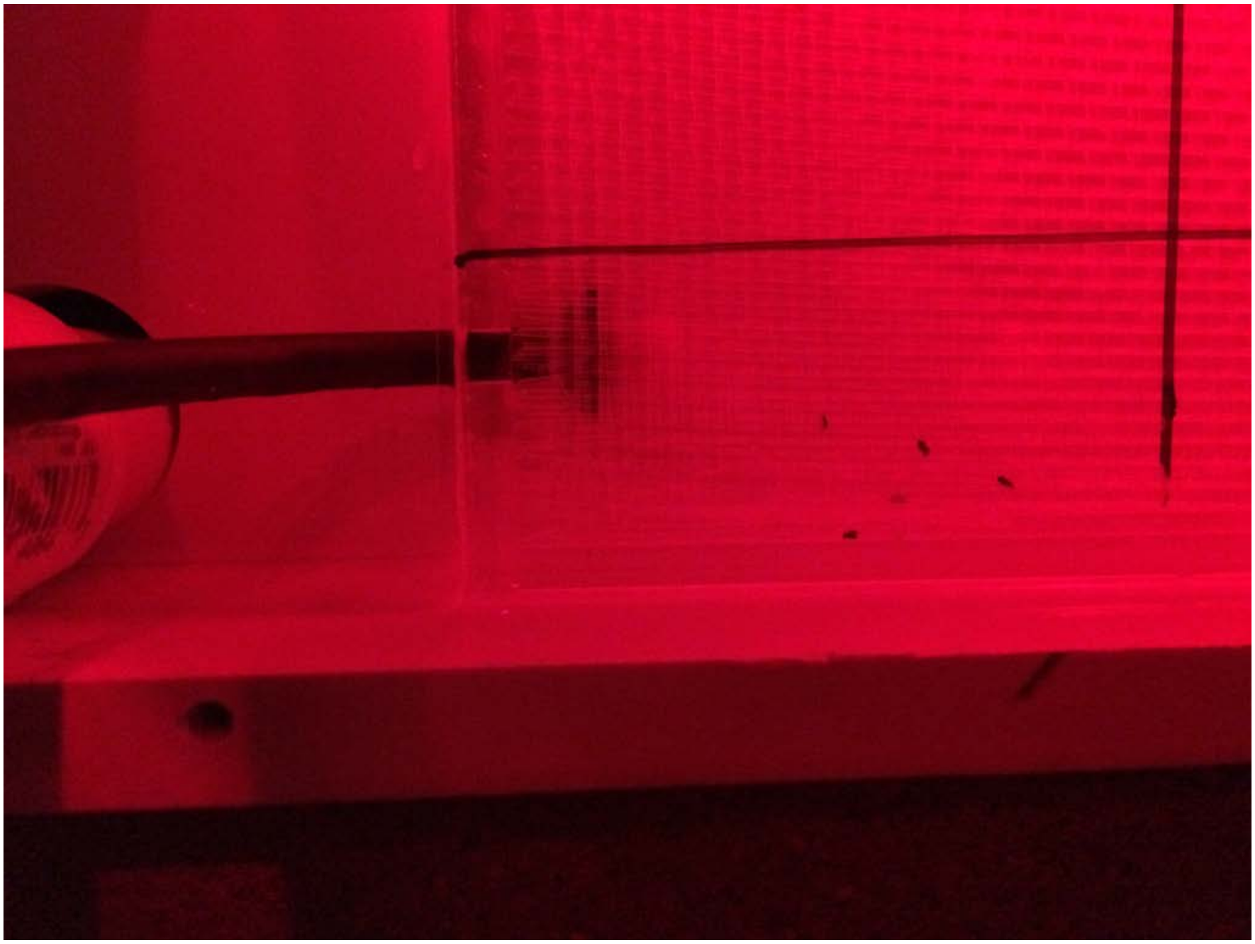
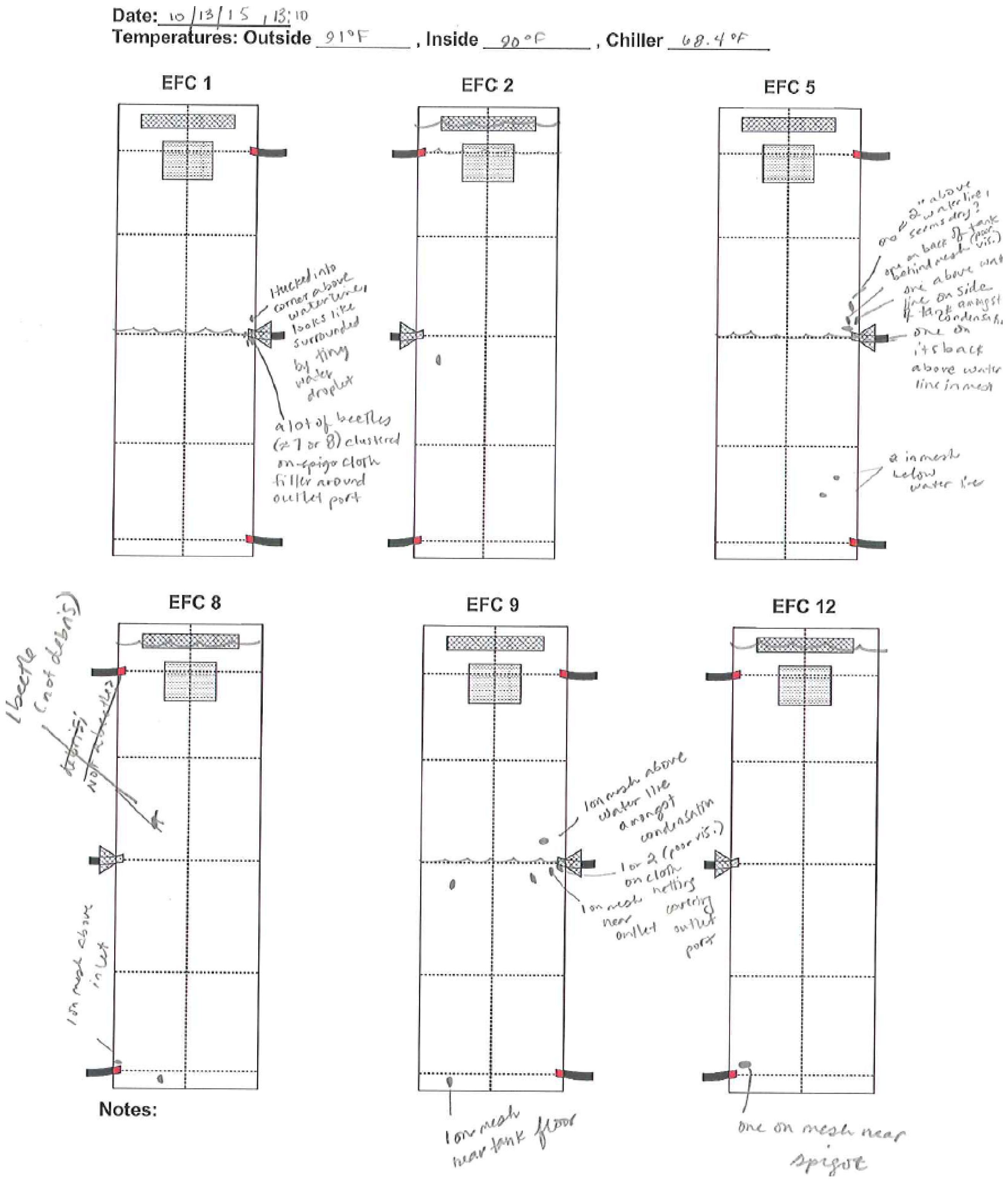


Figure 15. Location documentation conducted under the cover.

In general, qualitative observations and documentation over the course of the experiment suggest that beetles in EFCs with flowing water connected to the SOL tended to explore the entire tank, but often returned to the SOL. As such, a lot of the documented observations for these treatments show very few beetles outside of the SOL. In contrast, the beetles in the EFCs that were disconnected from the SOL spent the majority of time in the concentrated flow around the inlet and outlet in their respective tanks. We speculate that this might have occurred for a couple of reasons. The first is that the screen material blocking the outlet did build up a small amount of organic material over the course of the study. We mention this in that our 2014 riffle beetle work with *H. glabra* (BIO-WEST 2014) often had beetles attaching themselves above the water line to fine mesh that had organic material on it. Another possible explanation is that in the absence of organic matter, the beetles were simply attracted to areas of water flow / current.



Date: 10/13/15, 13:10

Temperatures: Outside 91°F, **Inside** 90°F, **Chiller** 68.4°F

Notes:

Figure 16. Beetle spatial location observations from October 13, 2015.

4.4 Lateral Habitat Connection and Diet Study

Passive Sampling Pit Study
In the passive sampling pit study, the installed wells collected relatively few organisms over the three-week period (Table 4). Although this time period is consistent with other studies (e.g., Clinton et al. 1996), the numbers of organisms was relatively low (1 – 4 individual taxa collected per pit). In total, pits only collected three taxonomic groups or species (*Stygobromus pecki*, *Psephenus* sp., and Lumbriculid worms) from depths of 20 – 25 cm. Although differences in the number of each species and the total number of invertebrates among pits with and without lures did not significantly differ (one-way ANOVA; $F_{1,5} \geq 3.00$, $p \geq 0.116$ for all analyses), organisms were only collected from pits without cotton-poly lures. Most importantly, however, we did not collect any adult or larval riffle beetles in sampling pits over the three-week period. Although all of these sites are commonly associated with the presence of riffle beetle adults and larvae, the low densities of individuals we collected in samplers and the lack of riffle beetles in this portion of the study (the initial pilot study), we elected to not continue with a more complex and labor intensive monitoring of riffle beetle lateral and vertical movement within the benthos.

Table 4. The mean number (min – max) of invertebrates collected over a 3-week period in passive sampling pits without cloth lures and with cloth lures in Spring Run 3.

	W/O Lure (n = 3)	W/ Lure (n = 3)
S. pecki	0.7 (0 - 1)	0 (0)
Psephenus	2.0 (0 - 4)	0 (0)
Lumbriculidae	0.7 (0 - 1)	0 (0)

Bou-Rouch Collection Study
In the portion of the study that preliminarily examined the efficacy of using a Bou-Rouch sampler to assess vertical and lateral movement of riffle beetles within substrates, we collected eight samples from locations in which adult and larval beetles are frequently encountered. Much like the collections made with the passive sampling pits, the numbers of organisms collected per sample was relatively low (Table 5). The specific taxa collected with the Bou-Rouch were similar to those collected with the sampling pits, but overall number of taxa per sample was in general greater in Bou-Rouch samples. Overall, we detected a total of 5 species/taxa within samples (*Stygobromus* sp., *Psephenus* sp., Lumbriculid worms, *Lircelolus* sp., and *Tarebia granifera*). There were no significant differences between the number of individual taxa and the total number of individuals across all taxa when the samples collected with CO_2 and those collected without CO_2 were compared (one-way ANOVA; $F_{1,7} \geq 0.18$, $p \geq 0.356$ for all analyses).

Table 5. The mean number and range (min – max) of invertebrates in Spring Run 3 collected with a Bou-Rouch sampling without the addition of CO_2 prior to pumping and with the addition of CO_2 prior to pumping.

Treatment	Taxon	Mean	Range
No CO_2	*Stygobromus* sp.	0.25	(0 - 1)
	Psephenus sp.	0.25	(0 - 1)
	Lumbriculidae	0.5	(0 - 1)
	Lirceolus spp.	0.25	(0 - 1)
	Tarebia granifera	0.25	(0 - 1)
	Total invertebrates		(0 - 3)
CO_2 added	*Stygobromus* sp.	0.25	(0 - 1)
	Psephenus sp.	0.5	(0 - 2)
	Lumbriculidae	0.25	(0 - 1)
	Total invertebrates	1	(0 - 4)

Although we took into account and standardized several of the critical variables that can influence the capture/collection rate of Bou-Rouch samples (e.g., volume collected, pore size of the sampler), we did not directly account for other variables that might affect collection efficiency, such as variation in sediment porosity and a direct estimate of the rate of sample collection (i.e., pumping rate) (Hunt and Stanley 2000). Our estimated pumping rates (approximately 4-6 L/min) were relatively high and should have produced higher invertebrate yields (Hunt and Stanley 2000). Most importantly, however, we did not collect any adult or larval riffle beetles in samples. Given the absence of riffle beetles in the samples we collected, we elected to not continue with a more complex and labor intensive monitoring of riffle beetle lateral and vertical movement within the benthos using the Bou-Rouch method. The lack of beetles in Bou-Rouch samples could be a consequence of several morphological or physiological adaptations of the beetles, including the ability of beetles to cling tightly to flowing surfaces and an ability to tolerate relatively high CO_2 levels in an already CO_2 saturated environment.

General Comments on Passive Collection Methods and Future Riffle Beetle Studies

Current techniques for collection of *H. comalensis* are limited to two main methods: the use of cotton-poly lure cloths and through hand-picking them from surfaces. Intensive searching efforts indicate that riffle beetles in the Comal system tend to be of relatively low abundance (a few individuals per square meter; R. Gibson, *pers. obs.*), so digging by hand through substrates and hand picking individuals is very time and labor intensive. Thus, this technique is likely not the most efficient when trying to collect riffle beetles for monitoring or population/density estimates. Indeed, it may take several hours of searching and disturbing the substrates around spring openings to collect a few individuals (W.H. Nowlin, *pers. obs.*). However, hand-picking may be the preferred method if there is a need to collect a few individuals or if an investigator is not concerned with disturbing substrates.

The use of cloth lures is now the “established” method for monitoring beetle numbers in the Comal system because beetles tend to congregate on the lures at densities higher than the surrounding environment. However, the lure presumably serves as an attractant and therefore concentrates beetles in a given location, thereby affecting the ability of investigators to make reliable estimates of population density or size. Thus, there has been an increased interest in trying to find "passive" sampling methods for beetles that would allow researchers and management personnel to gain increased ability to make reliable density estimates. Based upon the two preliminary studies we conducted, it appears that passive sampling pits or wells and Bou-Rouch samples are not likely to be efficient methods to estimate beetles.

Stable Isotope Study

As a part of the stable isotope study, we collected individuals of 13 different taxa for analysis, including several species that are of concern in the EAHCP (i.e., *Stygobromus pecki*, *Stygoparnus comalensis*, and *H. comalensis*) (Table 6). The suite of taxa included a diversity of different feeding types, so that we could elucidate most of the major feeding groups within the invertebrates found in the Comal system. We initially assessed the food web at the two sampling locations separately (Spring Run 3 and Spring Island). We analyzed a total of 10 taxa in both Spring Run 3 and Spring Island.

The food web of Spring Run 3 included a diversity of consumers that utilized differing proportions of the three *a priori* identified basal food resources (i.e., periphyton, conditioned leaf litter, and partially rotted wood). In terms of the basal food resources that likely support the food web, in Spring Run 3, both the δ^{13}C and δ^{15}N values differed among the basal food resources (δ^{13}C: one-way ANOVA, $F_{2,9} = 15.18$, $p = 0.001$; δ^{15}N: one-way ANOVA, $F_{2,9} = 3.98$, $p = 0.047$) (Figure 17). Periphyton δ^{13}C in periphyton was relatively deplete (-31.5‰) when compared to wood (-27.31‰). However, well-conditioned leaf material exhibited δ^{13}C values (-30.62‰) that did not significantly differ from that of periphyton (Tukey HSD, $p > 0.05$), but was significantly different from that of wood (Tukey HSD, $p < 0.05$). In contrast, the δ^{15}N values of periphyton and wood were not different from one another (Tukey HSD, $p < 0.05$), while leaves had a significantly enriched δ^{15}N when compared to wood and periphyton (Tukey HSD, $p < 0.05$).

The consumers in Spring Run 3 appeared to be grouped into two general food chains within the overall food web. One group of consumers, which was composed of *Psephenus* sp., *M. pusillus* (adults and larvae grouped together), baetid mayfly nymphs, and helicopsychid caddis fly larvae, were likely feeding directly on periphyton. In contrast, the other main food chain was likely feeding on wood-based biofilms and was composed of *H. comalensis* (adults and larvae grouped together) and *Hyalella*. Elevated above these two groups (as indicated by relatively enriched δ^{15}N values were snail species (*Elimia*, *Tarebia*, and *Melanoides*). Snails in stream systems often exhibit somewhat elevated δ^{15}N values (e.g., Pound et al. 2011) because they may be less selective grazers than other algivorous invertebrate groups, such as psephenids (Anderson and Cabana 2007). *Stygobromus pecki* was the consumer exhibiting the highest trophic position in the SR 3 community, indicating that it is likely predatory on other invertebrates in the system.

Table 6. **Mean and ± 1 SE values, and the number of samples analyzed to determine δ13C and δ15N of consumers and potential basal food resources collected at the two sites (Spring Run 3 and Spring Island) in the Comal system. Consumers with an * after their name had an *n* = 1 and were not analyzed within an individual site, but were combined into the same taxonomic group when the entire Comal system food web was analyzed. Species with (all) indicates the mean value of adults and larvae were pooled for the individual site, but adult and larvae were split out for the entire Comal system analysis.**

Spring Run 3

Consumer	δ13C ‰	± 1 SE	*n*	δ15N ‰	± 1 SE	*n*
Baetidae	-34.814	0.223	4	4.682	0.153	4
Elimia sp.	-32.855	0.516	3	7.771	0.069	3
Helicopsychidae	-34.042	0.367	5	4.535	0.247	5
Heterelmis comalensis (all)	-26.674	1.618	3	4.965	0.430	3
Hyalella azteca	-27.997	0.971	4	5.330	0.094	4
Melanoides tuberculata	-28.066	0.683	3	7.682	0.275	3
Microcylloepus pusillus (all)	-35.067	0.152	5	4.471	0.241	5
Psephenus sp.	-35.142	0.202	4	3.277	0.303	4
Stygobromus pecki	-27.761	0.726	4	9.404	0.225	4
Tarebia granifera	-31.045	0.509	3	8.218	0.206	3
Source						
Leaves	-30.619	0.360	3	5.670	1.018	3
Perphyton	-31.591	0.722	4	3.729	0.198	4
Wood	-27.086	0.132	6	4.026	0.069	3

Spring Island

Consumer	δ13C ‰	± 1 SE	*n*	δ15N ‰	± 1 SE	*n*
Baetidae*	-31.053	--	1	6.994	--	1
Elimia sp.	-32.214	0.400	4	6.910	0.148	4
Helicopsychidae	-33.117	0.452	4	4.570	0.114	4
Heptageniidae	-30.574	0.972	4	6.036	0.172	4
Heterelmis comalensis (adult)	-26.559	0.441	3	4.571	0.089	3
Heterelmis comalensis (larvae)	-27.349	0.321	5	4.817	0.151	5
Hyalella azteca	-32.005	0.191	3	5.596	0.182	3
Microcylloepus pusillus (adult)	-33.711	0.504	5	4.434	0.065	5
Microcylloepus pusillus (larvae)*	-32.438	--	1	4.683	--	1
Psephenus sp.	-33.699	1.610	5	2.940	0.353	5
Stygobromus pecki	-30.339	0.462	4	9.275	0.293	4
Stygoparnus comalensis	-24.230	0.059	4	5.564	0.115	4
Tarebia granifera	-31.246	0.260	4	6.937	0.166	4
Source						
Leaves	-29.314	0.077	3	3.966	0.096	3
Perphyton	-31.435	1.192	4	4.138	0.144	4
Wood	-27.086	0.132	6	4.026	0.069	3

The food web in the Spring Island section of the Comal system was similar to the patterns found in Spring Run 3 (Figure 17). Again, the basal resources (periphyton, leaves, and wood) exhibited significantly different $\delta^{13}C$ values (one-way ANOVA, $F_{2, 12} = 13.04$, $p = 0.002$). Again, wood exhibited significantly enriched $\delta^{13}C$ values when compared to periphyton (Tukey HSD, $p < 0.05$), but was not significantly different from leaves (Tukey HSD, $p > 0.05$). In addition, leaf periphyton $\delta^{13}C$ values were intermediate to those of periphyton and wood and not significantly differ from those of periphyton $\delta^{13}C$ values (Tukey HSD, $p > 0.05$). In contrast, the $\delta^{15}N$ values did not differ among sources in the Spring Island site (one-way ANOVA, $F_{2, 9} = 0.570$, $p = 0.590$). The consumers in the Spring Island food web exhibited a similar configuration to SR3. Again, *Psephenus*, *M. pusillus*, and Helicopsychidae were associated with periphyton and larval and adult *H. comalensis* were more closely associated with wood. In addition, *Stygoparnus comalensis* was also closely associated with wood, as indicated by its enriched $\delta^{13}C$ value. Heptageniid mayfly nymphs and the snails *Tarebia* and *Elimia* were more associated with periphyton (as indicated by $\delta^{13}C$ values), but exhibited a higher estimated trophic position as estimated by $\delta^{15}N$ values. Again, less-selective grazers Heptageniidae and snails often exhibit higher $\delta^{15}N$ values than other grazers such as psephenids (Anderson and Cabana 2007).

However, in contrast to the SR3 site, the two amphipods (*Hyalella* and *Stygobromus pecki*) appeared to shift over more toward the periphyton-based food chain at the Spring Island site, with the $\delta^{13}C$ values of both of these consumers getting significantly more deplete (and therefore more reflective of an algal-based signature) in the downstream Spring Island site (*Hyalella*: one-way ANOVA: $F_{1, 7} = 16.40$, $p = 0.007$; *S. pecki*: one-way ANOVA: $F_{1, 7} = 8.97$, $p = 0.024$). The shift in basal resources for these two species may indicate some spatial variation and plasticity in their feeding strategy as the canopy cover opens up downstream at Spring Island.

We then combined the data from both sites to create the food web for the overall Comal system (Figure 18), but still reflected the shift in basal resource food chains associated with *S. pecki* and *Hyalella*. Again, in the overall Comal system food web, the two main food chains based upon two most differing basal food resources (periphyton versus wood) were apparent, with *H. comalensis* adults and larvae, *Stygoparnus comalensis*, *Hyalella* at SR3, *Melanoides*, and *Stygobromus pecki* at SR 3 associated with the wood biofilm based food chain. The remaining consumers in the food web and *Hyalella* and *S. pecki* at Spring Island were largely associated with the periphyton-based basal resource food chain.

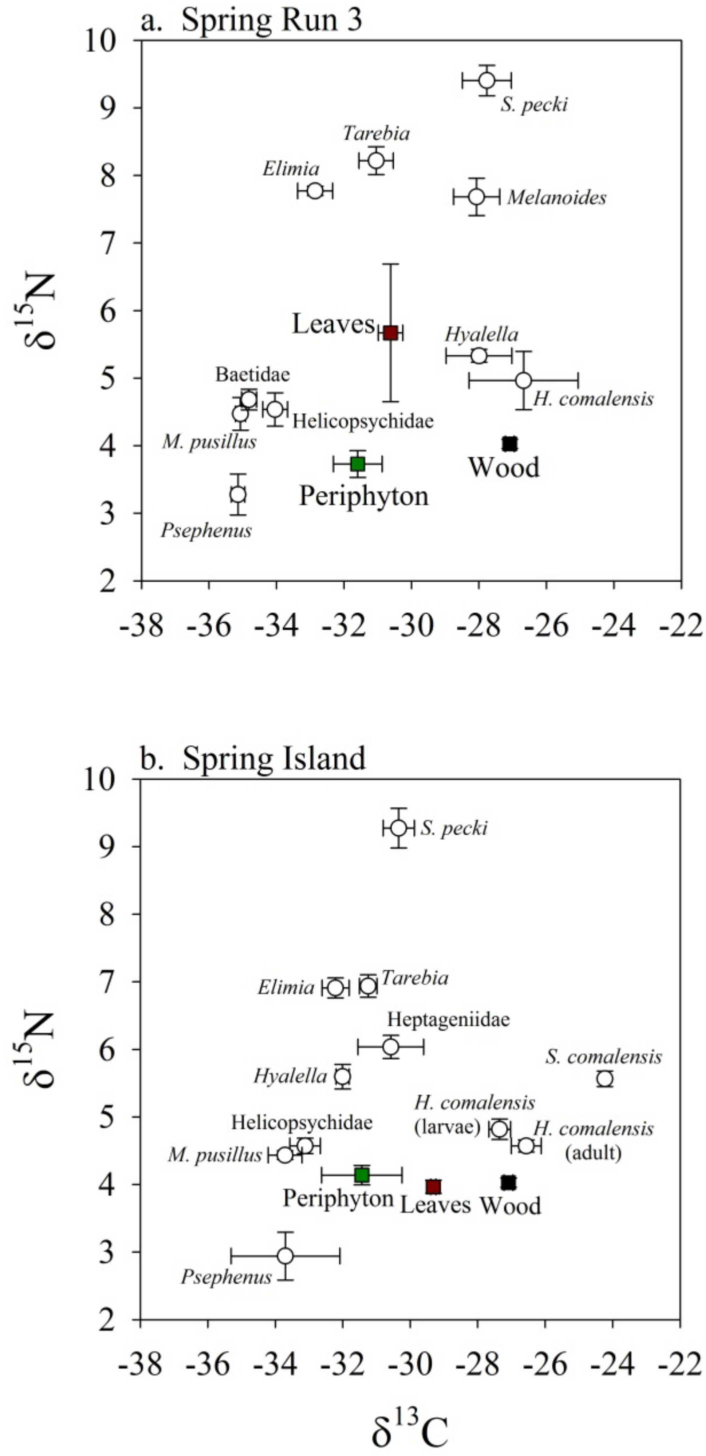


Figure 17. **Isotope bi-plots for consumers and basal food resources in (a) Spring Run 3 and (b) Spring Island. Each point represents the mean δ13C and δ15N values for each consumer or source (bars are ± 1 SE). See Table 6 for raw data.**

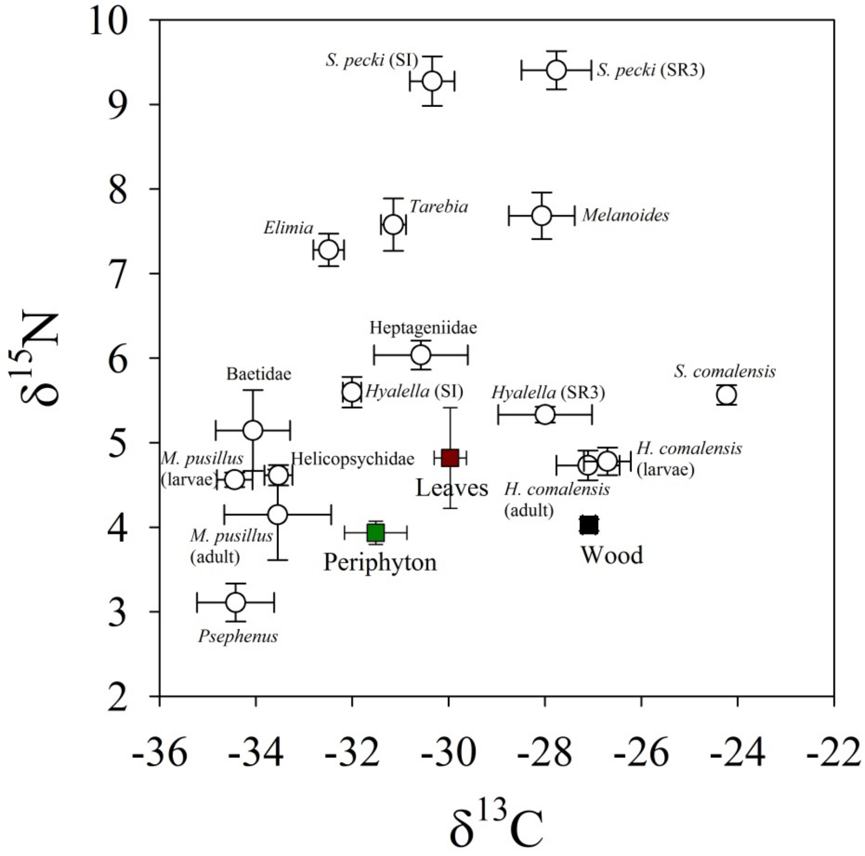


Figure 18. **Isotope bi-plots for consumers and basal food resources in the entire Comal system. Each point represents the mean δ13C and δ15N values for each consumer or source (bars are ± 1 SE). Mean δ13C and δ15N values for *Hyalella* and *Stygobromus pecki* in Spring Island (SR3) and Spring Island (SI) have been plotted separately because δ13C for these two species (and presumably the basal food resource) differed significantly between SR3 and SI (see text for analysis).**

When the combined data set was run in SIAR, the model output for each of these species generally reflected the differences that were apparent in the isotope bi-plot (Table 7). In this report, we provide the mean, mode and 95% credibility intervals for the model outputs for each consumer in the Comal food web; the actual posterior probability distributions and matrix plots are available upon request. The mean of the posterior probability distributions for *Psephenus*, helicopsychid caddis flies, *Tarebia*, and *M. pusillus* adults indicated that these consumers had diets that consisted of materials that was >50% of periphyton origin; this result clearly suggests that a majority of the diet of these consumers is periphyton-derived. In addition, there were several other consumers (*Hyalella* in Spring Island, *S. pecki* in Spring Island, *Elimia*, Heptageniidae, Baetidae, and *M. pusillus* larvae) who's posterior probability distributions indicated that periphyton-derived matter made up the largest single contributor of their diet (mean values ranging from 36 – 45% of the diet). In contrast, SIAR runs for several consumers indicated a large contribution of terrestrial-derived materials (wood and leaves combined) was the resource base supporting their diets. Posterior probability distributions indicated that the majority of the diets of both *Hyalella* and *S. pecki* in SR3 were derived from terrestrial detritus (64 and 72%, respectively). *Stygoparnus comalensis* had a proportional diet contribution of 44% from only wood and 71% from both types of terrestrial materials. Finally, model results indicate that *H. comalensis* adults and larvae derive 80% and 73% of their diet from terrestrial-derived materials (wood and leaves combined). In particular, *H. comalensis* larvae were the consumer in the Comal system which exhibited the highest proportional contribution of wood-derived materials to its diet (mean contribution of 59%).

Table 7. Summary output data from SIAR model runs examining the percent contribution of various basal resources to the isotopic signatures of consumers in the Comal system food web. Summary results presented are the mean and mode percent contribution of each source to each consumer after the model run for each consumer (n of iterations = 30,000), as well as the upper and lower values of the 95% credibility intervals. Posterior probability distributions and matrix plots for each consumer are available upon request.

	Leaves		Periphyton		Wood	
Consumer	Mean, Mode	95% CI	Mean, Mode	95% CI	Mean, Mode	95% CI
Baetidae	28%, 35%	0 - 60%	45%, 42%	3 - 86%	25%, 5%	0 - 56%
Elimia sp.	32%, 36%	2 - 56%	47%, 44%	15 - 84%	21%, 4%	0 - 46%
Helicopsychidae	19%, 4%	0 - 49%	68%, 87%	32 - 99%	13%, 3%	0 - 40%
Heptageniidae	28%, 31%	0 - 56%	47%, 45%	11 - 87%	25%, 6%	0 - 52%
Heterelmis comalensis (adult)	25%, 5%	(0 - 53%)	27%, 25%	0 - 51%	48%, 46%	19 - 80%
Heterelmis comalensis (larvae)	21%, 5%	0 - 48%	20%, 16%	0 - 44%	59%, 64%	31 - 89%
Hyalella azteca (SR3)	28%, 32%	0 - 58%	36%, 38%	2 - 66%	36%, 38%	3 - 64%
Hyalella azteca (SI)	27%, 31%	0 - 58%	49%, 45%	11 - 91%	24%, 5%	0 - 53%
Microcylloepus pusillus (adult)	25%, 4%	0 - 55%	55%, 46%	18 - 96%	21%, 4%	0 - 49%
Microcylloepus pusillus (larvae)	33%, 35%	0 - 65%	36%, 39%	0 - 69%	31%, 34%	0 - 62%
Psephenus sp.	28%, 35%	0 - 58%	50%, 44%	12 - 93%	22%. 5%	0 - 50%
Stygobromus pecki (SR3)	38%, 39%	0.5 - 72%	28%, 32%	0 - 53%	35%, 37%	3 - 61%
Stygobromus pecki (SI)	39%, 42%	0.5 - 71%	47%, 47%	7 - 84%	15%, 3%	0 - 41%
Stygoparnus comalensis	27%, 7%	0 - 57%	29%, 36%	0 - 60%	44%, 40%	3 - 85%
Tarebia granifera	31%, 33%	0.8 - 58%	55%, 50%	21 - 92%	14%, 3%	0 - 38%

These results indicate that inputs of terrestrial-derived materials are likely to be particularly important for the diet (and thus conservation) of *H. comalensis* and *S. comalensis*. Both of these species are commonly found on decaying woody material in the field and individuals can be maintained in the lab by supplying well-conditioned leaf material (W.H. Nowlin and R. Gibson, *unpublished data*). Reliance on wood-associated biofilms is not a new observation in riffle beetles. Seagle (1982) found that the gut contents of larvae and adults of three riffle beetle species (*Stenelmis crenata*, *Stenelmis mera*, and *Optisoservus trivittatus*) were dominated by detritus-like materials, including wood xylem, unidentified organic matter, and mineral particles, while algal material was consumed to a much lesser extent. Thus, it has been suggested that elmids as a whole should be reclassified as detritivores-herbivores rather than as strictly herbivores, with the exception of known xylophagus genera (i.e., *Lara*) (Seagle 1982).

The results of this study suggest that a lateral connection to terrestrial matter sources may be particularly important for two of the species listed under the EAHCP: *Heterelmis comalensis* and *S. comalensis*. Lower spring discharge and declining flows can lead to disconnection of the aquatic environment from the bank and to lower water surface areas of aquatic habitats, potentially leading to decreased inputs of terrestrial material that may support populations of these wood-associated taxa. These results also support the importance of a well developed riparian area essential for the conservation of the endangered Comal beetles stated as Primary Constituent Element (PCE) 4 in the critical habitat designation (USFWS 2007) and PCE(3) in critical habitat revision (USFWS 2013). In addition, decreased flow rates and water velocities could also lead to lower rates of downstream transport of terrestrial materials from more canopy-covered upstream areas (e.g., the more canopy-covered Spring Runs) to more open areas with lower rates of terrestrial inputs (e.g., Spring Island area).

Results from our study indicate that *Stygobromus pecki*, another spring-associated consumer covered under the EAHCP may have a more plastic feeding strategy than *H. comalensis* and *Stygoparnus comalensis* and may be able to switch to an alternate basal resource food chain as environmental conditions vary spatially. However, our results indicate that the feeding strategy of *H. comalensis* and *S. comalensis* appear to be largely dependent upon wood- and leaf-based biofilms, and this dependence doesn't change between sites. Therefore, the feeding strategy of these two species do not appear to respond to spatial changes in the canopy cover and presumably amount of terrestrial detritus inputs. Thus, downstream populations of *H. comalensis* and *S. comalensis* may be particularly sensitive to lower inputs of terrestrial detritus material. Our results also suggest that *M. pusillus* may not be an appropriate surrogate for *H. comalensis* in that the diet of *M. pusillus* was much more reliant upon periphyton and therefore feeds on an entirely different food chain.

5.0 Conclusions

In conclusion, this study conducted water quality evaluations and survival trials to establish a laboratory location for 2015 riffle beetle experimentation, a subsequent evaluation of potential surrogate beetle species, and a series of laboratory and field investigations examining key components of upwelling and lateral habitat connectivity of *H. comalensis*.

Although both SMARC and FAB exhibited fairly similar water quality conditions with similar lack of temporal variability, there were striking differences in the survival of riffle beetle adults with extensive mortality at FAB. As such, all subsequent 2015 riffle beetle laboratory studies associated with this project were conducted at SMARC. In response to the unknown causes for this mortality concern, Dr. Nowlin and Texas State University (both outside of this project) have been working closely with the Edwards Aquifer Authority to determine the cause for the adult mortality and how to manage the issue. It is currently established that the downstairs FAB Holding House water does not cause mortality in adult beetles and that the Wet Lab water does not cause mortality for adults when it is first passed through a flow-through activated charcoal filter. Assurances from Texas State University Facilities have been granted that they will work to determine the source of the problem, fix the problem, and that they will supply whatever charcoal filtration needs are required so that researchers can successfully conduct any future riffle beetle research at FAB.

The surrogate evaluations demonstrated that the relatively long-term temperature-dependent survival thresholds were consistent with previous work that examined upper temperature limits in *H. comalensis* and *H. glabra* (Nowlin et al. 2014). Additionally, the "thresholds" found for all three species tested in the present study are also in line with thresholds reported for other plastron utilizing beetle species (i.e., Harpster 1941, 1944). In the present study, we found that *M. pusillus* exhibited a slightly higher mortality threshold temperature than the two *Heterelmis* species. These results make sense in light of the types of habitats these species are associated with in natural systems. It has been hypothesized that organisms living in thermally stable environments, such as subterranean systems and spring-influenced ecosystems should be stenothermal (having a narrow thermal tolerance range) (Mermillod-Blondin et al. 2013). Our results are consistent with this hypothesis and indicate that *M. pusillus* is less sensitive to longer-term temperature changes than *H. comalensis* and *H. glabra*. These results also suggest that *M. pusillus* is likely not the best candidate to serve as a surrogate species in physiological stress studies associated with variation in environmental conditions, especially water temperature.

The use of cloth lures is now the "established" method for monitoring beetle numbers in the Comal system because beetles tend to congregate on the lures at densities higher than the surrounding environment. However, the lure presumably serves as an attractant and therefore concentrates beetles in a given location, thereby affecting the ability of investigators to make reliable estimates of population density or size. Thus, there has been an increased interest in trying to find "passive" sampling methods for beetles that would allow researchers and management personnel to gain increased ability to make reliable density estimates. Based upon the two preliminary studies we conducted, it appears that passive sampling pits or wells and Bou-Rouch samples are not likely to be efficient methods for monitoring beetles.

Results from the upwelling habitat connectivity study conducted in SMARC laboratory using the RBASS revealed a statistically significant difference with greater survival in treatments connected to organic matter than in treatments that were disconnected via water flow to organic matter over the course of the study. Additionally, qualitative observations of beetle movement over the course of the upwelling experiment suggest that beetles in EFCs with flowing water connected via flow through organic material tended to more active and often frequented the organic material provided near the surface of the upwelling.

Results from the field investigations indicate that inputs of terrestrial-derived materials are likely to be particularly important for the diet (and thus conservation) of *H. comalensis* and *S. comalensis*. In fact, model results indicate that *H. comalensis* adults and larvae derive 80% and 73% of their diet from terrestrial-derived materials (wood and leaves combined). The results of this study suggest that a lateral connection to terrestrial matter sources may be particularly important for two of the species listed under the EAHCP: *H. comalensis* and *S. comalensis*. Lower spring discharge and declining flows can lead to disconnection of the aquatic environment from the bank and to lower water surface areas of aquatic habitats, potentially leading to decreased inputs of terrestrial material that may support populations of these wood-associated taxa. In addition, decreased flow rates and water velocities could also lead to lower rates of downstream transport of terrestrial materials from more canopy-covered upstream areas (e.g., the more canopy-covered Spring Runs) to more open areas with lower rates of terrestrial inputs (e.g., Spring Island area).

Results from our study indicate that *Stygobromus pecki*, another spring-associated consumer covered under the EAHCP may have a more plastic feeding strategy than *H. comalensis* and *S. comalensis* and may be able to switch to an alternate basal resource food chain as environmental conditions vary spatially. Backing up results from the water temperature surrogate testing study, our stable isotope results also suggest that *M. pusillus* may not be an appropriate surrogate for *H. comalensis* in that the diet of *M. pusillus* was much more reliant upon periphyton and therefore feeds on an entirely different food chain.

6.0 Recommendations for future applied research

The EAHCP Science Committee and National Academy of Science committees have both recommended additional life history and environmental stimulus work to be conducted with *H. comalensis.* Our study team concurs with that assessment and are actively engaged with 2016 EAHCP applied research directly assessing life history requirements from egg to adult. Additional research is being conducted on the endangered Comal invertebrates relative to rearing in captivity via on-going and upcoming refugia applied research.

Relative to this study, it was clear that the long-term cumulative stress experiments provide useful initial information on thermal thresholds for riffle beetles and their potential limits of acclimation for increasing environmental temperatures, but there are potentially a large number of additional studies that should be considered. Experiments for consideration could include examining the performance of beetles (both *H. glabra* and *H. comalensis*) to environmental stimuli (water temperature, organic matter connection or disconnection, levels of siltation, etc.) over extended periods of time (months) in order to establish “preferred” conditions for maintenance of both wild and refuge populations. It may also be extremely insightful to monitor physiological responses (instead of behavioral responses) to increasing temperature, such as respiration rates, immune function, and the concentration of various biomolecules (e.g., Mermillod-Blondin et al. 2013). The RBASS again proved to be a viable research tool to consistently control environmental variables in a simulated upwelling environment. To expand on the upwelling habitat connectivity assessment, it might prove valuable to conduct a study evaluating three scenarios of organic matter connectivity; the two tested in this study (connected and disconnected) with a third which would consist of a disconnected treatment that received infrequent rainfall events filtering over surface organic matter.

Finally, based upon the two preliminary studies we conducted, it appears that passive sampling pits or wells and Bou-Rouch samples are not likely to be efficient methods to estimate beetles. However, the efficiency of these and other methods should be considered for more thorough investigation in a systematic study that explicitly examines and compares various collection methods in the field. In addition to these comparisons, other data should be considered for collection in the lab, including the dispersal or movement ability of beetles, the ability to mark and re-capture beetles in small-scale settings, and the ability of beetles to hold onto surfaces at different flow or suction rates.

7.0 Acknowledgments

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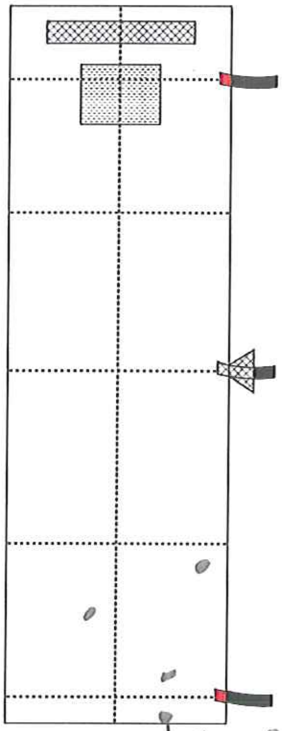
APPENDIX A

Comal Springs riffle beetle location data sheets

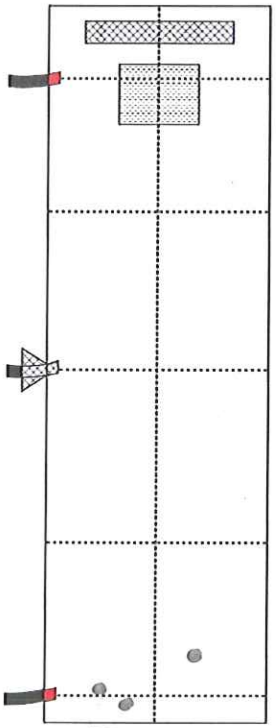
Date: 09/22/15, 12:20
Temperatures: Outside 90°F , **Inside** 90°F , **Chiller** 68.9°F

EFC 1

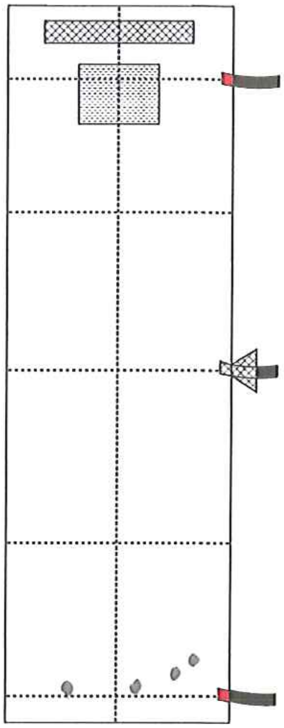


clump of 5? stuck in eddy

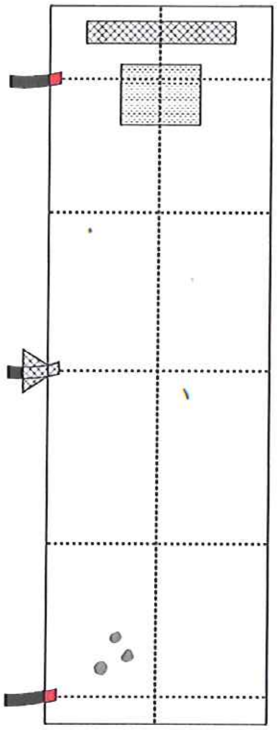
EFC 2



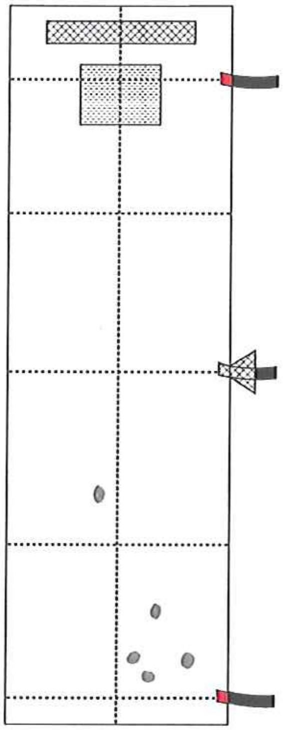
EFC 5



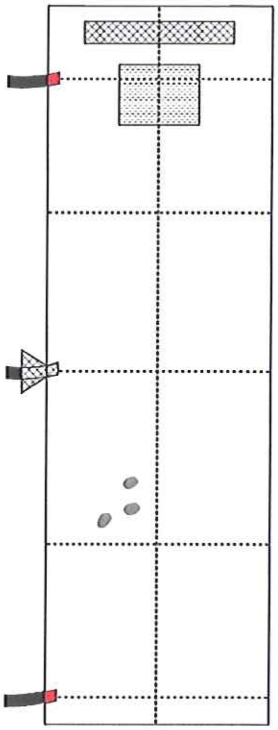
EFC 8



EFC 9



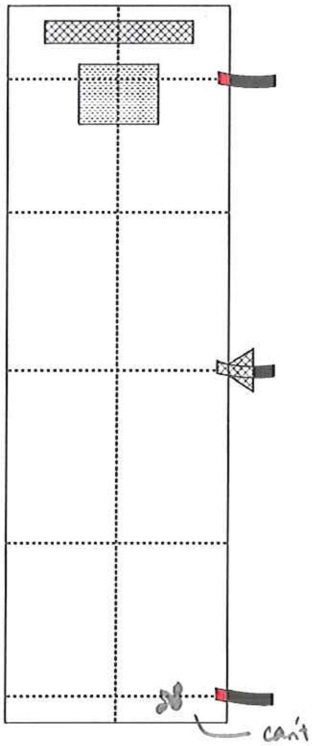
EFC 12



Notes:

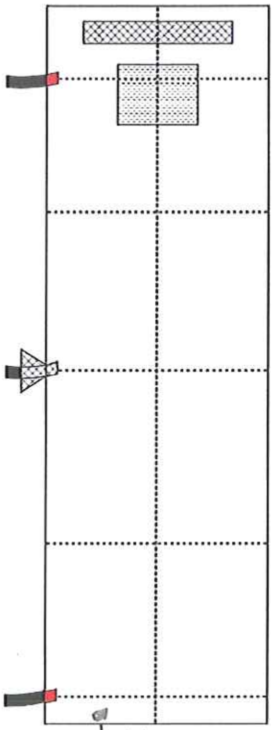
Date: 09/26/15, 18:30
Temperatures: Outside 90°F, Inside 92°F, Chiller 68.1°F

EFC 1



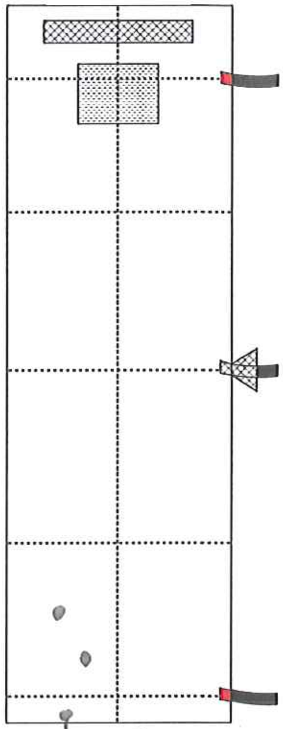
can't tell if 4 ? 5 ?

EFC 2



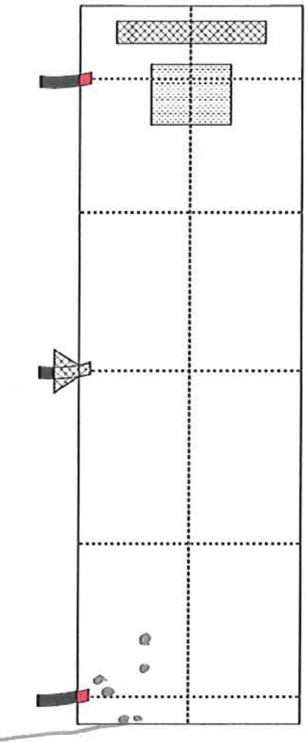
1 beetle on a small stick

EFC 5



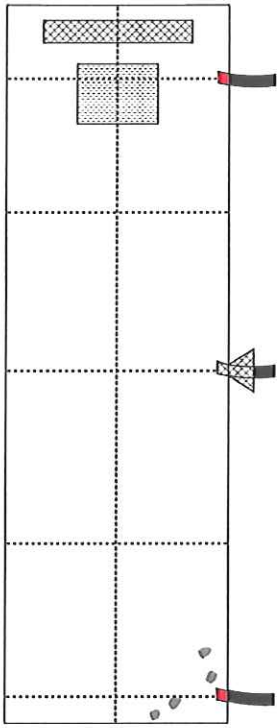
on floor, small stick with beetle

EFC 8

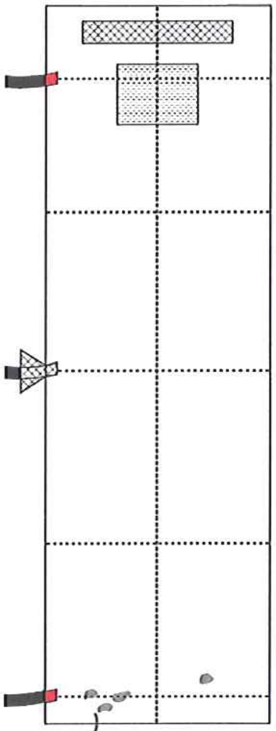


clump of two

EFC 9



EFC 12

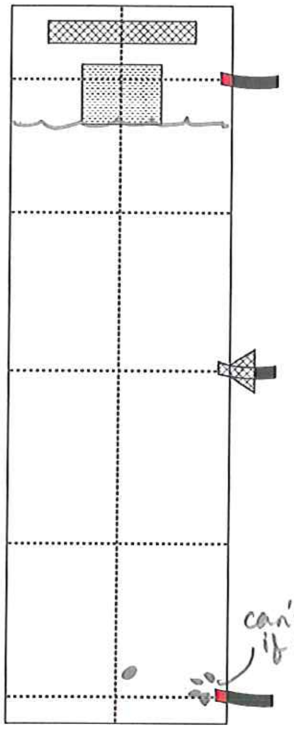


clump of 2 or 3

Notes:

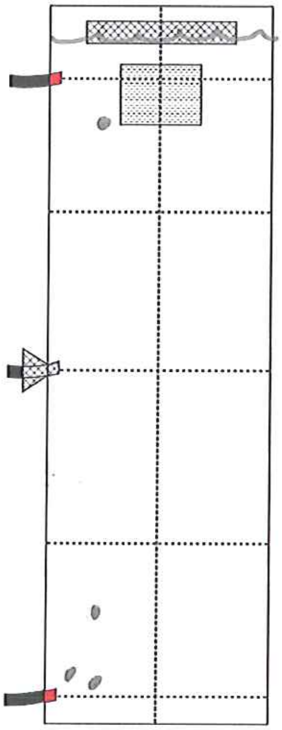
Date: 09/28/15, 18:07
Temperatures: Outside 84°F , **Inside** 86°F , **Chiller** 68.3°F

EFC 1

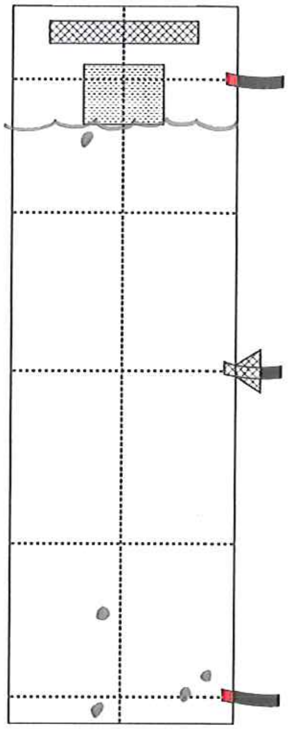


can't tell if 3 or 4

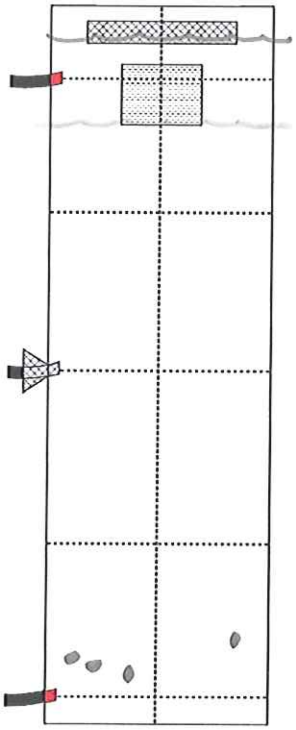
EFC 2



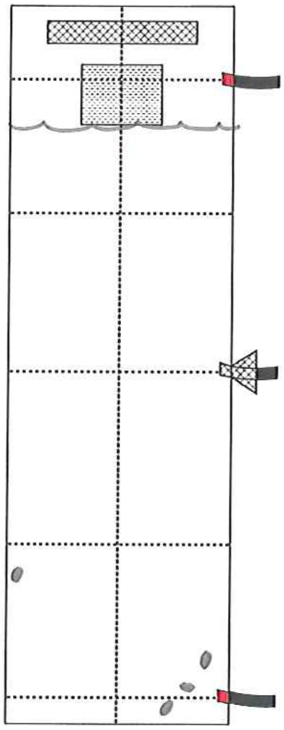
EFC 5



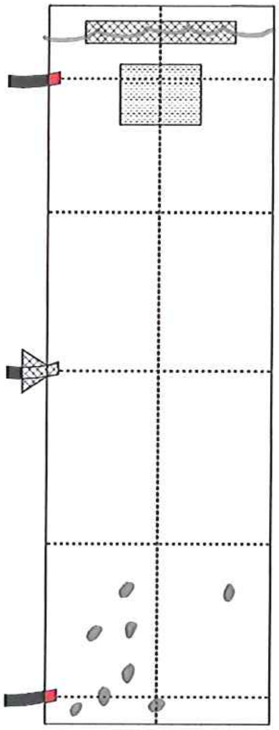
EFC 8



EFC 9



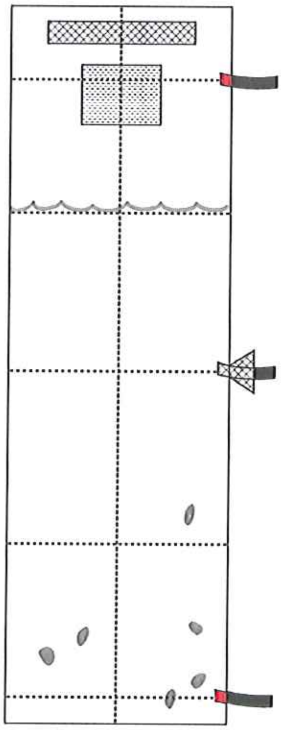
EFC 12



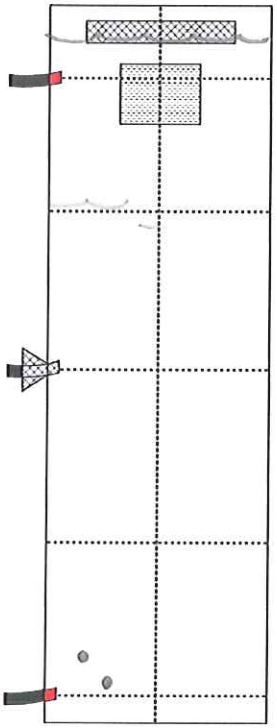
Notes:

Date: 09/29/15, 14:15
Temperatures: Outside 91 °F , **Inside** 88 °F , **Chiller** 68.3 °F

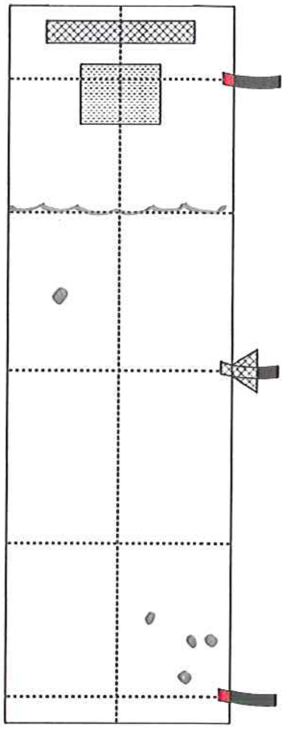
EFC 1



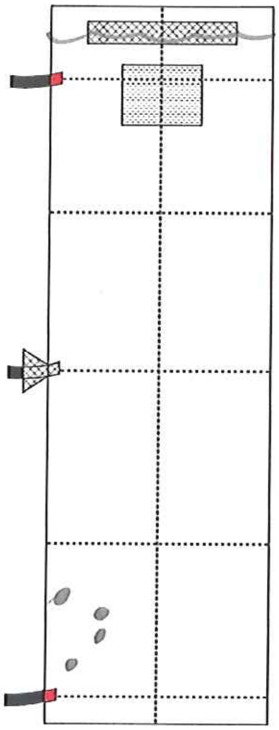
EFC 2



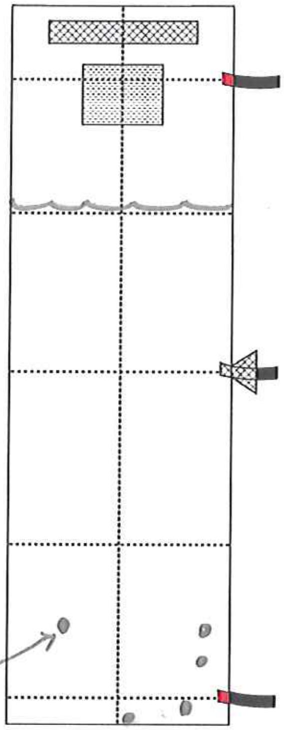
EFC 5



EFC 8

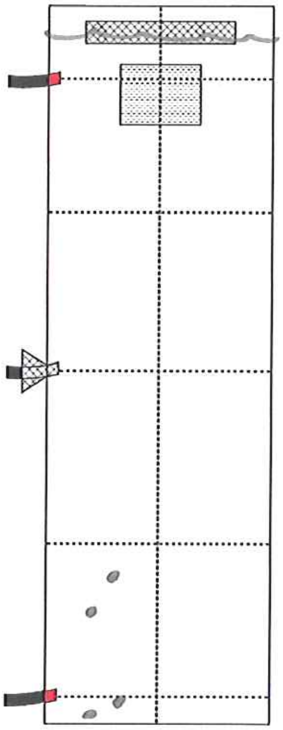


EFC 9



when this beetle saw my headlamp light he started crawling up on the mesh

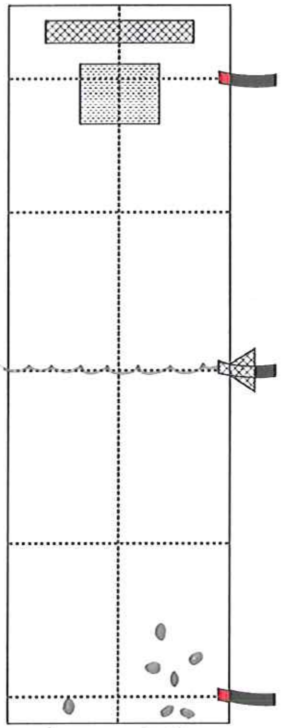
EFC 12



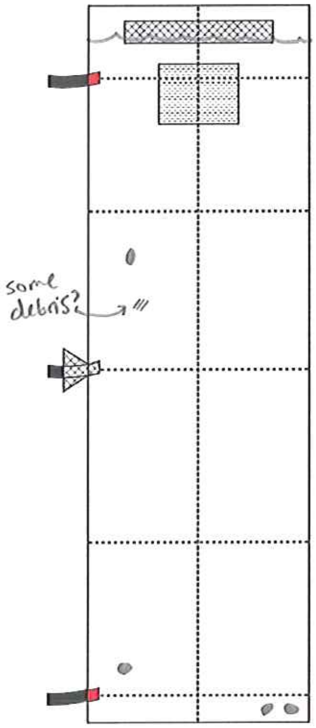
Notes:

Date: 10/01/15 15:17
Temperatures: Outside 85°F , **Inside** 95°F , **Chiller** 68.6°F

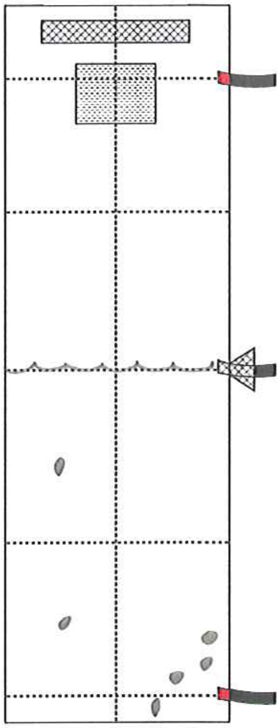
EFC 1



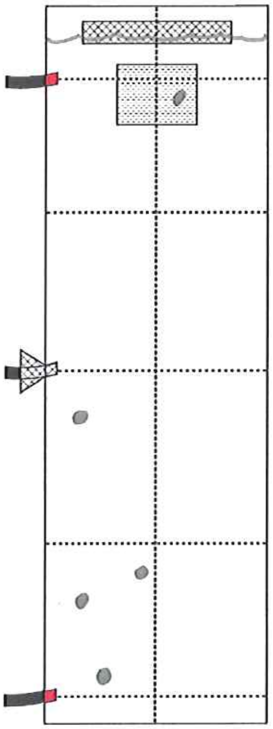
EFC 2



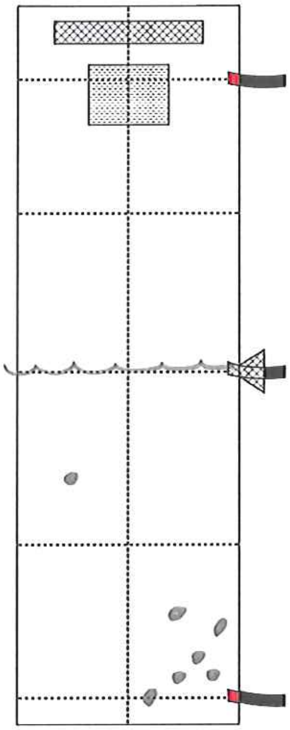
EFC 5



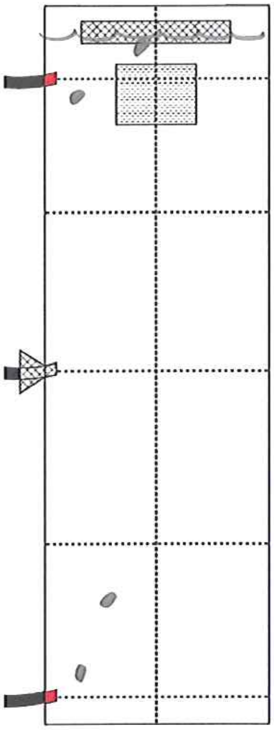
EFC 8



EFC 9



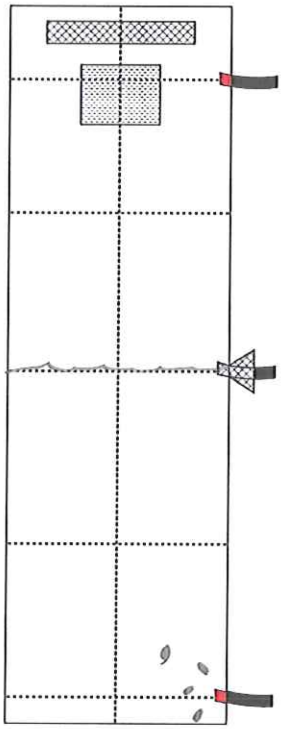
EFC 12



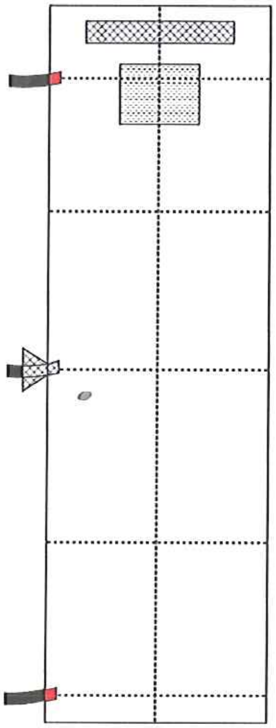
Notes: finished dropping water levels today at 15:37.

Date: 10/03/15 ,08:15
Temperatures: Outside 70 °F , **Inside** 64°F , **Chiller** 68.8°F

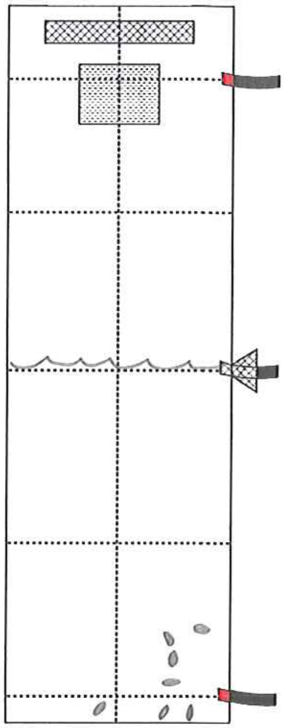
EFC 1



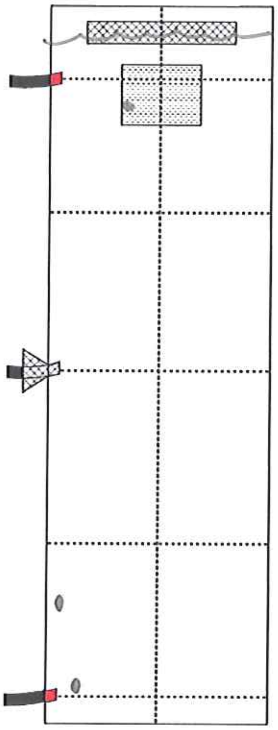
EFC 2



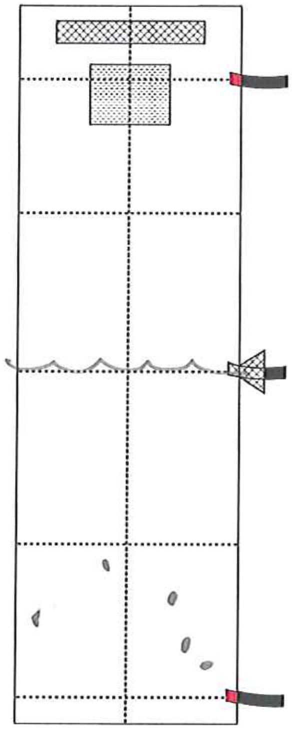
EFC 5



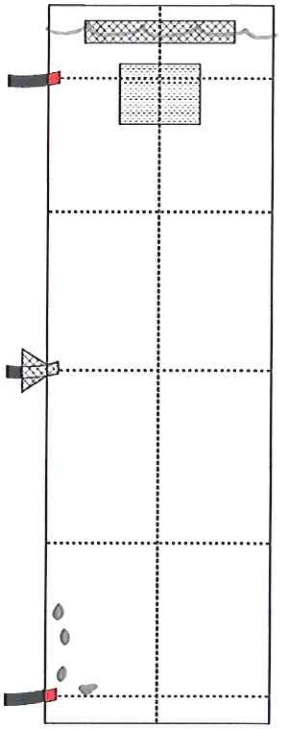
EFC 8



EFC 9



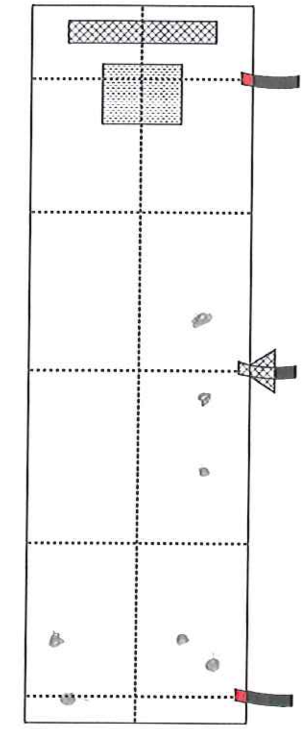
EFC 12



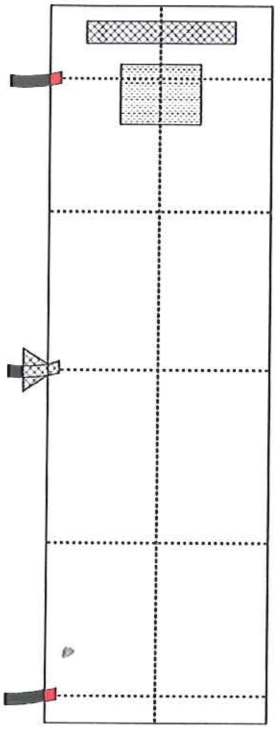
Notes:

Date: 10/5/2015 11:20 am
Temperatures: Outside 77°F **, Inside** 80 F **, Chiller** 68.2F

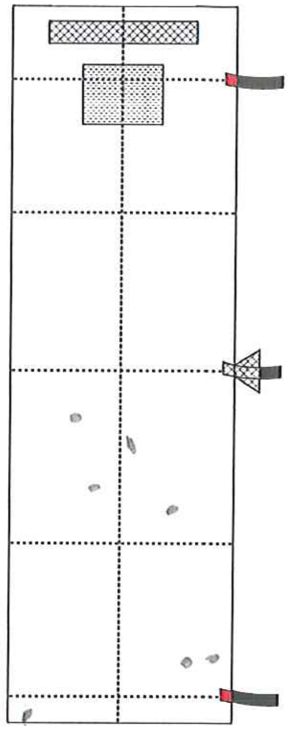
EFC 1



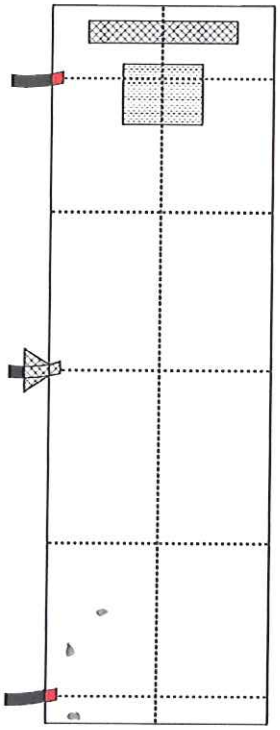
EFC 2



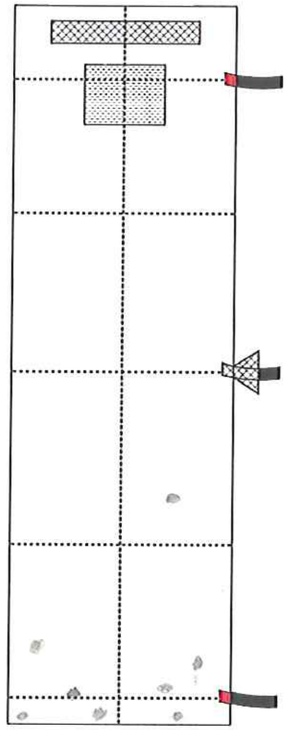
EFC 5



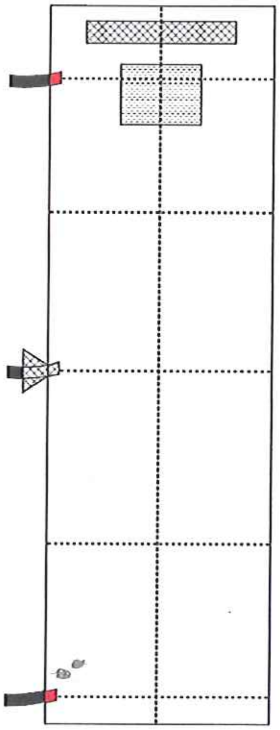
EFC 8



EFC 9



EFC 12

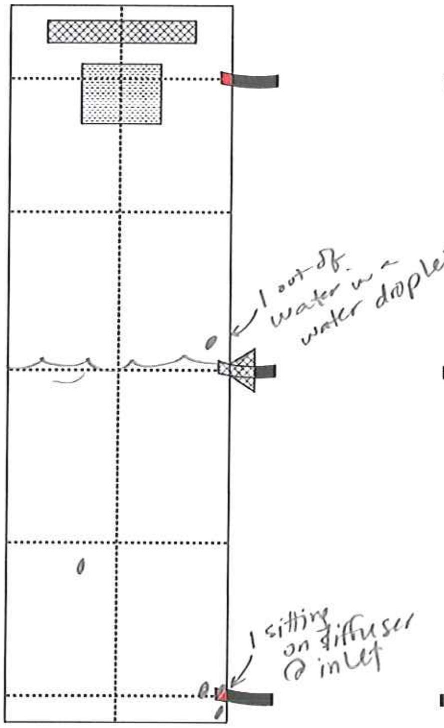


Notes:

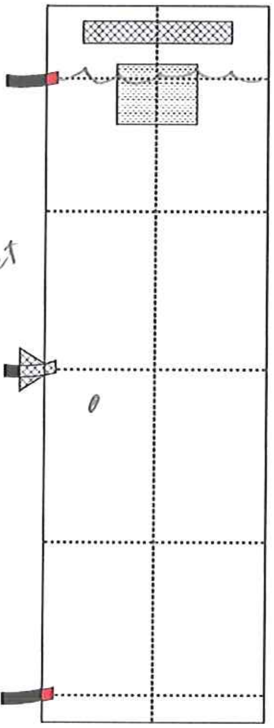
Date: 10-07-15

Temperatures: Outside 90 °F , **Inside** 90 °F , **Chiller** 68.5 °F

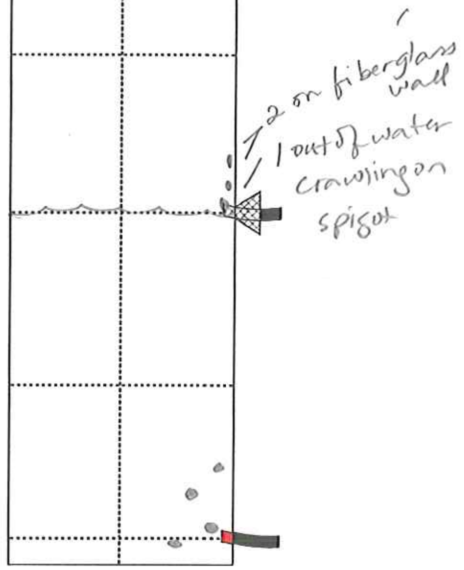
EFC 1



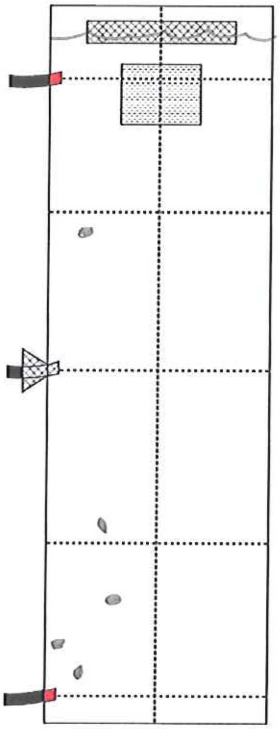
EFC 2



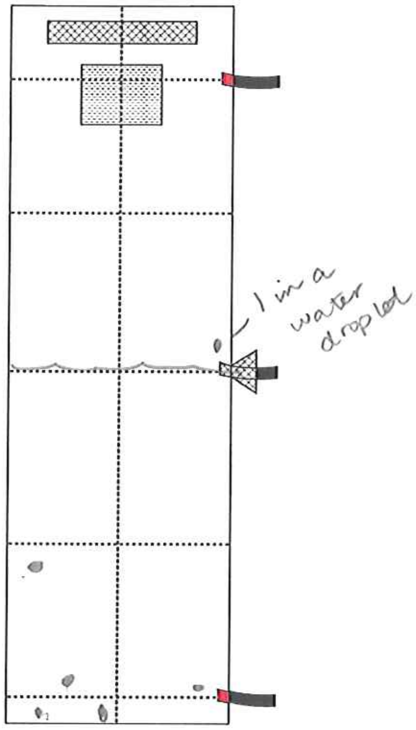
EFC 5



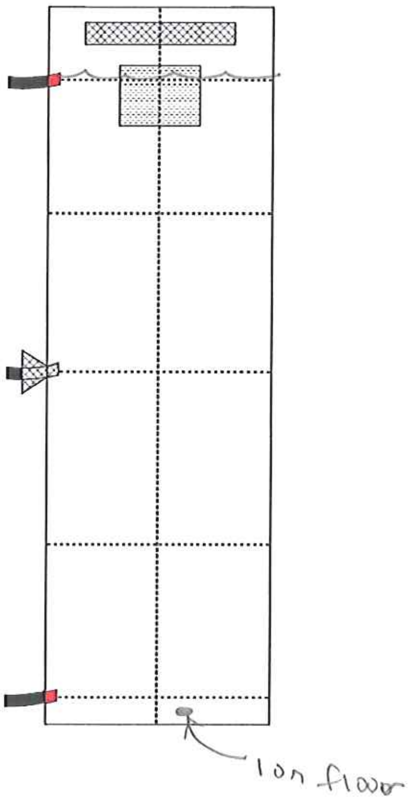
EFC 8



EFC 9



EFC 12

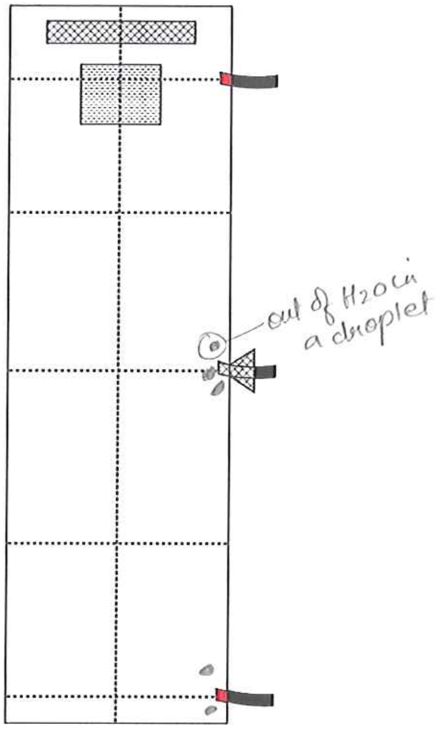


Notes:

Date: 10/08/05

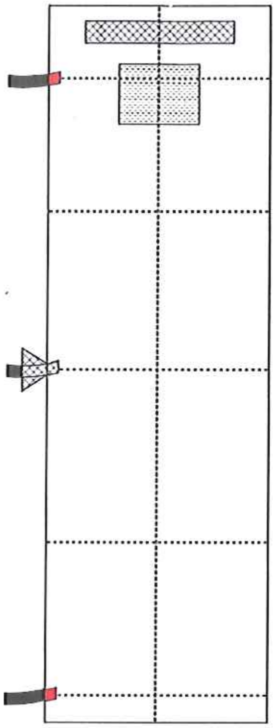
Temperatures: Outside 90°F **, Inside** 85°F **, Chiller** 68.5F

EFC 1

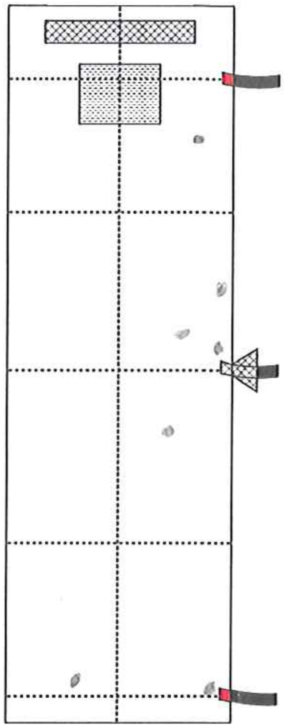


out of H2O in a droplet.

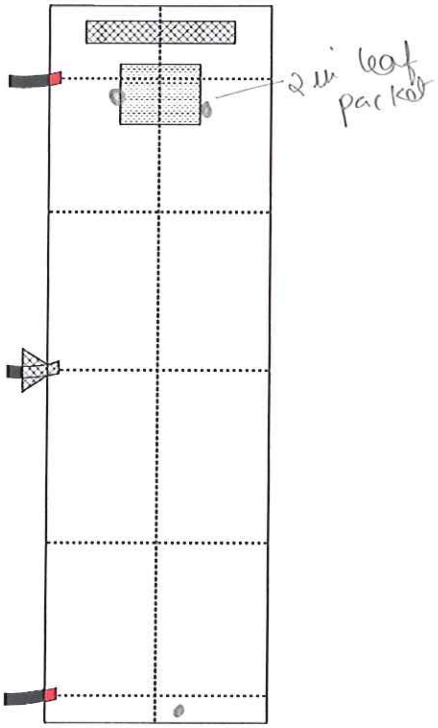
EFC 2 – None seen.



EFC 5

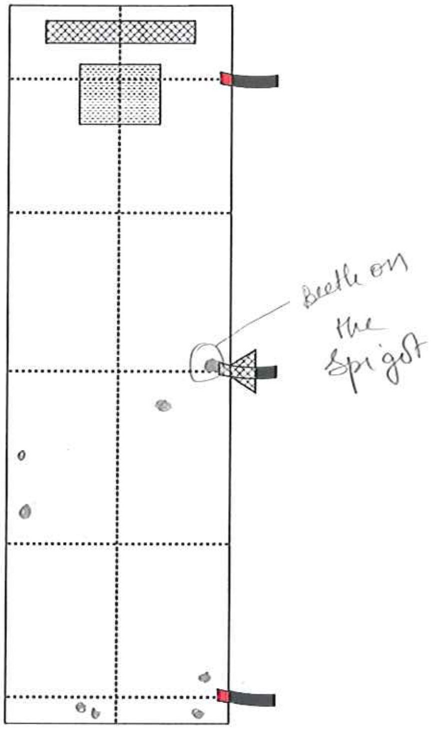


EFC 8



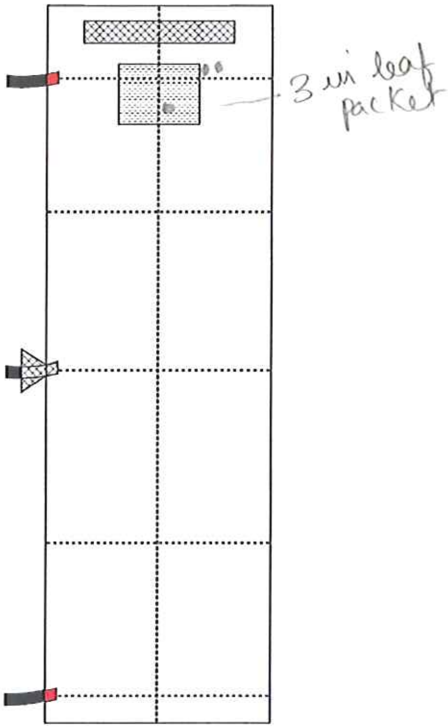
2 in leaf packet

EFC 9



Beetle on the Spigot

EFC 12



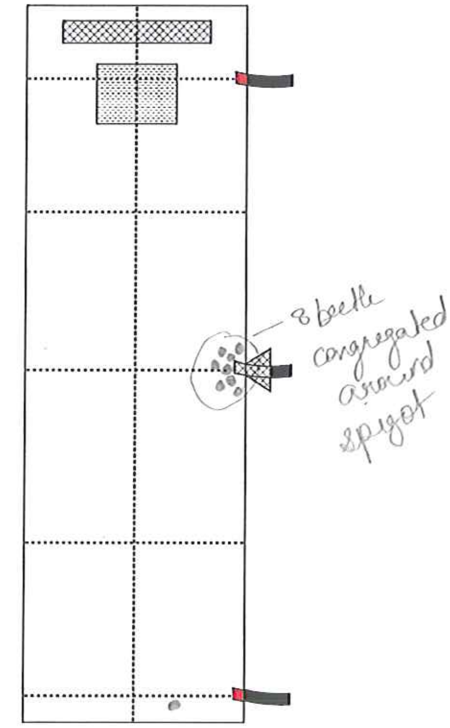
3 in leaf packet

Notes:

Date: 10/10/2015

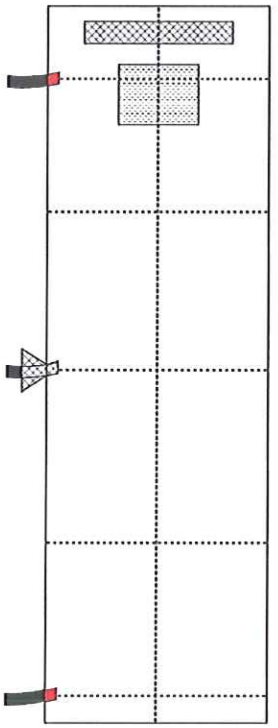
Temperatures: Outside 91°F **, Inside** 88°F **, Chiller** 68.5

EFC 1

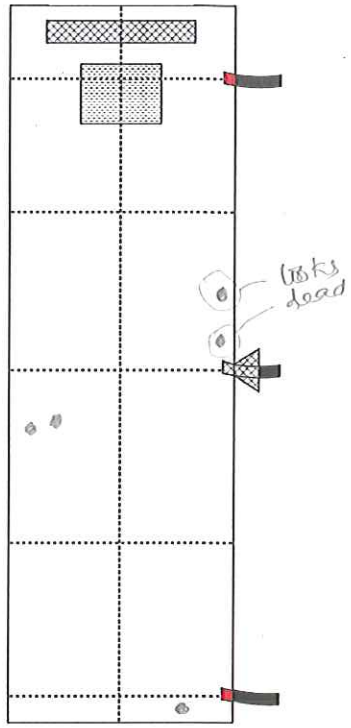


8 beetle congregated around spigot

EFC 2

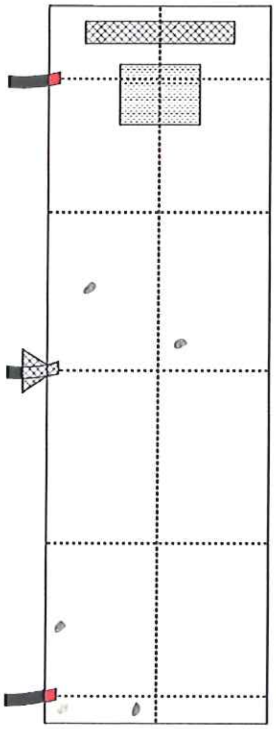


EFC 5

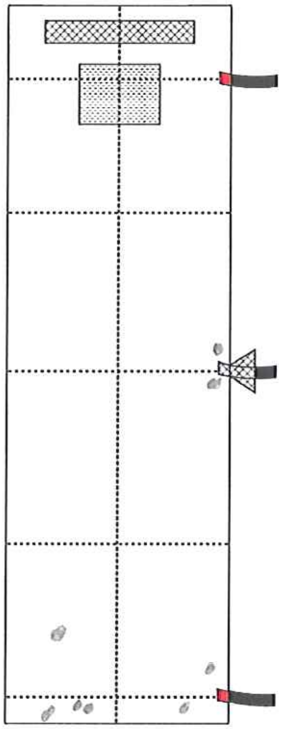


looks dead

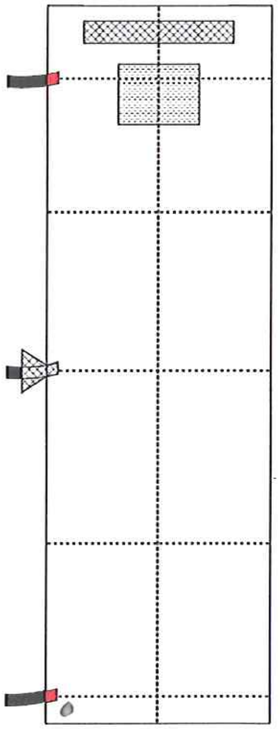
EFC 8



EFC 9



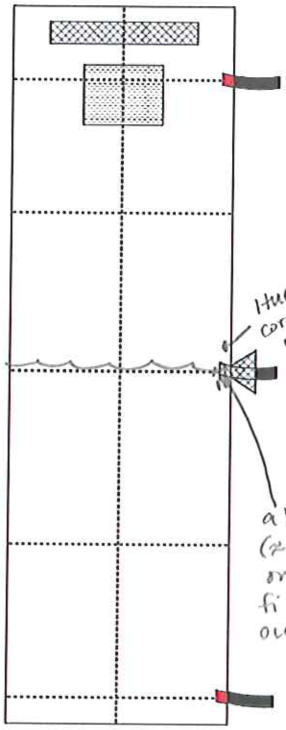
EFC 12



Notes:

Date: 10/13/15, 13:10
Temperatures: Outside 91°F , **Inside** 90°F , **Chiller** 68.4°F

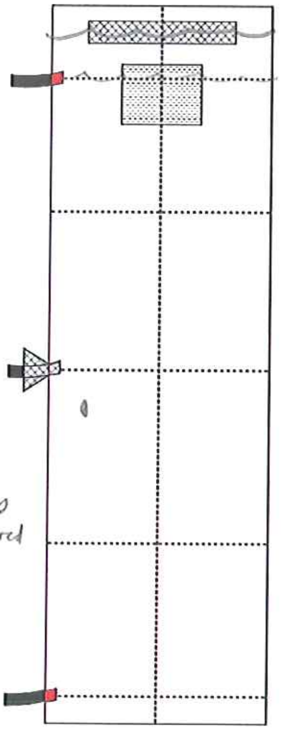
EFC 1



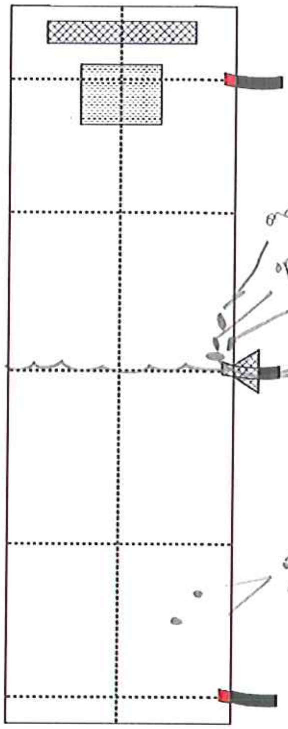
Hucked into corner above waterline, looks like surrounded by tiny water droplet

a lot of beetles (≈7 or 8) clustered on spigo cloth filler around outlet port

EFC 2



EFC 5



one ~2" above waterline, seems dry?

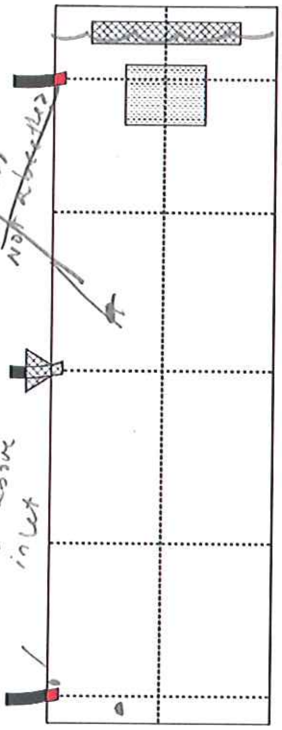
one on back of tank behind mesh (poor vis.)

one above water line on side of tank amongst condensation

one on it's back above water line in mesh

2 in mesh below water line

EFC 8

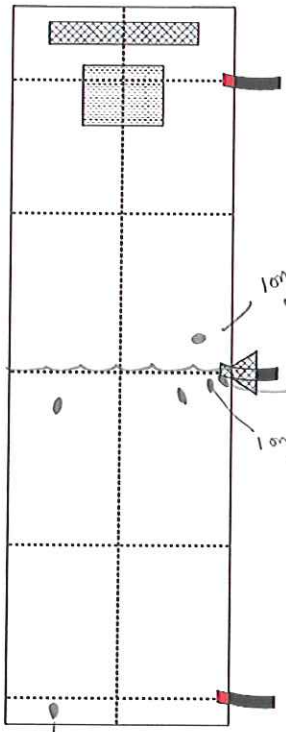


1 beetle (not debris)

~~debris; NOT a beetle?~~

1 on mesh above inlet

EFC 9



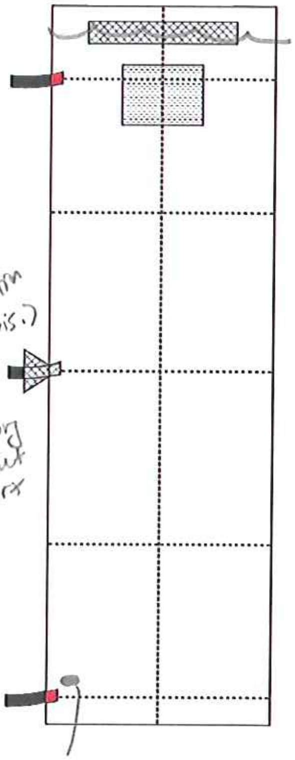
1 on mesh above water line amongst condensation

1 or 2 (poor vis.) on cloth netting covering outlet port

1 on mesh near outlet

1 on mesh near tank floor

EFC 12



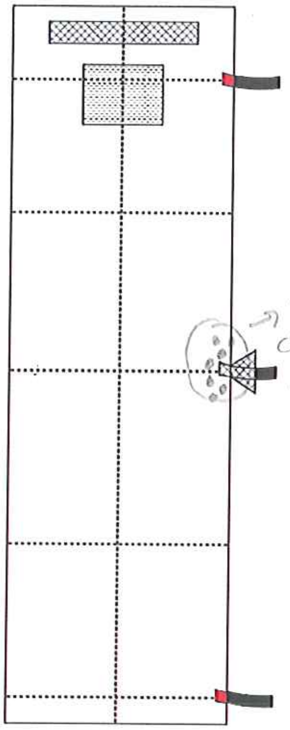
one on mesh near spigot

Notes:

Date: 10/15/2015

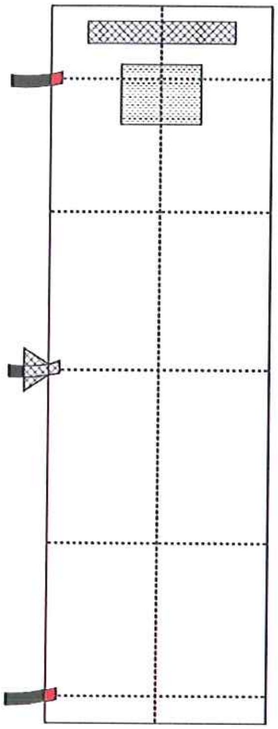
Temperatures: Outside 94°F , **Inside** 86°F , **Chiller** 68.4F

EFC 1

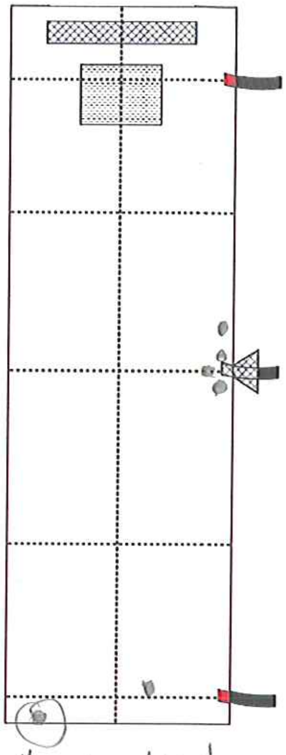


→ 7 congregated around spigot

EFC 2

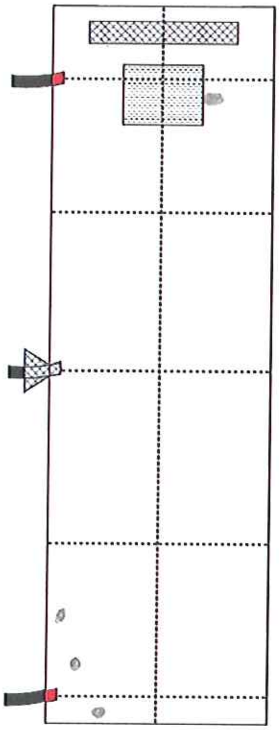


EFC 5

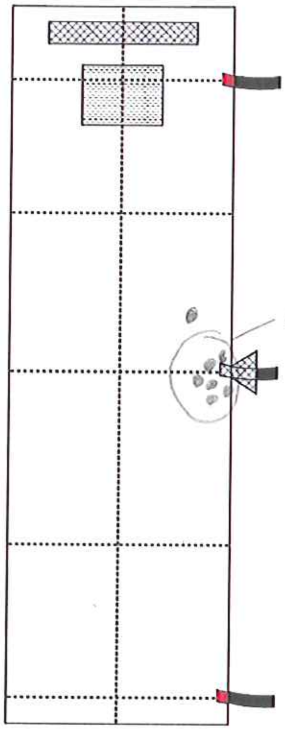


looks dead

EFC 8

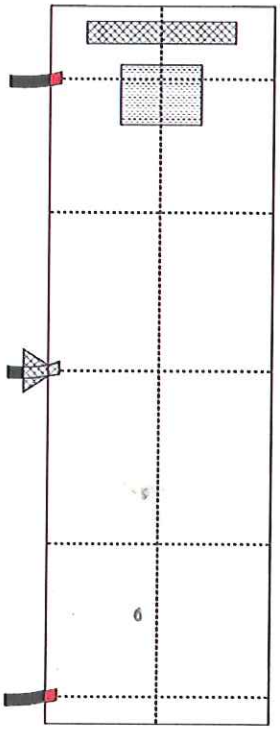


EFC 9



around spigot

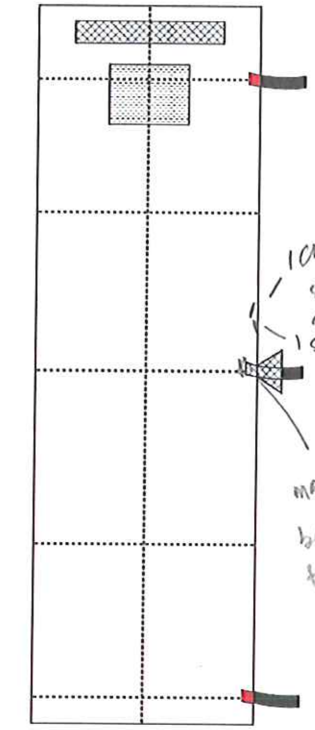
EFC 12



Notes:

Date: 10/17/2015 10:44
Temperatures: Outside 75 °F , **Inside** 81 °F, **Chiller** 68.2 °F

EFC 1

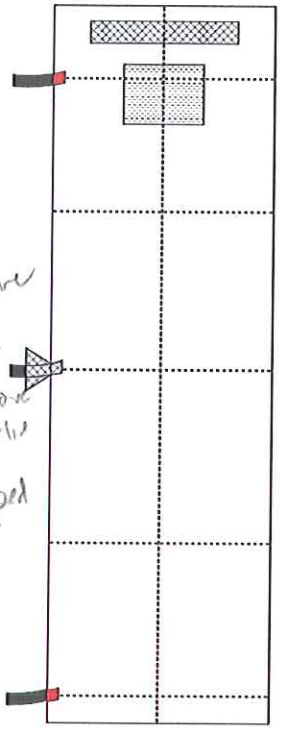


1 crawling on side of tank ≈2" above water line

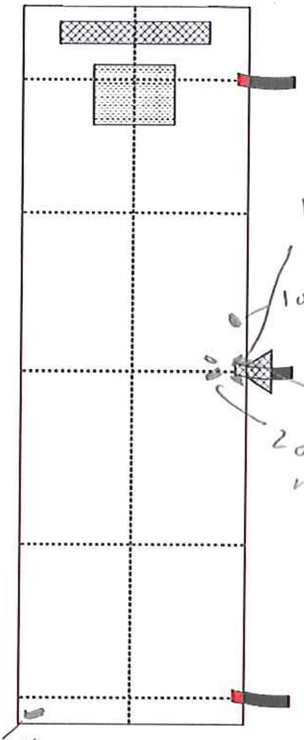
1 sitting on side of tank ≈1.5" above waterline

mass of beetles clumped together on outlet

EFC 2 - NONE visible



EFC 5



1 on tank wall very near outlet

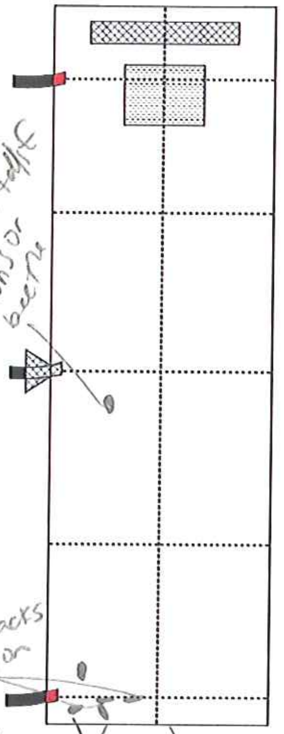
1 unmoving ≈ 2" above water line

2 on outlet

2 on mesh near outlet

1 on its back on bottom of tank; unmoving

EFC 8



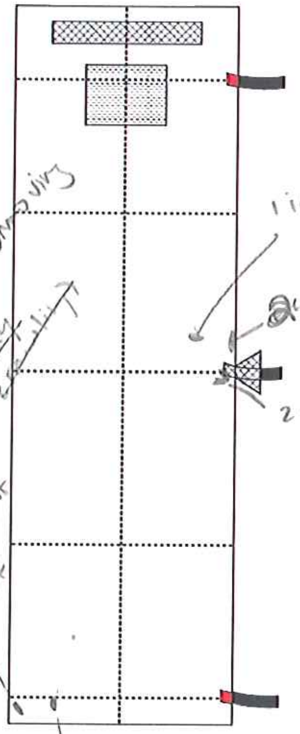
(on top of debris or beetle

2 on backs supine on floor unmoving

2 on mesh near bottom

quite a bit of organic debris

EFC 9



inactive/unmoving ~~dead~~ ~~likely~~ (seems to be on tank bottom of corner of tank

1 in corner of tank

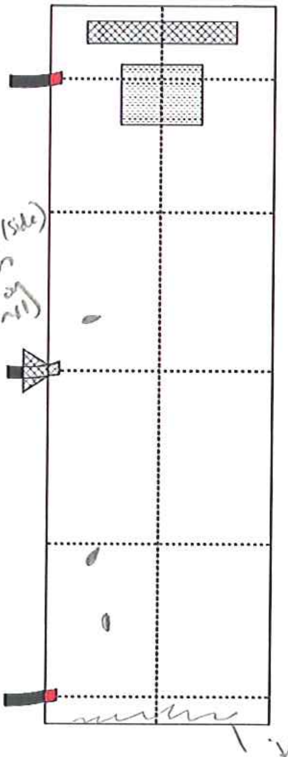
1 in mesh above water line

2 on tank wall (side) (condensation also on wall)

2 on outlet

1 on mesh @ bottom of tank

EFC 12



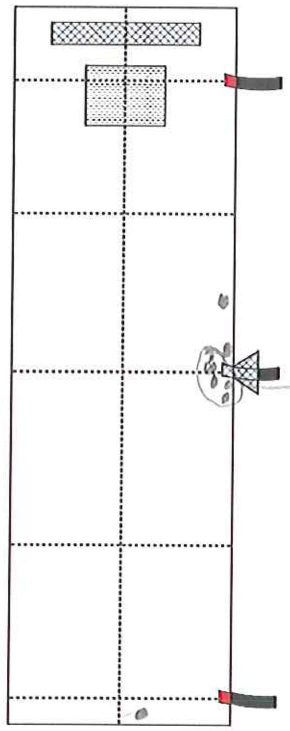
quite a bit of small-diameter organic debris

Notes:

Date: 10/19/2015

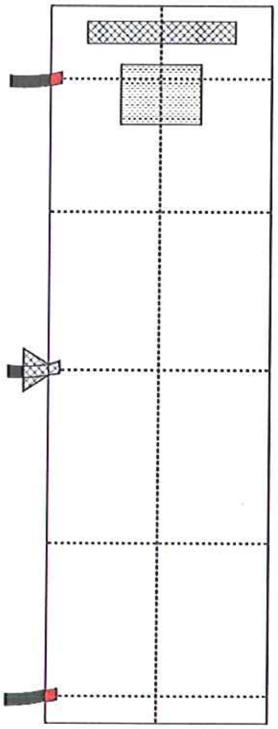
Temperatures: Outside 84°F , **Inside** 88°F , **Chiller** 68.5

EFC 1

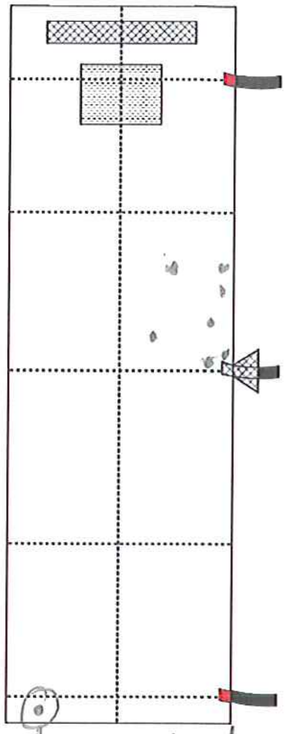


5 near spigot

EFC 2

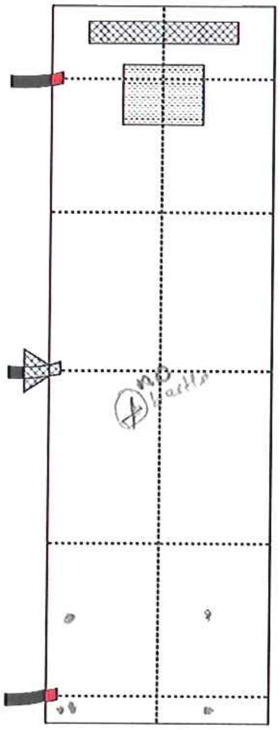


EFC 5



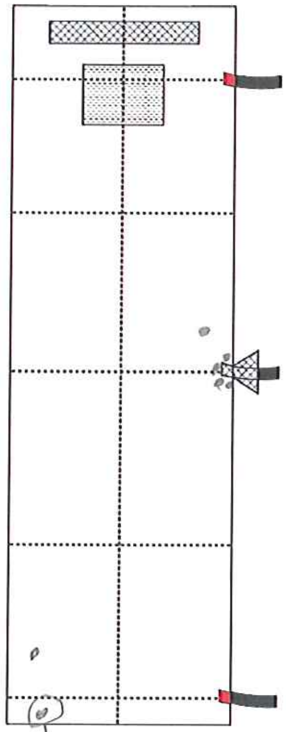
looks dead

EFC 8



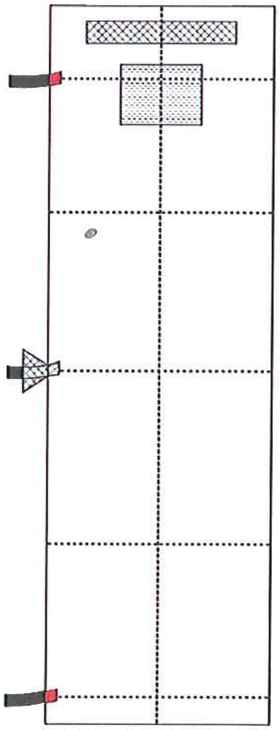
no beetle

EFC 9



looks dead

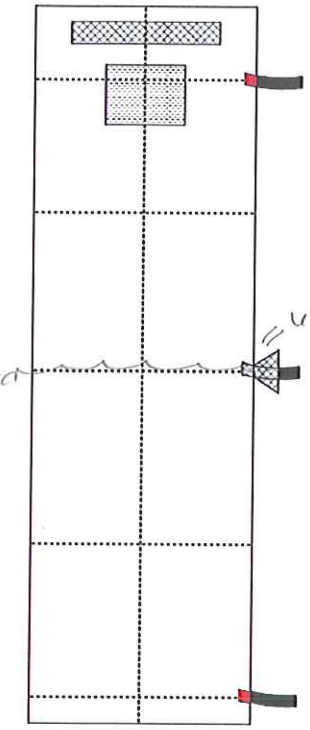
EFC 12



Notes:

Date: 10-21-15 10:30
Temperatures: Outside 79°F **, Inside** 81°F **, Chiller** 158°F??? Chiller seems broken, The water is still cool + running, but chiller is off

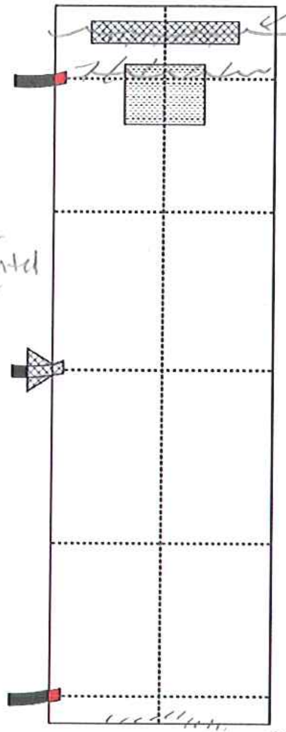
EFC 1



1 ← Approx. 6-8 beetles visible; Approx 1-3 beetles unaccounted for

≈ 6-8? beetles sitting on outlet cover

EFC 2



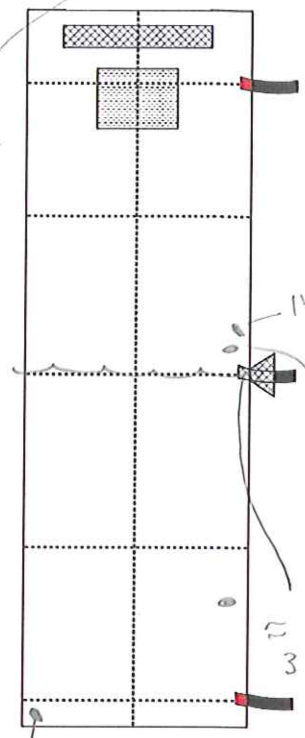
2 ← Approx 0 beetles visible; Approx 9 unaccounted

debris also in mesh of overflow

← No beetles visible

lots of debris

EFC 5



5 ≈ Approx. 7 beetles visible, Approx 2 unaccounted for

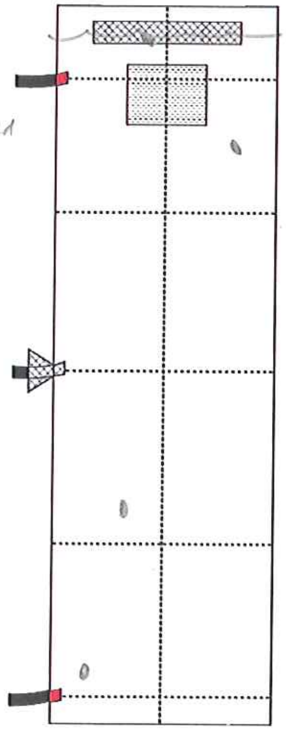
1 beetle motionless on tank wall above water line

1 beetle inactive, clinging to mesh above water line

≈ 3 beetles sitting on outlet cover

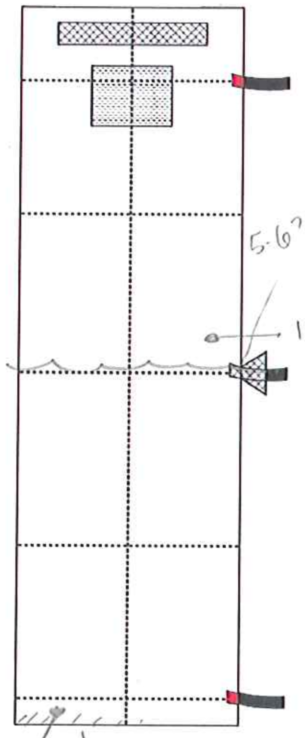
1 beetle on back of tank bottom

EFC 8



8 ← 4 beetles visible, 5 unaccounted for

EFC 9



9 ← Approx. 7 beetles visible, Approx 2 unaccounted for

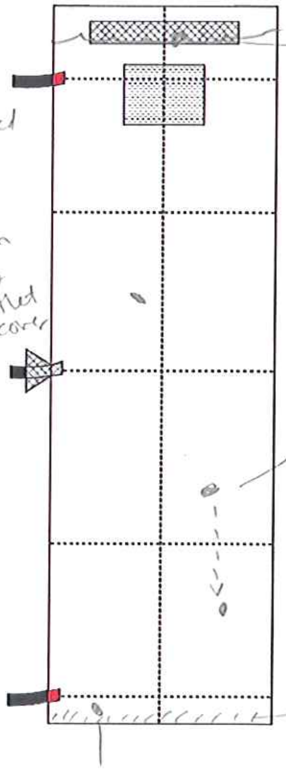
5-6? or more beetles sitting on top of outlet cover

1 clinging to mesh above water line

looks like one beetle inactive, supine on tank floor, legs folded

Some debris at tank bottom

EFC 12



12 ← 4 beetles visible, 5 unaccounted for

1 on mesh of outflow just below water line

~~can't determine if beetle or debris~~ beetle! ** moved here in 5 minutes after check

lots of debris on tank floor

1 beetle clinging to mesh

Notes:

* all debris at tank bottoms appears to be small POM

** the beetles seem to move a lot when exposed to light

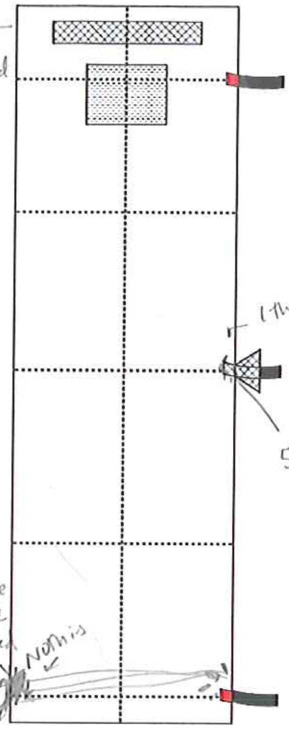
FINAL LOCATIONS

Date: 10-21-15
Temperatures: Outside ________ , **Inside** ________ , **Chiller** 68 °F

EFC 1

1 FINAL

- 9 accounted for, none in SOL.
- 5 of those had been on fabric mesh filter.
- very little POM on bottom of tank
- 8 placed in refugia



1 that had been above water column on side of tank was inactive but revived

5 beetles had been sitting on outlet; all were alive

4 looked inactive but ~~inactive~~ quickly revived

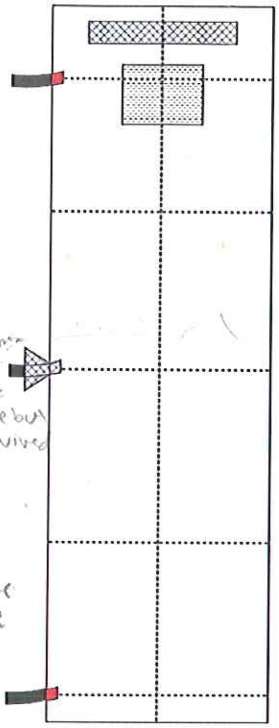
Not this

1 active

1 alive but died during extrication

These were alive but had been obscured by substrate

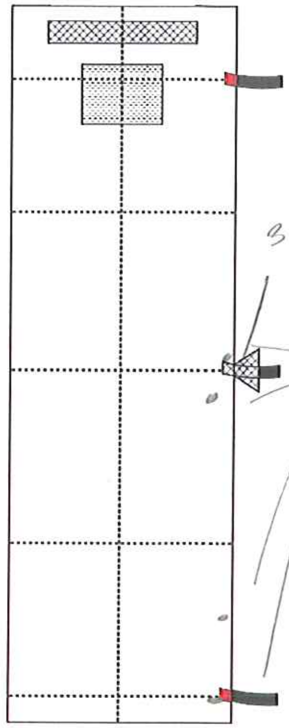
EFC 2



2 FINAL

NONE VISIBLE ON MESH EVEN AFTER CLOSE INSPECTION

EFC 5



3 beetles on outlet seemed inactive

3 of the ~~all~~ 4 beetl

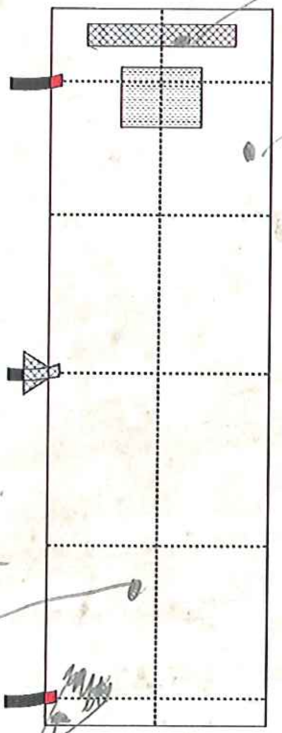
on mesh appar

alive;

1 inactiv

Seems to be 1 stuck above water line

EFC 8



alive

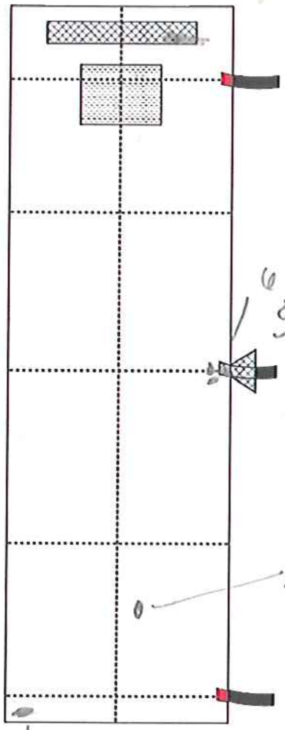
alive

alive

alive

the one on its back in the corner / bottom of the tank was alive

EFC 9

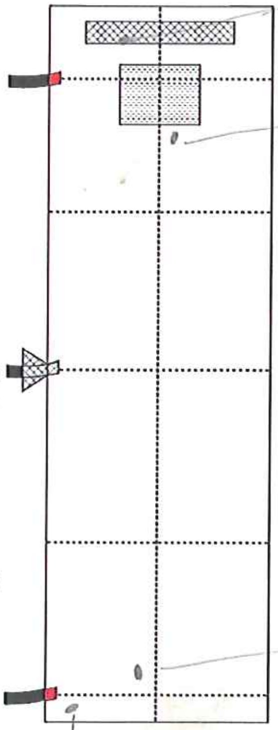


6 beetles 3 active in 3 inactive

1 alive, obscured by mesh

1 inactive

EFC 12



1 alive on mesh

seemed inactive

seemed inactive

alive.

Notes: