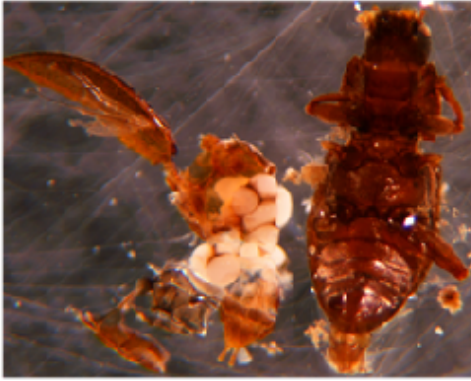# Evaluation of the Life History of the Comal Springs Riffle Beetle

Egg

to

Adult

Photographs by Randy Gibson and Mike Quinn

Year One Report

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TEXAS★STATE BIOLOGY

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BACKGROUND

Riffle beetles (Family Elmidae) are small aquatic beetles that occupy larger substrates in swift habitats of high quality, low temperature, streams and rivers. Riffle beetles respire through their plastron (Brown 1987, White and Roughley 2008, Elliott 2008a) and are typically sensitive to pollutants and environmental change (Elliott 2008a). The Comal Springs riffle beetle (*Heterelmis comalensis*) was Federally listed as endangered in 1997 due to threats caused by potential decreases in spring discharge attributed to drought or excessive groundwater extraction. It is also vulnerable to groundwater contamination from urban runoff, agricultural waste and toxicants, and leaking storage facilities and pipelines. *Heterelmis comalensis* are flightless, non-vagile, and associated with gravel substrates near spring sources (Bosse et al. 1988, Cooke et al. 2015). Adults are approximately 2 mm long and it is thought that they feed on decaying organic matter and aufwuchs (Brown 1987, White and Roughly 2008).

Applied research conducted over the first three years of the Edwards Aquifer Habitat Conservation Plan (HCP) in the Comal River system has demonstrated that aquatic vegetation and fountain darters are quite tolerant suggesting that *H. comalensis* may in fact be more useful as a sentinel species. This is extremely important in that the present HCP flow regime exhibits periods of extended drying of the spring runs, and areas along the western shoreline and Spring Island (these areas are the presumed strong hold for the riffle beetle in the Comal system). The HCP flow regime is not projected to reach the minimum levels recorded in the drought of record (DOR). However, the HCP projects extended periods of <100 cfs, which is beyond what was observed during the DOR.

Conservation of *H. comalensis* requires strategies to maintain aquatic habitats that provide for growth, reproduction, and population viability. These strategies are developed by determining habitat preferences (e.g., water temperature, depth, current velocity, etc.) of the species, or by experimentally examining the effects of different environmental conditions on body condition. Information from these types of studies can provide insight into the importance of a variety of habitat metrics, and the range in habitat conditions required of *H. comalensis*. The limited distribution, sensitivity of habitats, small body size, and difficulties involved in sampling the three-dimensional habitat of *H. comalensis,* have proven to make determining habitat preferences through field studies problematic.

This applied research is striving to evaluate the life history and developmental stages through the life cycle of *H. comalensis*. Thus far (2016), the focus has been on gaining a better understanding of factors contributing to production of eggs and larval development. Building off the considerable knowledge gained in 2016, the focus will shift next year to exploring factors and methods for achieving pupation and completion of the full life cycle. To compliment the life cycle studies a concurrent investigation is being performed to assess the factors that contribute to riffle beetle condition and health.

LITERATURE REVIEW

Population Distributions and Habitat Associations of the Comal Springs Riffle Beetle

Heterelmis comalensis is a crenophilic riffle beetle that is endemic to eucrenal habitats of Comal Springs, Comal County and San Marcos Springs, Hays County, both in Texas. Collection of *H. comalensis* using cotton cloth lures in the surface habitat of the Comal system was determined to be highly restricted spatially to within approximately 80 cm from spring openings throughout the primary

and smaller spring outlets along the spring runs (Cooke 2012). *Heterelmis comalensis* is found at the depths of 2-10 cm in hard-packed gravel habitats commonly found adjacent to spring openings (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 microhabitat associations. Data indicate that riffle beetles prefer spring water characterized by high carbon dioxide (CO_2), low dissolved oxygen (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 cease flowing and the habitat associated with the presence of the Comal Springs riffle beetle dries. 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 2012), it is likely that the water temperature will increase and DO concentrations will drop. A recent study conducted for the HCP 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 (undeveloped 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 2008). 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 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 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/or the lack of interstitial habitat due to clogging of interstices by fine sediments (Bo et al. 2006). In is also 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.

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 across the Western 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 among the remaining 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), it 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, how or if 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 (Gibson et al 2008). Presumably, Comal Springs riffle beetles are attracted to the lures to gain access to the biofilms that grow there. 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*) was 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 detritivorous 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.

Hypothesized life history

Prior to the present study, the only observation of eggs was dissected females in captivity which were found to carry around 10 relatively large eggs (Figure 1). No information existed on deposition sites, hatch time, and diapause for *H. comalensis*; however, it was speculated by Brown (1987) "that most elmids glue their eggs singly or in small clusters to undersides of submerged rocks, wood, or plant stems, depending on habitat preference of the species." It remained to be seen if *H. comalensis* lay eggs individually or in clutches and where females prefer to lay their eggs. Presumably, *H. comalensis* eggs do not overwinter or display diapause (dormancy) as in other aquatic inverts with seasonal patterns because it has been shown that *H. comalensis* exhibit no seasonality in the wild (Bowles et al. 2003) or in captivity [San Marco Aquatic Resource Center (SMARC) refugium data]. Riffle beetle eggs typically

have short incubation times of 5-15 days depending on temperature (Brown 1987) which was expected to be the case in *H. comalensis*.
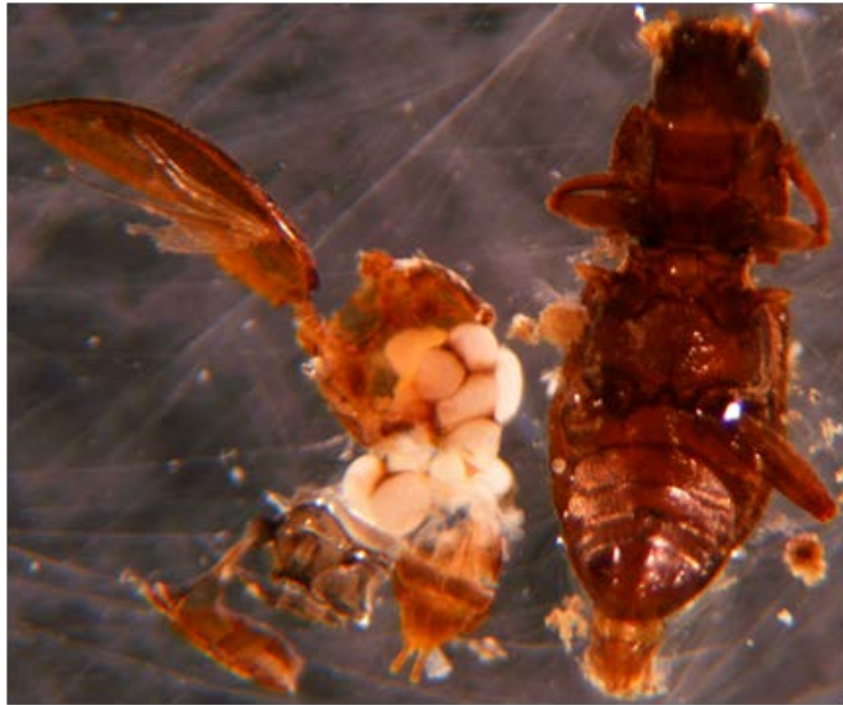

Figure 1. Photograph of a dissected single female in captivity that was found to be carrying around 10 relatively large eggs

After hatching, it was hypothesized that *H. comalensis* larvae undergo several molts between instars. Other genera of elmids studied have 6-8 larval instars across a duration of 6-36 months with developmental rate varying with temperature and food availability (Brown 1987, White and Roughley 2008). Cooke (2012) estimated *H. comalensis* to have approximately seven instars by measuring preserved larvae. Aside from this, no other information on larval development is known.
Like other elmids, *H. comalensis* larvae have gills and are aquatic, often inhabiting similar habitats as adults subsisting on microorganisms and debris scraped from substrate (Brown 1987). Later instar riffle beetle larvae develop tracheal air sacs which might aid in drifting to suitable pupation sites or escaping poor environmental conditions (Brown 1987).

Typically, elmids pupate above the water line in moist soils, under rocks, or in rotting wood (Brown 1987, White and Roughley 2008). Above water pupation might help in establishing a functional plastron in the adult when shifting from gill respiration of larvae. However, pupation in *H. comalensis* has been observed to take place under the surface of the water in captivity at the SMARC (Huston and Gibson 2015) and in the wild (pers. obs.). Currently, the requirements for pupation of *H. comalensis* are still unknown.

As it is unknown how long it takes for larvae to maturate into pupae, how long pupae must incubate before molting into adults, and how long beetles can live as adults, it is impossible to estimate the typical life span of *H. comalensis*. However, wild caught adults have been maintained in captivity at SMARC for up to 16 months and other species of elmids have survived for several years in captivity

(Brown 1973, White and Roughley 2008) therefore suggesting the lifespan of *H. comalensis* may be quite long.

METHODS AND RESULTS

EGG PRODUCTION AND MATING

Subtask 1.1 – Collection of study organisms

Adults were collected using cotton lures (Gibson et al. 2008, BIO-WEST Inc. 2004-present). Lures were retrieved approximately four weeks after they were set in spring openings in Spring Run 3, Western Shoreline, and around Spring Island. All larvae collected were returned to the spring they were collected from and a subset of adults (not exceeding 50% of collections) were brought to the SMARC alive where all subsequent phases of experimentation were conducted. Beetles were collected on three occasions, March 7th, April 5th, and May 24th 2016. Once at SMARC, all individuals were placed into quarantine and held for two weeks prior to being used in any subsequent research; this duration also served to acclimate beetles to captivity.

Subtask 1.2 – Noninvasive/nonlethal method of sexing wild-caught adults

The proposed experimental design called for the pairing of one male with one female to be held together to mate and then produce eggs. In order to produce pairs of males and females, a method was developed to reliably sex individuals to ensure that no same sex pairs were formed unintentionally. Prior to this research no established method existed to reliably determine the sex of riffle beetles. Most methods that have been employed thus far require the euthanasia and subsequent dissection of individuals to determine sex; unfortunately, these methods are not useful for sexing live individuals. Therefore, it was necessary to sex individuals on the basis of external morphology exclusively, without causing them any harm.

To begin studies on sexual dimorphism, archived specimens preserved in 95% EtOH held at SMARC were photomicrographed at various magnifications using Olympus Cellcens camera and software system at the standard shutter speed of 3.395 milliseconds. After capturing an image of various aspects of a specimen, the specimen was dissected to determine sex. These preliminary photos were used to measure and study various morphological characteristics and were studied for correlation with sex. A total of 21 archived specimens were dissected. This method was subsequently applied to 37 live specimens collected from Comal Springs because at least *n*=30 beetles were necessary to estimate the reliability of sexually dimorphic characteristics. Because the success of all subsequent phases of experimentation depended on this phase of study, this was considered necessary take.

Previous research suggested there may be a relationship between size and sex in adult *H. comalensis* (Bosse et al. 1988); however, this was not found to be the case in our data sets. Size did not vary consistently by sex; in fact, some of largest individuals were found to be male when it was previously thought that females were larger than males. Of all the morphological attributes measured, only the length, width, and ratio thereof of the 5th (posterior most) abdominal sternite was found to vary consistently by sex; sternite length was found to be the most robust predictor of sex (Table 1, Figure 2).

Table 1. Result of model selection procedure. Of the variables found to correlate with sex, length of sternite 5 was found to be the most effective measure of external morphology for sex determinations.

Model	*H. comalensis* F ratio	*p*	AIC weight	*H. glabra* F ratio	*p*	AIC weight	*H. vulnerata* F ratio	*p*	AIC weight
Length	139.7	<<0.001	1	43.35	<<0.001	0.846	95.81	<<0.001	1
Ratio	81.86	<<0.001	0	38.01	<<0.001	0.154	33.66	<<0.001	0
Width	9.794	0.00267	0	1.419	0.238	0	32.58	<<0.001	0
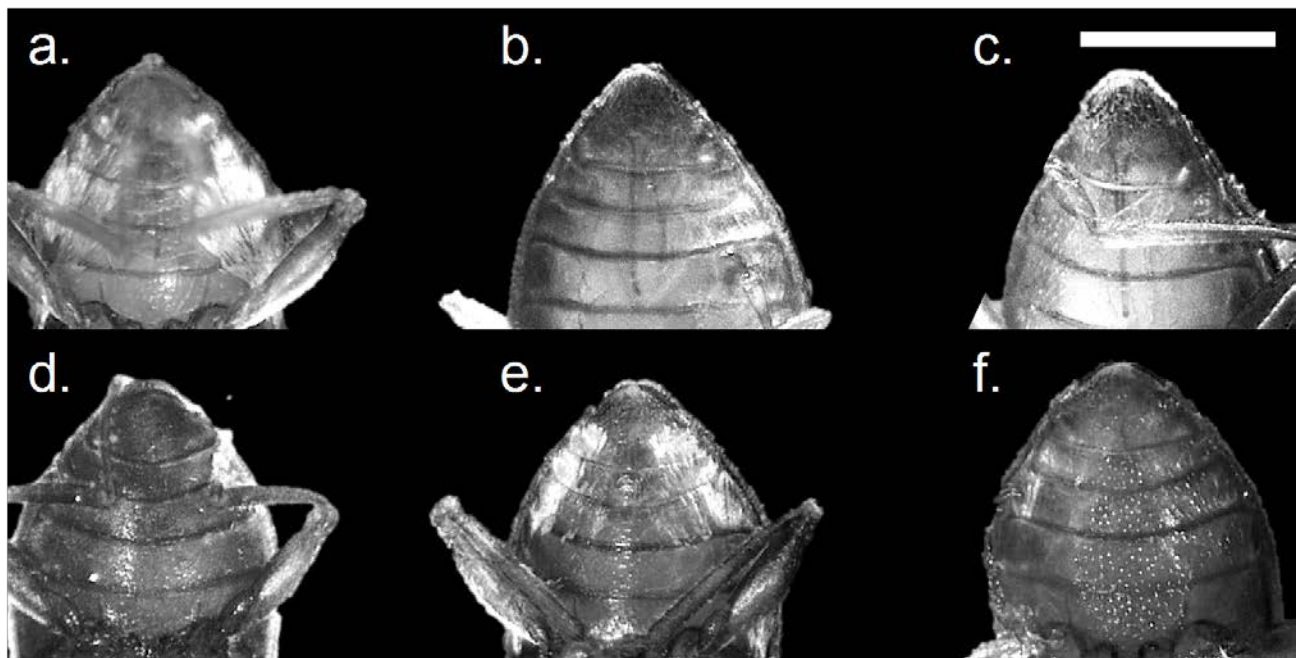
Figure 2. Photo micrographs of female (a-c), and male (d-f), *H. comalensis* abdomens showing variation in length of sternite 5. Females are recognizable by noticeably more elongate 5th sternites. b. is a representative example of the typical female while e. is a representative example of the typical male. Scale bar is 500-μm.

A bootstrap analysis of this data suggested that there was very little error using this methodology (Figure 3). The bootstrap method was subsequently applied to a total of 295 live specimens of *H. comalensis*. Based on this methodology, it was found that beetles in Comal Springs have male biased sex ratios; roughly 2 to 1 males to females (Table 2).
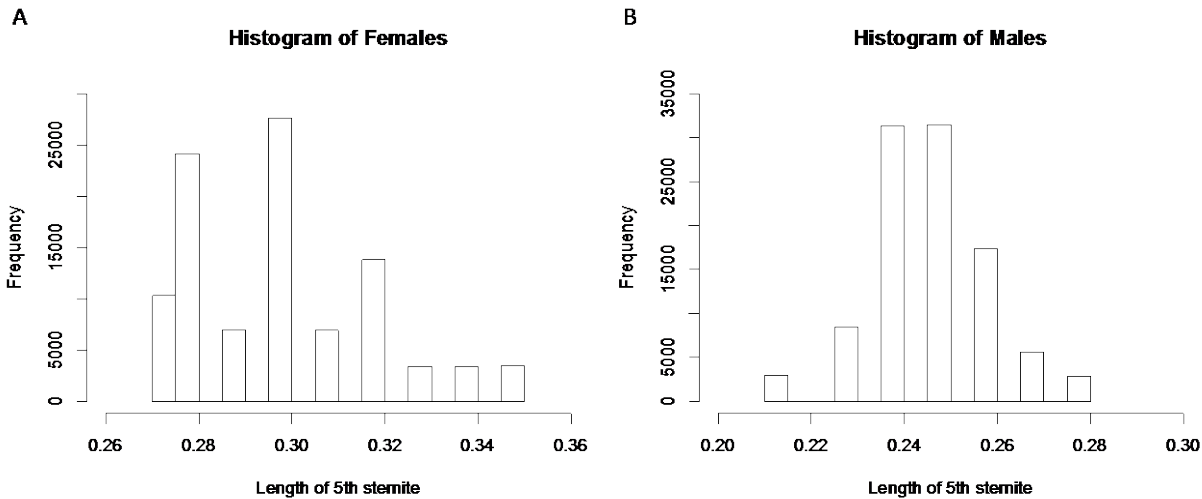
Figure 3. Bootstrap frequency distributions of sternite 5 length for *H. comalensis*; A) females, B) males.

Table 2. Observed sex ratio of each sampling event for *H. comalensis*.

Sampling event	*n* female	*n* male	Total *n*	Sex ratio (f/m)
March 8, 2016	29	60	89	0.33/0.67
April 5, 2016	23	44	67	0.34/0.66
May 10, 2016	8	16	24	0.33/0.67
May 24, 2016	12	28	40	0.30/0.70
June 7, 2016	32	43	75	0.43/0.57

Subtask 1.3 – Mating and egg production

Using the above method, male-female pairs were formed and one pair each was placed into a 55-μm pore size upwelling system to prevent loss of eggs due to their small egg size (≈150 μm).

Thirty-two male-female pairs were formed in total. Each pair was assigned a unique accession number and then randomly assigned to one of eight experimental groups with four replicates per experimental group. The eight experimental groups are as follows:
- Leaves
- Leaves + cotton cloth
- Leaves + cotton cloth + rock
- Cotton cloth + rock
- Cotton cloth
- Leaves + rock
- Rock
- Control (none of the above factors present)

To start experimental mating, four of each of the above treatments were given the appropriate treatment substrate and placed into a flow-through upwelling system (Figure 4). The substrate in these inserts were allowed to condition for 2 – 3 weeks prior to further experimentation. After this conditioning period, one pair of beetles was introduced to each upwelling. These pairs were allowed to run for 3 weeks prior to

the collection of any eggs. This was done for two reasons: 1.) when pairs were initially formed it would be impossible to know anything about which male fathered the eggs; and 2.) when pairs were initially formed, any eggs that were produced would have been developed within the females on a nutritional substrate in group holding instead of the treatment substrate in the upwelling systems. After this initial three-week period of adult pairing in upwelling systems, every upwelling was searched thoroughly for eggs. Any eggs found were placed into a group holding system and these larvae were set aside for future study. Afterwards, upwellings were checked weekly for eggs. Any eggs found were counted and it was recorded what substrate they were found on. Eggs were then placed into a separate upwelling with a small portion of the same combination of treatment substrate. This was repeated for up to six weeks.

Figure 4. Flow-through upwelling system in use with mesh inserts.

Analysis of location of egg deposition
Substrate of egg deposition was studied for the initial two weeks of egg production. During this time period a total of 65 eggs were laid, of which 82% were laid on leaves (Figure 5). It is important to point out that though the majority of the eggs laid were attached to leaves, more than half of the eggs produced were from treatment groups including cloth (Figure 6), suggesting that the biofilms that grow on cloth are important nutrition for egg development by female beetles.
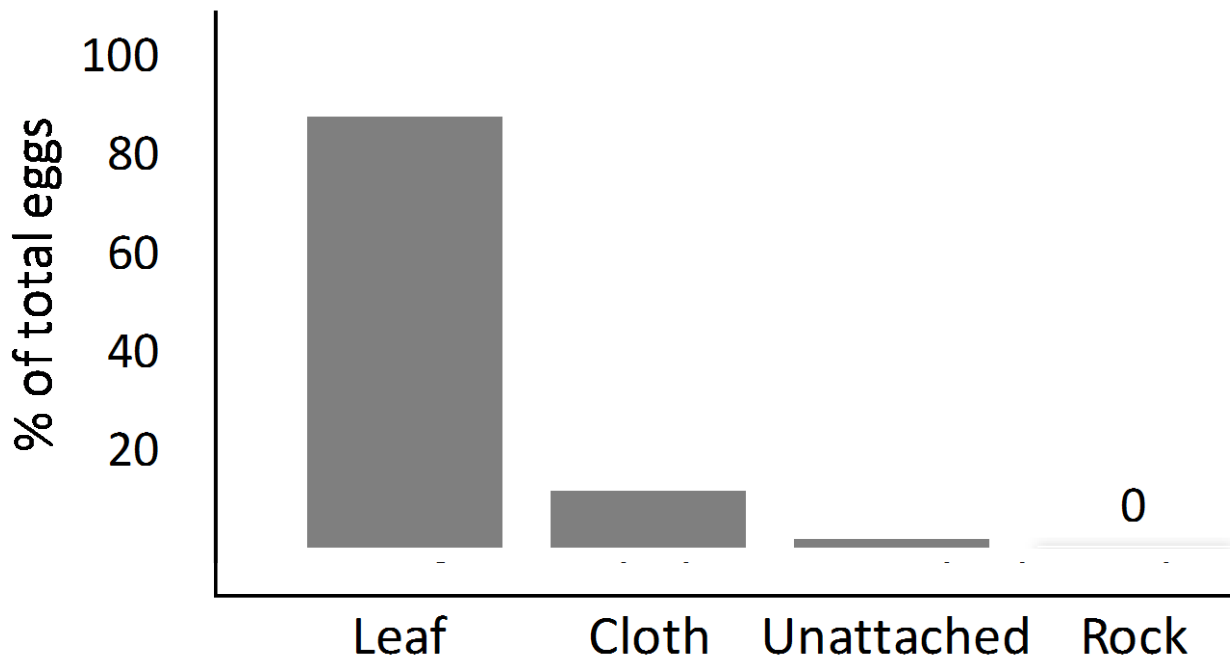
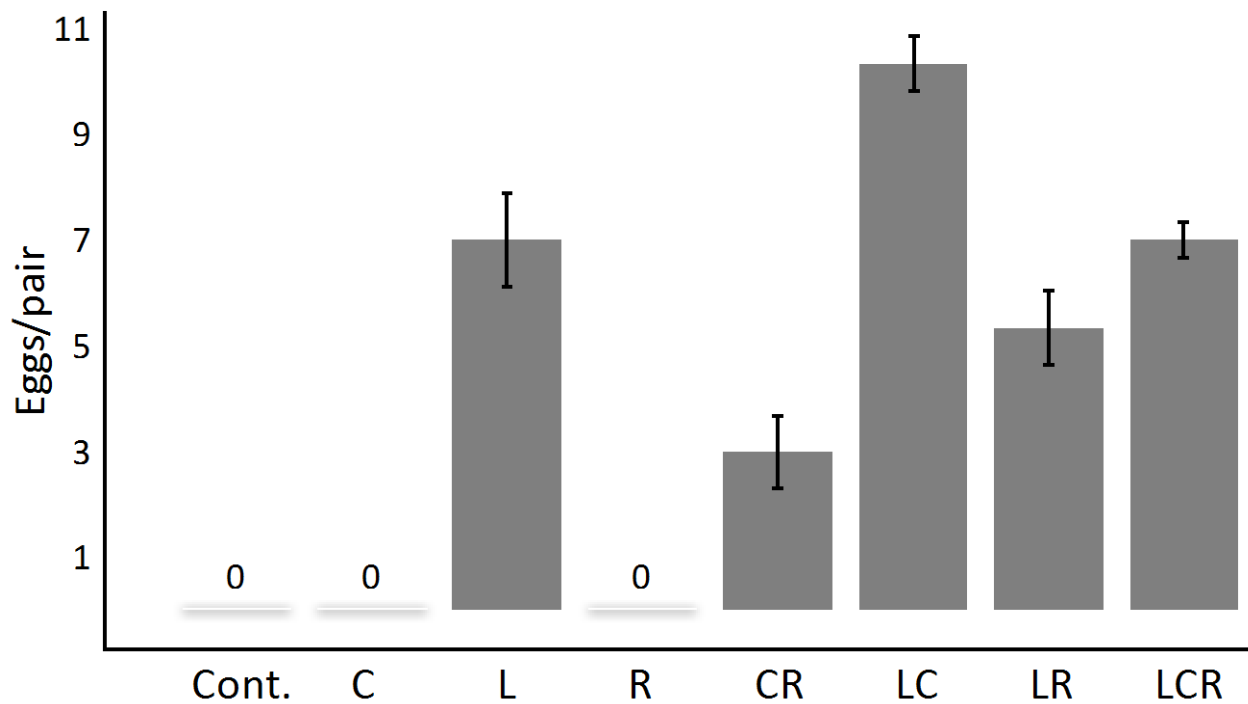**Figure 5.** Egg substrate deposition results by percent across all treatment groups.

Figure 6. Preliminary egg production results by treatment group. Note that only combinations including cloth or leaf produced eggs. Cont.=control; C=cloth; L=leaf; R=rock.

Analysis of number of eggs produced
A summary of egg production results can be found in Table 3. Factors that contributed to differences in the number of eggs produced and the survival of adults were analyzed using a combination of ANOVAs (Table 4). It was found that the duration in captivity and survival of parents greatly contributed to egg production. The survival of parents was found to be strongly influenced by treatment substrate [$F = 3.733$, $df = 7$, $p = 0.004$] suggesting that although age and survival of parents explained more variation in egg production than treatment substrate, treatment substrate may be responsible for variation in these two factors.

Table 3. Summary of egg production results. Cont.=control; C=cloth; L=leaf; R=rock; *n*=number of pairs; survival is duration female survived.

Substrate	*n*	Mean weeks of survival	Total clutches	Mean clutches per pair	Total eggs	Mean eggs per pair	Var. eggs per pair	Var./mean ratio
Cont.	7	3.29	1	0.14	2	0.29	0.57	1.97
R	6	5	3	0.5	4	0.67	1.07	1.61
L	4	3.5	3	0.75	9	2.25	14.92	6.63
L + R	7	4.43	9	1.29	21	3	11.34	3.78
C + L	6	3.17	10	1.67	54	9	40.4	4.49
C + R	5	3.6	12	2.4	72	14.4	353.3	24.53
C	9	5.75	26	2.89	132	14.67	239.75	16.35
C + L + R	4	6	15	3.75	71	17.75	177.6	10.01

Table 4. Results of ANOVAs on factors contributing to egg production.

SOV	*df*	*F*	*p*
Duration in captivity	1	15.09	<<0.001
Survival of parents	1	12.566	<<0.001
Treatment substrate	1	2.35	0.01

To determine which treatment factors contributed most to the observed results, a combination of multiple factor ANOVAs was used to test for differences in treatment substrate combinations. It was found that cloth was the most important factor contributing to the number of clutches laid (Table 5) and the total number of eggs (Table 6).

Table 5. Results of ANOVAs on substrate factors contributing to number of clutches laid. C=cloth; L=leaf; R=rock.

SOV	*df*	*F*	*p*
R	1	1.22	0.28
L	1	0.494	0.48
C	1	17.76	<<0.001
R + L	1	3.66	0.064
R + C	1	0.027	0.97
L + C	1	122	0.28
R + L + C	1	3.02	0.09

Table 6. Results of ANOVAs on substrate factors contributing to the total number of eggs laid. C=cloth; L=leaf; R=rock.

SOV	df	F	p
R	1	0.532	0.47
L	1	0.083	0.77
C	1	18.45	<<0.001
R + L	1	1.131	0.29
R + C	1	0.561	0.46
L + C	1	0.532	0.47
R + L + C	1	0.864	0.36

Subtask 1.4 – Incubation of eggs

Hatching success

Factors that contributed most to hatching success was analyzed with a combination of multiple factor ANOVAs. Of the factors analyzed, only the treatment of the parents was found to affect hatching success (Table 7). Clutch size was analyzed because it was thought that there may be some relationship between the number of eggs being produced by a female and the relative quality of nutrition received by each individual egg. Duration that parents were in captivity was analyzed because it was thought that productivity may decline with time in captivity or age. However, no effect was found for either of these two factors. Treatment was found to have an effect on hatching success suggesting that the nutrition a female receives while developing eggs affects the quality of the eggs produced.

Table 7. Factors analyzed for effect on hatching success.

SOV	df	F	p
Clutch size	1	0.021	0.7
Parents duration in captivity	1	<<0.001	0.99
Treatment	7	2.39	0.031

Treatment substrate was further studied to determine which type of substrate contributed most to producing healthy eggs. An AIC analysis was used to select between models with all possible substrate combinations. It was found that the presence of cloth explained variation in hatching success (Table 8) which once again suggested biofilms that grow on cloth are important nutrition for egg development by female beetles in captivity.

Table 8. Factors contributing to hatching success of eggs.

Model	df	F	p	AIC weight
C	1	7.09	<0.01	0.750
R + C	1	0.014	0.907	0.089
L + C	1	0.011	0.912	0.080
R	1	0.644	0.425	0.032
L	1	0.534	0.467	0.031
R + C + L	1	0.039	0.843	0.010
L + R	1	0.411	0.524	0.009

Development

A subset of eggs was monitored closely throughout incubation until hatching. Eggs were found to have translucent shells making it possible to observe development without damaging the eggs. Early in development, eggs were found to have few relatively large globular bodies, believed to be undifferentiated cells of the zygote (Figure 7.)
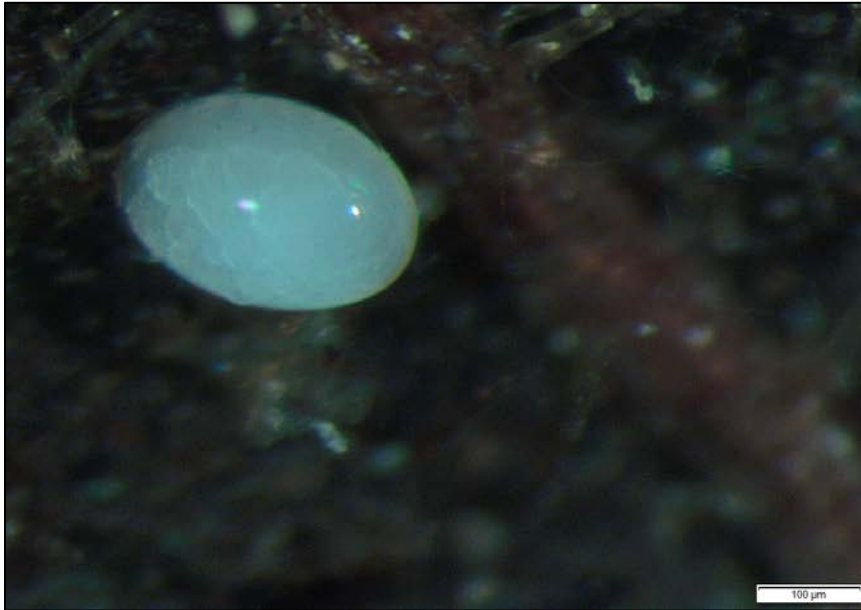

Figure 7. A ≈3-day old egg. Note few large cells.

Approximately one week into development, much more cellular division had occurred resulting in numerous smaller cells (Figure 8).
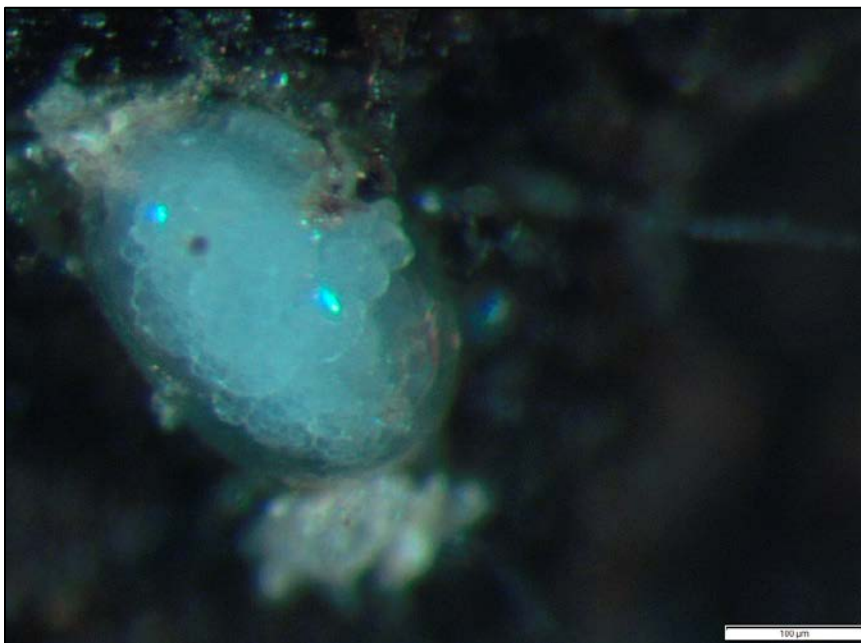
Figure 8. A ≈7-day old egg. Note numerous smaller cells.

After approximately 2 weeks, tissue differentiation could be observed. A linear embryo with budding appendages was visible (Figure 9).

Figure 9. A ≈14–18-day old egg. Note linear embryo with budding appendages.

After approximately three weeks, nearly fully developed larvae could be observed in eggs, as well as a faintly visible eye (Figure 10).
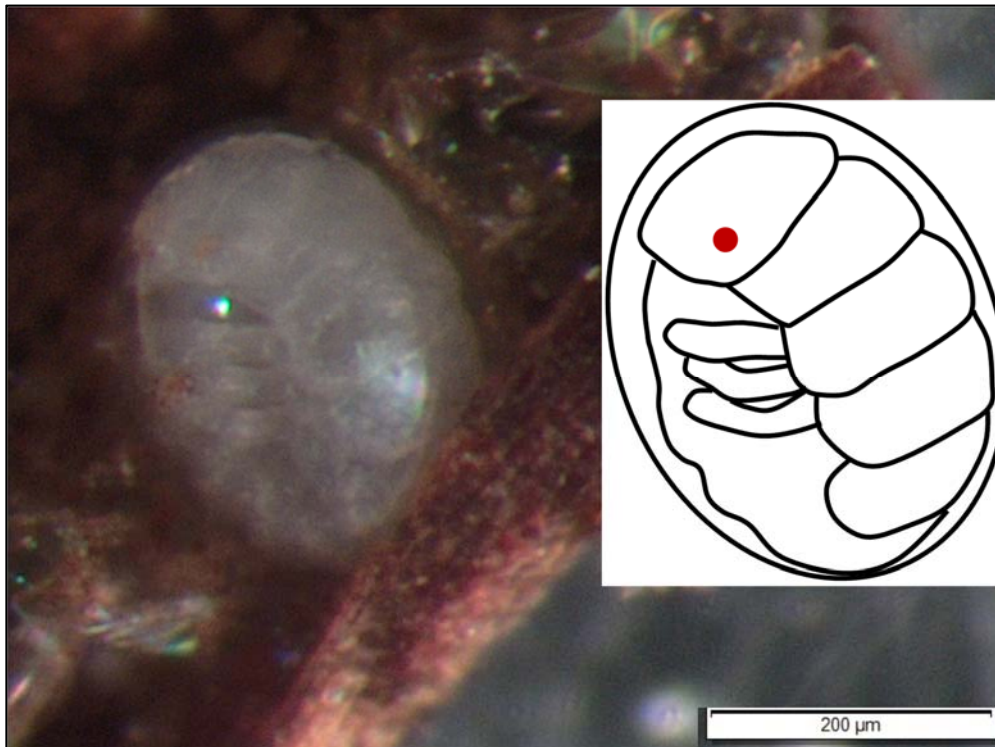

Figure 10. A ≈21-day old egg. Note nearly complete development of larvae, including the presence of an eye. Schematic inset for clarity.

Larvae hatched from eggs after approximately three and a half weeks (Figure 11). First instar larvae were morphologically unlike later instar larvae development, with long tail filaments and much more setose.

Figure 11. Newly hatched 1st instar larva after ≈25 days of incubation.

LARVAL DEVELOPMENT

Complete development into adults from small larvae has been observed to take place as quickly as 4 months at the SMARC; however, single larvae have been held as along as at 1.5 years at the SMARC without development into larger instars. This stagnate stage may be due to nutritional requirements of *H. comalensis* and thus, various sources of nutrition were tested on the development of the larvae. Additional larval studies were conducted at both the Texas State University Freeman Aquatic Building (FAB) and the SMARC in order to maximize space, resources, and provide replication of data as well as a backup in case of equipment failure.

General design for this task is as follows:

Subtask 2.1 – Individual holding - development
A repeated measures ANOVA was used to determine if survival of larvae varied across treatment groups. Four counts were taken at one month intervals on larvae of varying age and hatching dates. No treatment effect was found [$F = 1.685$, $df = 3$, $p = 0.208$]; however, it is important to note that this analysis only included treatment groups for which larvae were successfully produced. These treatment groups were leaves + cotton cloth, leaves + cotton cloth + rock, cotton cloth + rock, and cotton cloth.

Developmental instar of larvae was estimated on larvae of known age by measuring head capsule width once monthly for four months. The instar was then inferred using the frequency distribution of Cooke (2012). Growth rate was found to fit a linear trend across time for all treatment groups (Figure 12).
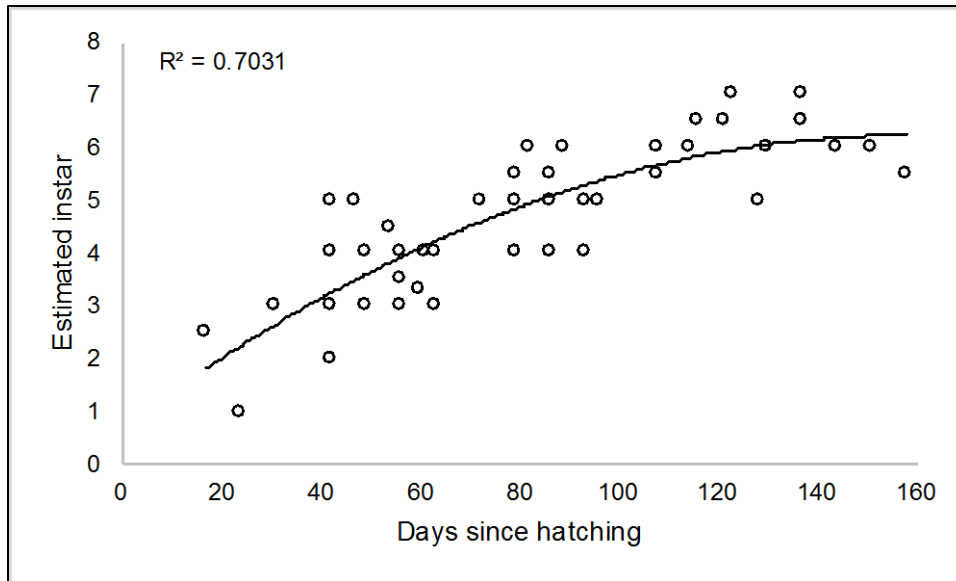
Figure 12. Scatterplot of larval instars across five months; at approximately three months a plateau in growth was observed.

Subtask 2.2 – Group holding - habitat preference

Heterelmis comalensis in culture at SMARC readily produce numerous larvae. Therefore, at any given time there are usually hundreds of larvae of various instars in culture at SMARC. Because these larvae are of unknown age and instar, they are not useful for the study of larval development. However, these larvae are useful for the study of larval physiological tolerances and habitat preferenda. To study habitat preferenda, larvae were taken from refuge culture tanks and placed into four vertically oriented 6-in. PVC cylinders with 55-µm mesh bottoms partially submerged into tanks with flow through water (Figure 13). These cylinders were sectioned into eight equal sized sectors with one combination of treatment substrates in each. Sectors were divided to prevent the movement of substrate material between sectors but still allow for the movement of larvae. Each cylinder had one section for each of the following substrate types:

- Leaves
- Leaves + cotton cloth
- Leaves + cotton cloth + rock
- Cotton cloth + rock
- Cotton cloth
- Leaves + rock
- Rock
- Control (none of the above factors present)

The arrangement of the different treatment groups across sectors was randomized for each cylinder in an effort to ensure that artefacts of the distribution of the different substrate types does not confound results. Each cylinder received 40 larvae taken from refuge culture; 5 randomly assigned to each sector to start experimental trials. Afterwards, each sector was examined once monthly for larvae; larvae in each sector were counted in each of the four cylinders. We assessed differences among substrate types in terms of the number of larvae in each section using a single factor ANOVA. Relative abundance was found to vary significantly with treatment substrate [$F = 4.583$, $df = 7$, $p = 0.0002$]. A Tukey HSD analysis indicated that C + L, and C + L + R were preferred substrates.

Figure 13. Habitat preference study cylinders submerged in flow through system.

CONDITION INDEX

The purpose of the condition index task is to determine analytical methods and to assess relationships between environments and habitat associations on *H. comalensis* body condition. We hypothesize that the length-mass relationship of *H. comalensis* will be indicative of body condition, whereby the condition of slender individuals will be lower than more robust animals. Hence, environments producing slender individuals will be considered less suitable to *H. comalensis* than conditions that produce more robust animals. Investigation into sample methods were initiated using seven *H. glabra* and nine *H. vulnerata* that were received at Desert Research Institute (DRI) frozen, in good condition, and encased in individual aluminum foil packages. All work during this initial evaluation was conducted by Dr. Don Sada. Technicians will be used in subsequent studies, after techniques are adequately determined.

Specimens were maintained in frozen condition until work was initiated. Each riffle beetle was placed in a sterile glass vial, assigned a number, and dried for 18 hours in an oven maintained at 105°C. Individuals were weighed using a Metrler Toledo Model XP56 analytical balance that measures to the microgram. The balance was calibrated before daily use. Following weighing, specimens were placed under a Luminera Infinity 2 camera mounted on an Olympus SZX 16 dissecting microscope. Total length (tip of head to the distal end of the elytron), length and maximum width of the elytron and the

pronotum were measured from an image that included a 3.0-mm standardized measure. Measurements were made to the nearest 0.05-mm using a Vernier caliper. Lengths were determined from the proportional relationship of each measurement to a 3.0-mm standard measure.

Length-weight regressions were calculated for *H. glabra* and *H. vulnerata* to determine if this relationship could be measured and quantified (Figures 14 to 21). There was a positive relationship between length and weight for all measurements. All regressions were statistically significant $p < 0.05$, but some relationships were stronger than others (pronotum length was not measured due to the inability to precisely determine its boundaries). Calculated R^2 values for length/biomass relationships were generally higher for *H. glabra* (range 0.3062 to 0.9271) than *H. vulnerata* (range 0.251 to 0.707). Differences between the two species present opportunities for additional research into explanations about morphological variation. The total body length/biomass relationship for *H. glabra* was the strongest observed ($R^2 = 0.9271$; Figure 14). The elytron width/biomass relationship was strongest for *H. vulnerata* ($R^2 = 0.7070$; Figure 20).
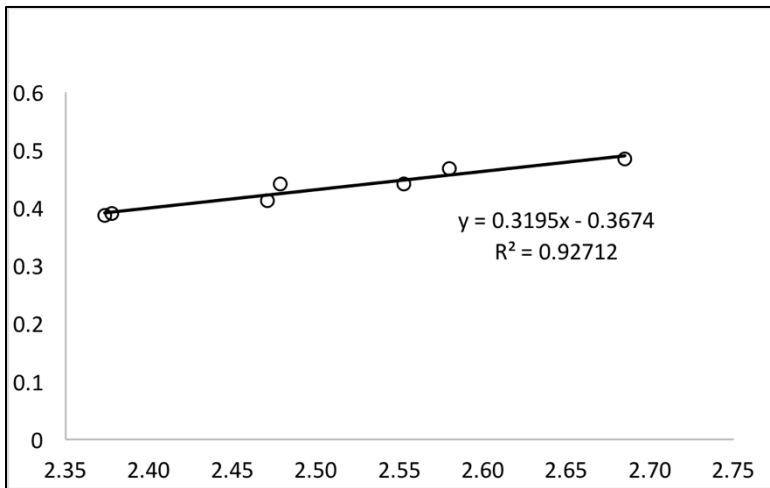

Figure 14. *Heterelmis glabra* total body length (mm)/biomass (micrograms) relationship.
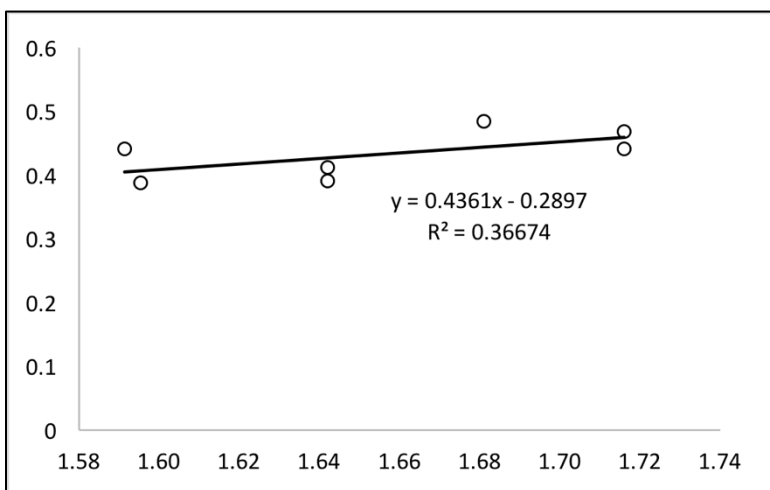

Figure 15. *Heterelmis glabra* eltyron length (mm)/biomass (micrograms) relationship.
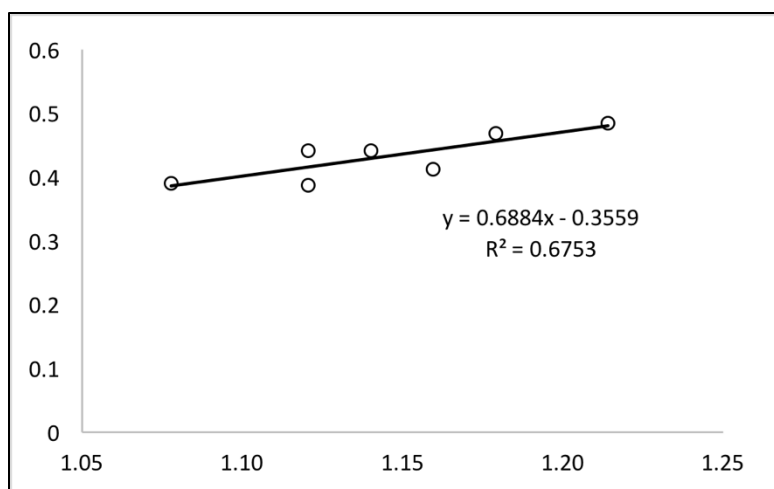
Figure 16. *Heterelmis glabra* eltyron width (mm)/biomass (micrograms) relationship.
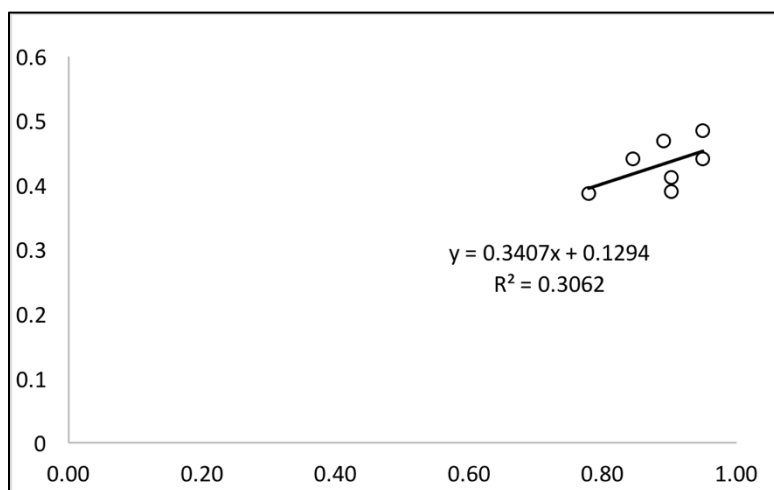

Figure 17. *Heterelmis glabra* pronotum width (mm)/biomass (micrograms) relationship.
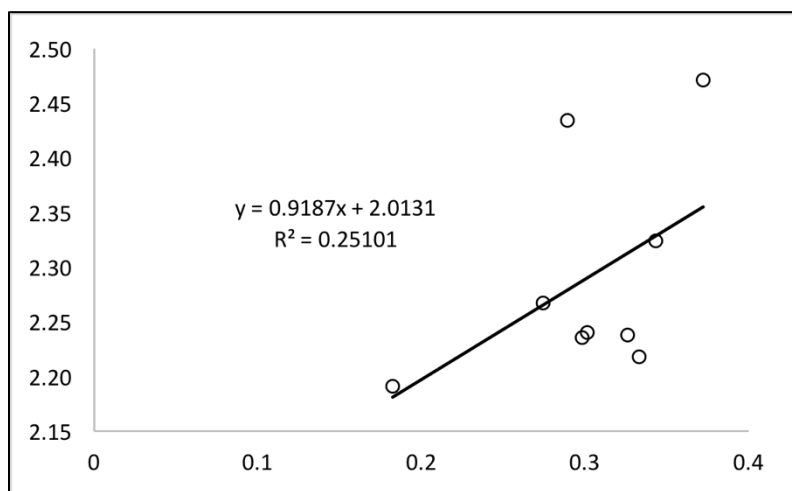

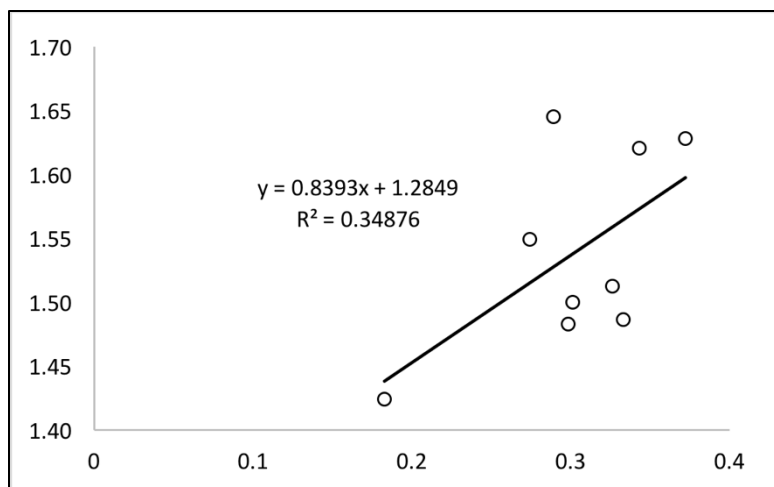**Figure 18.** *Heterelmis vulnerata* total body length (mm)/biomass (micrograms) relationship.

Figure 19. *Heterelmis vulnerata* elytron length (mm)/biomass (micrograms) relationship.
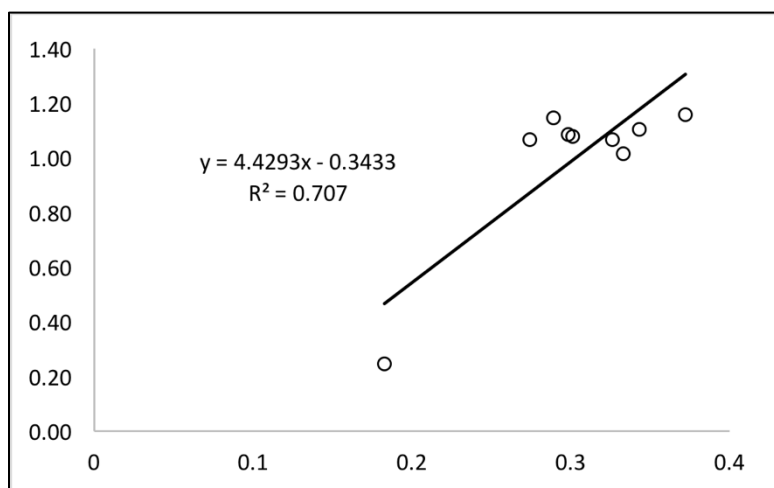

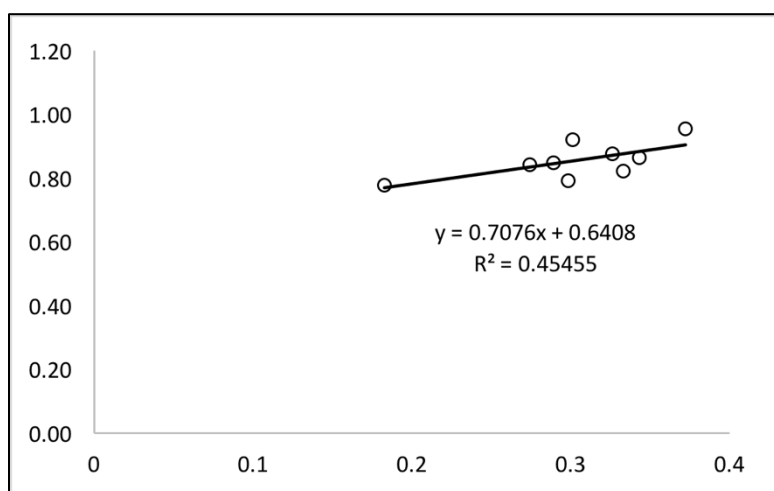**Figure 20.** *Heterelmis vulnerata* elytron width (mm)/biomass (micrograms) relationship.

Figure 21. *Heterelmis vulnerata* pronotum width (mm)/biomass (micrograms) relationship.

YEAR 1 SUMMARY

During our sexing experiments, we found that males and females do not differ in overall size as previously hypothesized (Bosse et al. 1988). However, the 5th sternite was found to vary predictably between males and females. Therefore, the methodology presented herein is the only method recommended for future sexing of *H. comalensis*. An unexpected result pertaining to sex ratios is that approximately 2/3rds of beetles sampled in Comal Springs were found to be male. At this time, it is unclear if and why sex ratios may be skewed in the wild.

The study of egg deposition indicates that of the substrates tested, females preferentially deposit eggs on leave substrates. Although eggs were predominantly deposited on leaves, it is important to note that eggs were produced in greatest abundance in treatment groups including cloth, suggesting that biofilms growing on cloth are a significant source of nutrition for egg production. However, cloth alone did not successfully produce eggs in great abundance suggesting that synergism with other substrates is important to biofilm growth and egg production. Eggs were found laid singly and in clutches. No trend was observed as to which pattern of deposition is predominant.

The amount of eggs produced declined with time in captivity as did the survival of adults. It is unclear if beetles were expiring due to age or inadequate nutrition in captivity. However, survival and egg production were strongly correlated with treatment substrate. Parental nutrition was also found to be influential to hatching success and is likely an important component to successfully cultivating *H. comalensis*. Egg production declined with duration in captivity; however, this was not found to significantly affect hatching success.

At approximately 3 weeks of incubation before hatching, *H. comalensis* have relatively long incubation durations compared to other elmids. Throughout our study of egg incubation, no evidence of diapause was observed. Larval development fit a linear trend across time with larvae likely reaching their final instar at approximately 4 months. Results of the larval study show that larvae were found to prefer treatment substrates involving cloth. Larvae were also found to have the greatest survival on treatments involving cloth.

In summary, cloth was found to be the most important factor for nutrition at all stages of development. However, synergism with leaves likely promotes the growth of biofilms as treatment groups with exclusively cloth were not as successful as those involving cloth and leaves. Despite biofilms on cloth proving to be the most important of substrates for nutrition, leaves are the most important for egg deposition.

The condition index study determined that a length/weight relationship can be quantified for *Heterelmis* species and these relationships likely differ by species. Certain metrics of length proved to be more precise. Further experimentation is necessary to determine which metric of length/weight best estimates beetle condition.

YEAR 2 SCOPE OF WORK

The scope of work for the second year of study will include a more in-depth evaluation of larval development. At this time, it is thought that *H. comalensis* larvae undergo seven instars based on frequency distributions of wild-caught larvae (Cooke 2012). However, larvae have never been closely studied throughout their development and the occurrence of intermediates between designated instars

suggests that there may be more instars or size variation between individuals. The morphological development at each instar is also unknown though 1st instar larvae differ significantly from later instar. Therefore, we are proposing to isolate and closely study the development of a subset of larvae to understand the morphological development and number of instars.

The primary goal of the second year of study will be to identify factors contributing to entering pupation and achieve successful pupation in captivity. This has proven to be the major obstacle to successfully cultivating *H. comalensis* in captivity over the years and therefore will likely be the most difficult to achieve. Therefore, pupation will be the major focus of the second year of study.

The final goal of the second year of study will be to determine what factors contribute to the condition of beetles. This will be accomplished by taking length/weight measurements on beetles raised on various substrates and looking for a relationship between beetle condition and diet.

The proposed scope of work briefly described above is built directly off the knowledge obtained during Year 1 studies as well as contractual requirements. Specific studies and methodologies will be further developed and presented to the HCP Science committee for review and approval in early 2017.

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