Comal Springs Riffle Beetle (*Heterelmis comalensis*): Life History and Captive Propagation Techniques

Final Report

PREPARED FOR:

Edwards Aquifer Authority
900 E. Quincy Street
San Antonio, TX 78215

PREPARED BY:

BIO-WEST PROJECT TEAM
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EXECUTIVE SUMMARY

The Comal Springs riffle beetle (*Heterelmis comalensis*) is a Federally listed endangered species primarily found at Comal Springs, Comal County, Texas and is believed to be vulnerable to potential threats caused by decreases in spring discharge from drought and excessive groundwater extraction, groundwater contamination from urban runoff, and agricultural or industrial waste. This is important because the present Edwards Aquifer Habitat Conservation Plan (HCP) flow regime exhibits periods of extended drying of the spring runs, and areas along the western shoreline and Spring Island; areas documented to be strongholds for *H. comalensis*. Therefore, understanding their life history and physiological tolerances is vital to the conservation of the species through continued protection in the wild and for support of the HCP refugia program. Conservation efforts for *H. comalensis* should include strategies that maintain critical aquatic habitats that support growth, reproduction, and genetic diversity of healthy populations. Prior to the present study, little was known of the life cycle of *H. comalensis* thus this document represents the first full account of the *H. comalensis* life cycle. With this information we were also able to develop a cultivation strategy to guide the HCP refugia program.

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 abdominal sternite was found to vary predictably between males and females. Therefore, the sexing methodology presented herein is recommended for future use. An interesting note pertaining to sex ratios is that approximately 2/3rds of beetles collected for the sex ratio study from Comal Springs were found to be male. At this time, it is unclear why sex ratios are skewed.

Investigations on egg deposition indicated that females preferentially deposit eggs on leaves. This suggests that females deposit eggs on plant based materials in nature. Eggs were found laid singly and in clutches. No trend was observed as to which pattern of deposition is predominant. Though 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 on cloth.

The amount of eggs produced declined with the amount of time the parents were in captivity as did the survival of adults. It is unclear if beetles were expiring due to age, inadequate nutrition and/or reproductive strategy in captivity. However, adult survival and egg production were strongly correlated with treatment substrate. Parental nutrition was also found to be important to hatching success. This suggests that nutrition is an important component to successfully cultivating *H. comalensis*. Though it was found that egg production declined with duration in captivity, this was not found to significantly affect hatching success, suggesting that egg quality does not decline with time in captivity.

Heterelmis comalensis eggs incubate for approximately three weeks before hatching; a relatively long incubation duration 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 four months. Larvae were found to prefer treatment substrates containing cloth and also had the greatest survival rates 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. Prior to pupating, larvae were found to require four or more months in the 7th instar; during which time they were presumed to be assimilating nutrients necessary for successful metamorphosis. However, other potential explanations could include not all habitat or nutritional requirements (temperature, substrate, food, cover) being sufficiently met or possibly too much handling and disturbance taking place. Future applied research and ongoing captive propagation should continue to provide a better understanding of metamorphosis. Overall generation time was found to be two years.

The condition index work conducted at the Desert Research Institute developed several length/weight relationships for *Heterelmis* species, allowing for comparisons between wild-caught and captive-reared individuals. Certain metrics of length proved to be more precise. Further experimentation is necessary to determine which metric of length/weight best estimates beetle condition. The current dataset suggests that captive beetles have access to more food sources than wild. Differences were observed between wild and captively bred *H. comalensis*, indicating that beetles raised in different environments may exhibit different growth, hence length/weight and body conditions. The body condition of captively reared male and female *H. comalensis* consistently increased more rapidly with increased length than observed for wild individuals, suggesting that characteristics of body condition were different and that captive populations are as fit if not more so than wild populations.

Also presented herein is a detailed description of the current methodologies for *H. comalensis* captive propagation. Cultivation techniques describing equipment and facilities necessary to maintain proper conditions for each stage of the life cycle (e.g. mating and egg production, larval development and pupation) is included. Finally, an active applied research program focusing on *H. comalensis* nutrition and flow preferences at various life stages in captivity is recommended to support the long-term HCP refugia program.

BACKGROUND

Riffle beetles (Family Elmidae) are small aquatic beetles that generally occupy larger substrates in swift habitats of high quality, low temperature, streams and rivers. They respire through a plastron (Brown 1987, White and Roughley 2008, Elliott 2008a) and are typically sensitive to pollutants and environmental change. 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, incapable of swimming, and associated with gravel substrates near spring sources. Adults are approximately 2 mm long and they appear to be non-selective grazers whose diet originates from terrestrial derived coarse particulate organic matter inoculated more often with bacterial biofilms (Nowlin et al. 2017a).

This beetle's affinity for clean, clear spring fed waters is paramount because the present Edwards Aquifer Habitat Conservation Plan (HCP) flow regime projects periods of extended drying of the spring runs, and areas along the western shoreline and Spring Island (areas documented as strong holds for *H. comalensis* in the Comal System). Although the projected flow regime is not expected to be as severe as experienced during the drought of record, extended periods of < 100 cfs could threaten critical habitat and possibly even the survival of *H. comalensis*. As such, a thorough understanding of their life history, physiological tolerances and surface/subsurface interactions is vital to the conservation of this species.

Conservation efforts for *H. comalensis* should include strategies that maintain critical aquatic habitats that support growth, reproduction, and genetic diversity of healthy populations. These strategies are most effectively 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 hospitable to the livelihood 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 studies of habitat use and life history through field studies problematic.

This applied research intended to evaluate the life history and developmental stages of the life cycle of *H. comalensis*. Prior to the present study, the life cycle was documented to take more than a year in captivity (Randy Gibson, pers. comm.); therefore at least a two-year study was necessary to investigate important life history questions. A phased approach was implemented with Year 1 (2016) targeting factors contributing to production of eggs and larval development. The focus in Year 2 (2017) was to build off the available literature and Year 1 knowledge to further explore development and achieve pupation in order to complete the full life cycle. Documented herein is the first accurate account of the *H. comalensis* life cycle followed by guidance on successful captive propagation of this species. Also reported herein is data on Comal Springs riffle beetle condition and health which was collected concurrently with the life-history investigation.

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. The niche of *H. comalensis* in the Comal system was determined to be restricted spatially to within approximately one meter from spring openings throughout spring outlets along the spring runs (Cooke 2012). Data indicates that *H. comalensis* are restricted to spring water characterized by stable variation in physicochemistry (Cooke 2012), though the precise mechanisms for spatial restriction of *H. comalensis* within the Comal system are unknown. However, recent research conducted by Texas State University has suggested that narrow physiological tolerances drive microhabitat associations (Weston Nowlin, pers. comm.). Other species of more widespread elmids (i.e., *Stenelmis* spp.) also exhibit specific habitat associations (Phillips 1995). It is currently thought that reduced spring flow in Comal Springs may lead to habitat desiccation and/or degradation that may impact the livelihood of *H. comalensis*. A recent study conducted for the EAHCP (Nowlin et al. 2017b) found that Comal Springs riffle beetles could tolerate rapid changes in temperature and DO concentrations; for example, *H. comalensis* could withstand up to 45 °C before loss of function and could tolerate 0 mg DO/L for several minutes without displaying behavior indicative of stress. However, their tolerance ranges to short-term temperature changes were substantially narrower than a closely-related elmid species (*Heterelmis glabra*; Nowlin et al. 2014). Furthermore, the sensitivity of riffle beetles to long-term physicochemical fluctuations were found to be narrower for *H. comalensis* compared to more cosmopolitan elmids (Nowlin et al. 2017b).

Dispersal ability, habitat connectivity, and life history of the Comal Springs riffle beetle

Although many adult aquatic coleopterans emerge from aquatic habitats and disperse via flight to other aquatic habitats, most riffle beetle species typically cannot disperse great distances via flight (Hinton 1976, White and Roughley 2008). Other elmid species are considered flightless as adults (Elliott 2008a)

and the flight muscles of some species begin to degenerate upon re-entry into water (Hinton 1976). The loss of adult flight suggests that flight capability may not be compatible with utilization of plastron respiration because of the maintenance of sub-elytral air space required for functional wings (Thorpe and Crisp 1947). It is also likely that most dispersal events in the Edwards Plateau are evolutionary dead ends as springs are separated by great distance. Thus, selection favored *H. comalensis* evolving to be flightless. Most likely the main mode of locomotion for *H. comalensis* is by crawling slowly in 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 occur during drift events, as they are more susceptible to being washed away or carried to sub-optimal habitats by strong currents (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 degree of seasonal variation in environmental conditions, 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 stable environmental conditions found 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 has successfully housed both adult and larval *H. comalensis* to the extent that adults mate and oviposit in refugia, at the time the present study was commenced they had minimal success in pupation and adult emergence rates. Despite advances made over the last two years, there is still a clear need to better understand potential mechanisms and conditions leading to successful emergence of adult *H. comalensis*.

Surface-subsurface interactions for flowing water invertebrates

The subsurface hyporheos may act as refuge for benthic invertebrates during periods of low flow even if all water at the surface has dried (Williams and Hynes 1974). Interstitial spaces provide water relatively protected from evaporation which enables aquatic invertebrates to recolonize the surface once drought events have passed (Dole-Olivier et al. 1997). Drought-induced changes 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 to 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 desiccation of hyporheic sediments (Boulton and Stanley 1995), anoxia (Smock et al. 1994), and/or clogging of interstices by fine sediments (Bo et al. 2006). Currently, it is unknown whether a reduction in spring flow may disconnect *H. comalensis* from optimum food sources, such as properly conditioned terrestrial organic matter and detritus concentrated in the littoral zone. Additionally, the genetic variation of *H. comalensis* populations across the different meta-habitats throughout the Comal system suggests limited gene flow and connectivity among the different meta-populations (Gonzales 2008). Therefore, ensuring that micro-habitats remain wetted is essential to maintaining the species as loss of micro-habitat to desiccation could result in small scale local extirpation.

It remains unknown how *H. comalensis* 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 event. 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 into the hyporheos (Bowles et.al 2003). Previous research has shown that for semivoltine species of riffle beetles (i.e., individuals that live for multiple years) pool habitats connected via flowing surface water can serve as refugia for both larvae and adults during drought periods (Burk and Kennedy 2013).

Food habits and trophic ecology of Comal Springs riffle beetle

Potential food resources for *H. comalensis* were identified in the Comal system in Nowlin et al. 2017a. Previous literature sources support these recent observations describing 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 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). Although Nowlin et al. 2017b shed considerable light on riffle beetle food sources in the Comal system, the precise food sources and trophic ecology of *H. comalensis* in the Comal and San Marcos systems 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,

incubation duration, and possible diapause for *H. comalensis* though 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 Marcos 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*.

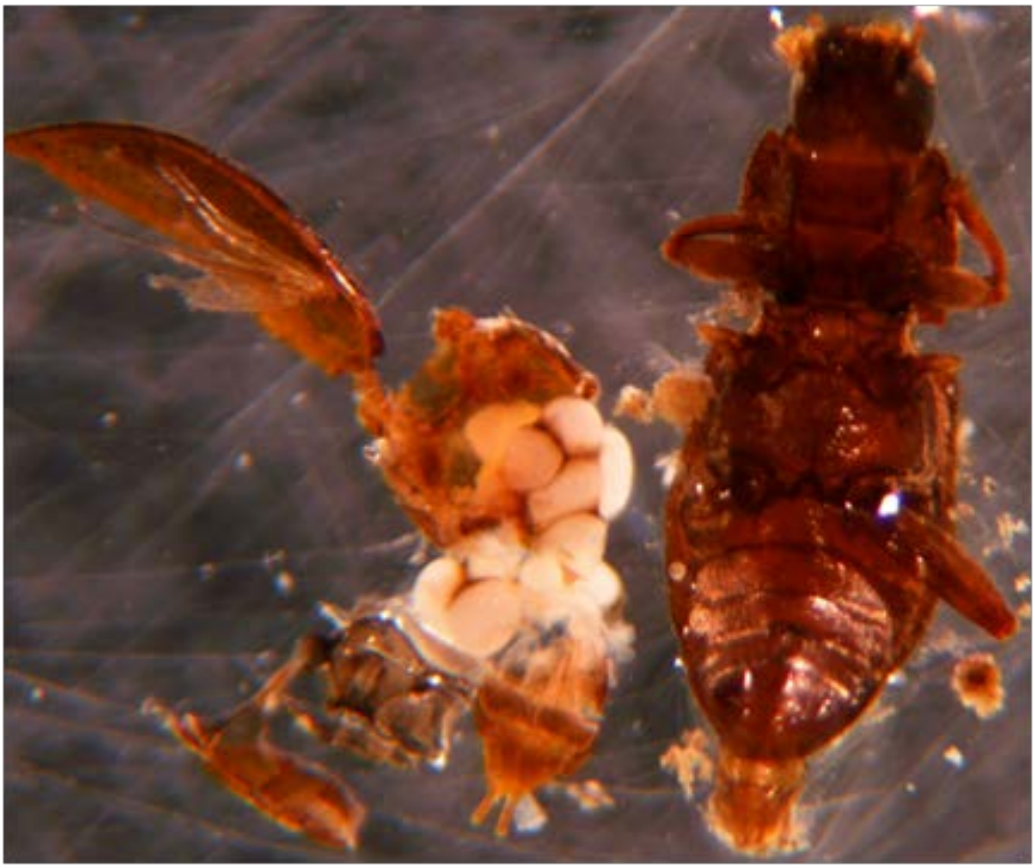

Figure 1. Photograph of a dissected single female in captivity that was found to be carrying around 10 relatively large eggs.

After hatching, it was hypothesized that *H. comalensis* larvae undergo several molts. 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* 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 specific requirements for pupation of *H. comalensis* are still unknown; however, the present study succeeded in producing several pupae, all of which were under water.

METHODS AND RESULTS

EGG PRODUCTION AND MATING

Collection of study organisms

Adults were collected using cotton lures (Gibson et al. 2008). 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.

Noninvasive/nonlethal method of sexing wild-caught adults

The experimental design called for the pairing of one male with one female to mate and produce eggs. In order to pair males and females, a method was developed to reliably sex individuals so same sex pairs were not 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 euthanizing and dissecting individuals to determine sex. For our purposes, it was necessary to sex individuals exclusively based on external morphology so as not to harm them.

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 an additional 37 live specimens collected from Comal Springs. Though this is substantial take, at least n=30 was necessary to estimate the reliability of sexually dimorphic characteristics especially considering that all subsequent phases of experimentation depended on this aspect of study. As a safe guard, this methodology was applied to two surrogate species prior to applying to live *H. comalensis.*

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 the 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 determination for all species.

	***H. comalensis* (*n*=37)**			***H.* cf. *glabra* (*n*=31)**			***H. vulnerata* (*n*=61)**		
Model	***F* ratio**	***p***	**AIC weight**	***F* ratio**	***p***	**AIC weight**	***F* ratio**	***p***	**AIC weight**
5th sternite length	58.68	<<0.001	1	66.60	<<0.001	1	174.00	<<0.001	1
5th sternite width	3.59	0.066	0	1.23	0.280	0	4.76	0.033	0
Head width	9.50	0.004	0	8.74	0.0061	0	0.59	0.446	0
Abdomen length	24.88	<<0.001	0	18.21	<0.001	0	26.63	<<0.001	0
Abdomen width	14.53	<0.001	0	5.23	0.030	0	2.59	0.113	0
Thorax width	6.07	0.019	0	3.80	0.061	0	6.17	0.016	0
Thorax length	3.90	0.056	0	0.36	0.550	0	1.52	0.223	0
Total length	11.58	0.002	0	3.66	0.066	0	7.95	0.006	0

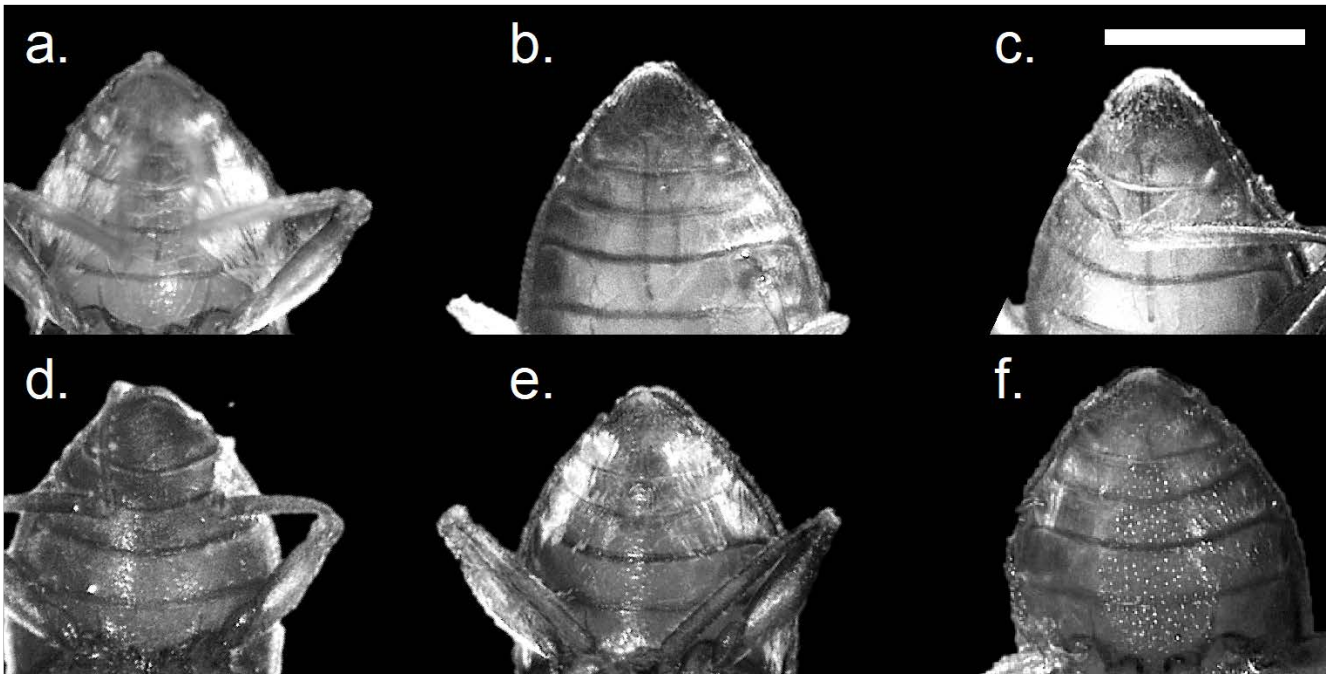

Figure 2. Photo micrographs of female (a-c), and male (d-f), *H. comalensis* abdomens showing variation in length of sternite 5. Females are recognizable by noticeably more elongate 5th sternites. b. is a representative example of the typical female while e. is a representative example of the typical male. Scale bar is 500-µm.

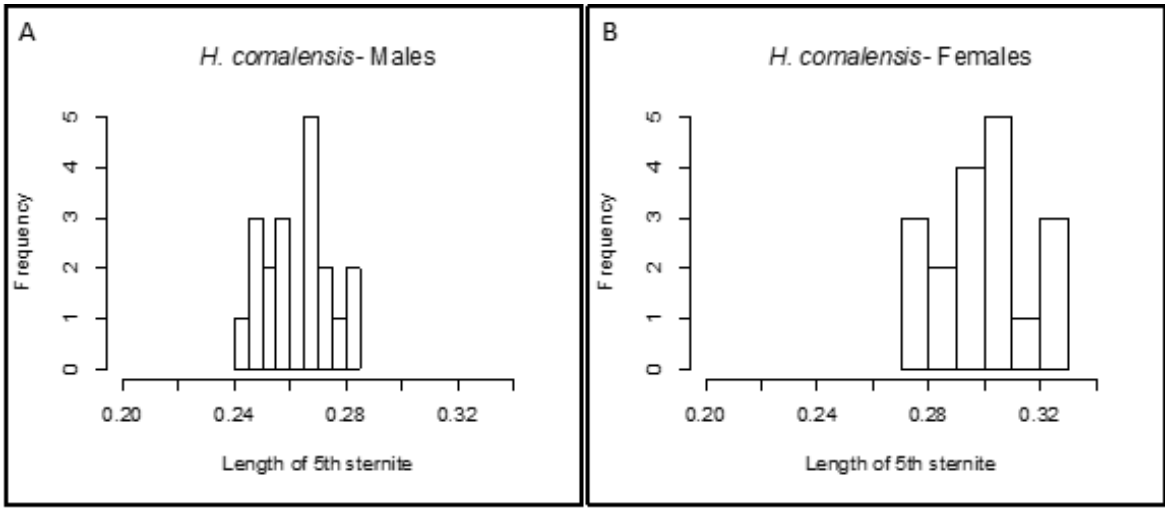

Figure 3. Observed frequency distributions of the 5th sternite length for *Heterelmis comalensis*. A) males and B) females.

The data generated from measuring the 5th sternite of $n = 37$ *H. comalensis* is shown in the frequency distribution plotted in Figure 3. This method was subsequently applied to a total of 295 live specimens of *H. comalensis*. Based on this methodology, additional beetles in Comal Springs were sampled where it was found that beetles in Comal springs have male biased sex ratios; roughly 2 to 1 males to females (Table 2).

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

Mating and egg production

Using the above method, male-female pairs were formed and one pair each was placed into 55-µm pore size upwelling system. This small pore size was necessary because eggs were found to be ≈150 µm in diameter thus a small mesh size was necessary to prevent loss of eggs. Forty-eight 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)

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 about two to three weeks prior to subject initiation. After the conditioning period, one pair of beetles was introduced to each upwelling. These pairs remained in the inserts for three 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, every upwelling was searched thoroughly for eggs. Any eggs found were placed into a group holding system and these larvae were set aside for other use. Afterwards, upwellings were checked weekly for eggs; any eggs found were counted and the substrate they were found on was recorded. 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

For the initial two weeks of egg production, the substrate of egg deposition was studied. 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, but the leaf substrate was important for oviposition. For further analysis of egg production by treatment group see next section of this report.

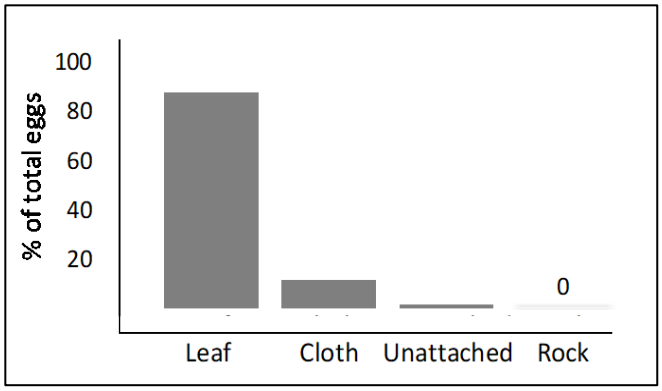

Figure 5. Egg substrate deposition results by percent across all treatment groups.

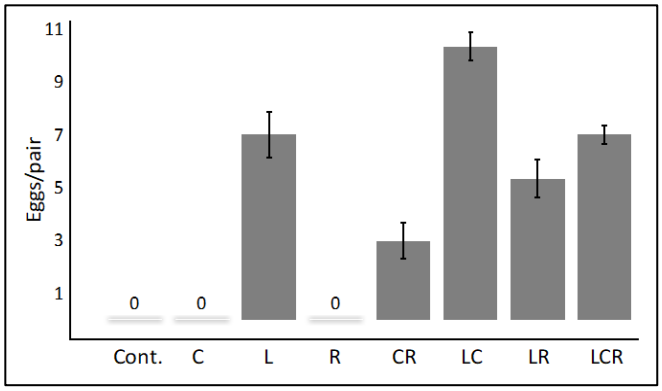

Figure 6. Egg production results by treatment group. Note that only combinations including cloth or leaf produced eggs. Cont.=control; C=cloth; L=leaf; R=rock.

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 explained by the 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 the treatment substrate, the 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.

Substrate	*n* (pairs)	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 = source of variation).

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 were 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

Incubation of eggs

Hatching success

Factors that contributed most to hatching success were analyzed with a combination of multiple factor ANOVAs. Of the factors analyzed, 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. The duration parents were kept 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. Substrate 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
Duration parents in captivity	1	<<0.001	0.99
Treatment substrate	7	2.39	0.031

Treatment substrate was further studied to determine which type of substrate contributed most to producing healthy eggs. 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 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

Egg development

Prior to the present study no information existed on deposition sites and incubation duration of *H. comalensis* eggs, 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. This speculation was found for *H. comalensis* (Figure 7) and to our knowledge is the first report of egg deposition from any elmid species.

A subset of eggs was monitored closely throughout incubation until hatching. Fortunately, eggs were found to have translucent shells making it possible to observe development without harming eggs. Early on in development, eggs were found to have very few relatively large globular bodies (Figures 7 and 8).

Figure 7. A pair of ≈3-day old egg.

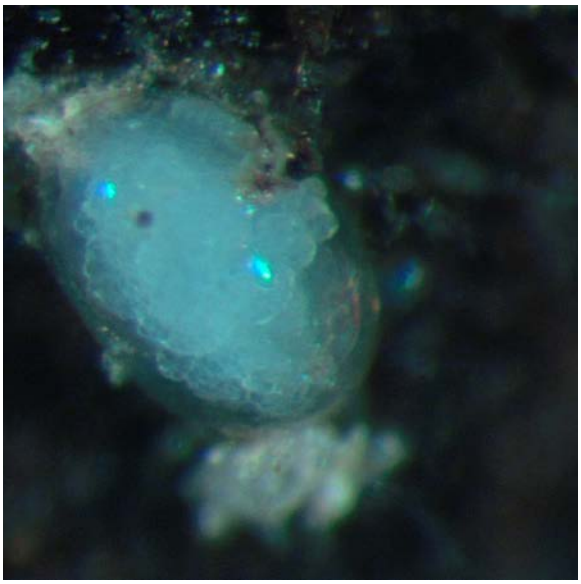

Figure 8. A ≈7-day old egg.

After approximately two weeks, tissue differentiation could be observed. A linear embryo with budding appendages was visible (Figure 9). After approximately three weeks nearly fully developed larvae were observed in eggs (Figure 10). At this point eyes became faintly visible.

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

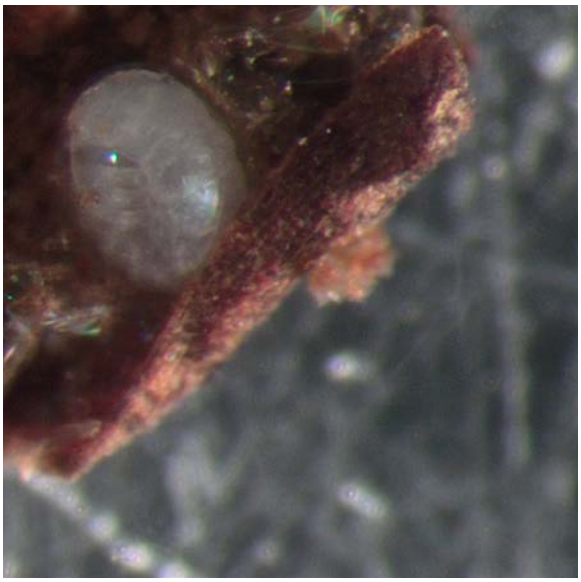

Figure 10. A ≈21-day old egg. Note nearly complete development of larvae including the presence of eye.

Riffle beetle eggs are thought to typically have short incubation times of 5-15 days depending on temperature (Brown 1987) which was expected to be the case with *H. comalensis*. It was found that *H. comalensis* eggs take 21-25 days to hatch which is appreciably longer than reports for other elmids and is curious considering there is no thermal fluctuation affecting incubation duration (i.e. long incubation durations are expected to be associated with colder temps in temperate waters). First instar larvae were quite small with long tail filaments and setose bodies (Figure 11), and morphologically unlike later instar larvae. 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].

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

LARVAL DEVELOPMENT

Individual holding - development

Growth rate and survivorship

A repeated measures ANOVA was used to determine if survival of larvae varied across substrate treatment groups that females originated on. Three counts were taken at one-month intervals on larvae of varying age and hatching dates. No treatment effect was found [$F = 1.685$, $df = 3$, $p = 0.208$, $n = 20$], 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.

Instar was estimated on larvae of known age by measuring head capsule width once monthly for four and a half months. Growth rate was found to fit a linear trend across time for all treatment groups with a plateau in growth around 12 weeks of age (Figure 12); again, it is important to note that this analysis only included treatment groups for which larvae were successfully produced. This analysis revealed that *H. comalensis* undergoes seven larval instars during its life cycle.

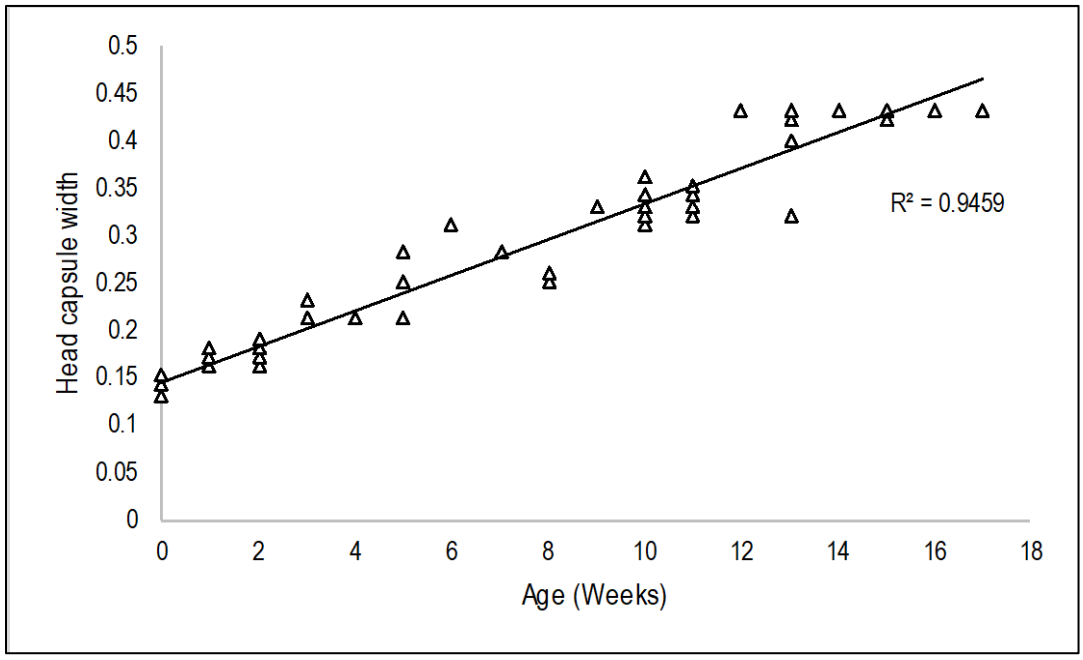

Figure 12. Scatterplot of larval head capsule width across four and a half months.

Mean head capsule width per age group was used to estimate developmental instar. Estimated instar was regressed across age which accentuated the plateau in development at 12 weeks of age (Figure 13). This plateau lasted for at least 16 weeks and indicates a pause in development by larvae in the last instar prior to pupation. This pause is thought to be necessary for assimilation of nutrients prior to successful pupation. Long durations spent in the last instar is not uncommon in elmids with many species remaining in the last instar for several years (Lesage and Harper 1976). Larval survival was found to be poor until the 5th instar (Figure 13). It is unclear if this survival rate is an artefact of inadequate conditions in captivity or is natural.

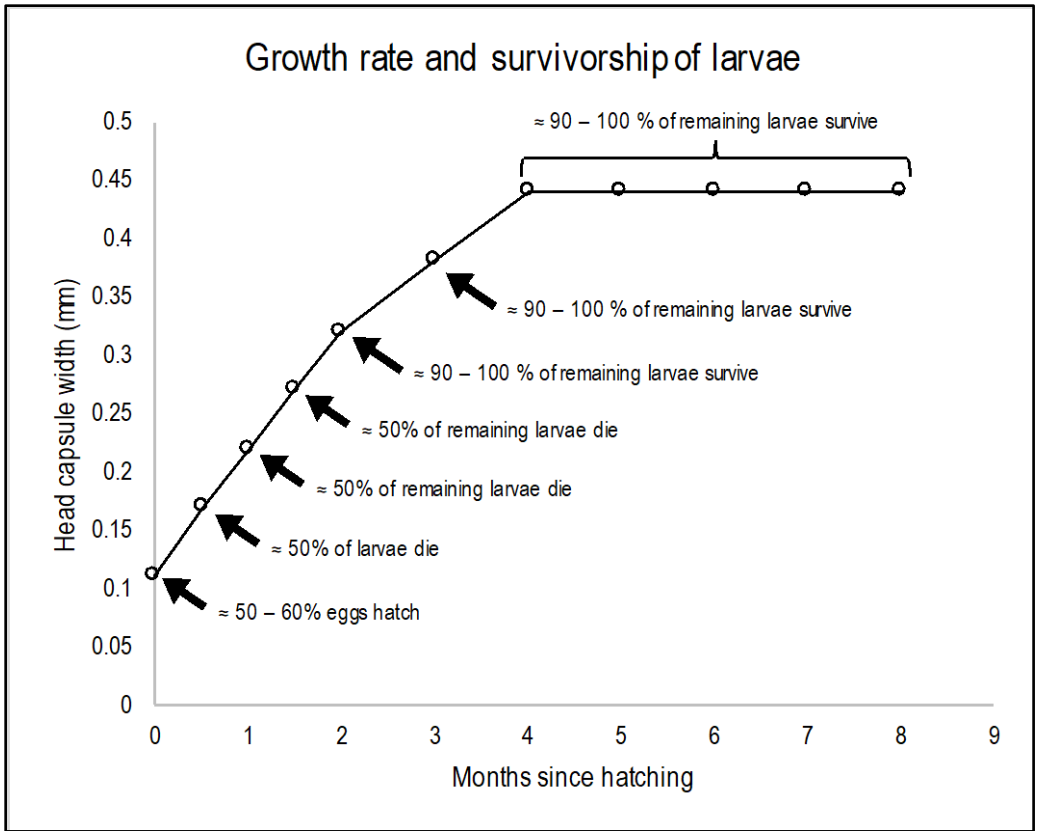

Figure 13. Mean head capsule width per age group. Note that growth rate plateaus in the 7th and final instar.

Group holding - habitat preference

Heterelmis comalensis in culture at SMARC readily produce numerous larvae. Therefore, at any given time there are usually hundreds 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 14). 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)

Figure 14. Habitat preference study cylinders submerged in flow through system.

The arrangement of treatment groups across sectors was randomized for each cylinder in an effort to ensure that artifacts of the substrate distribution did not confound results. Each cylinder received 40 larvae taken from refuge culture; five 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 15).

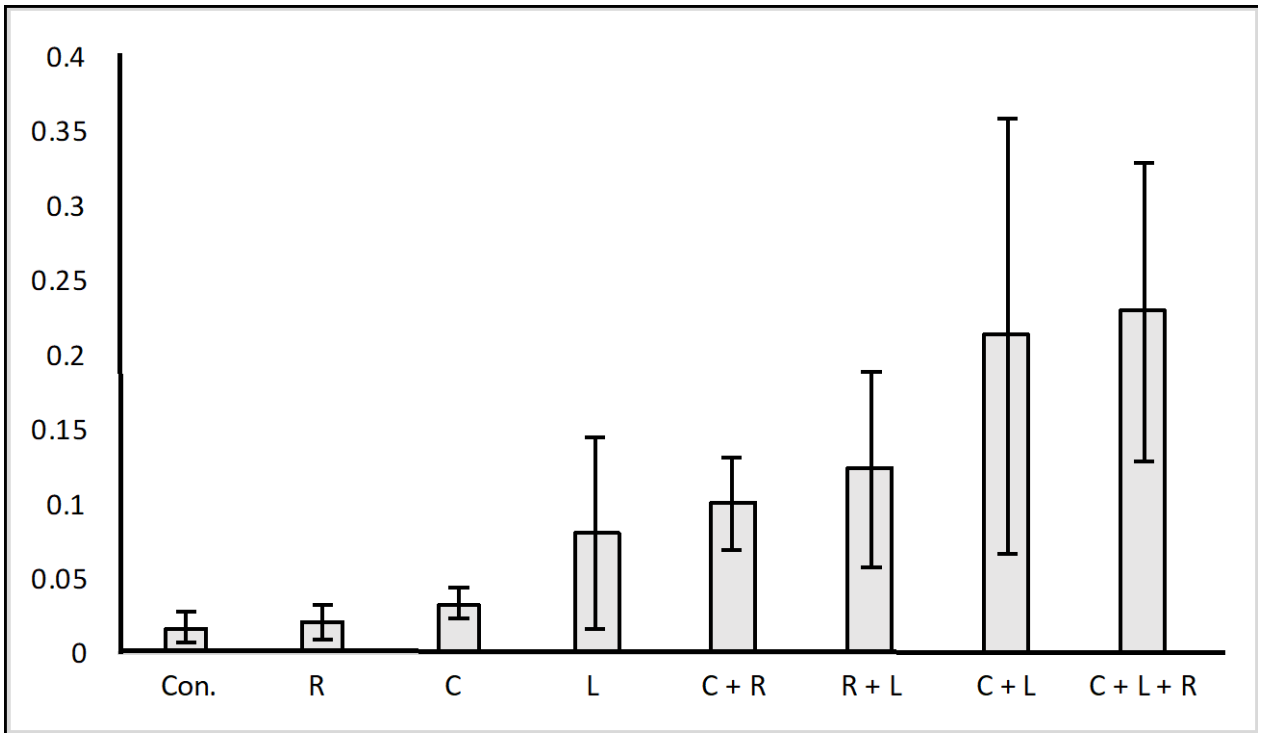

Figure 15. Relative abundance of larval distribution by substrate type in culture. Error bars represent 1 standard error around the mean. Larvae were of mixed age.

Thermal effect on larval survival

Despite the above-mentioned limitations of insight from other elmids, it has been indicated that thermal variation in Comal Springs can affect *H. comalensis* (Nowlin et al. 2017b). Though it is not expected that thermal variation in Comal Springs is of great enough magnitude to affect the survival or pupation of *H. comalensis* larvae, this possible effect has never been addressed. Therefore, we conducted a study on the effect of thermal variation on survival and possibly pupation at the Freeman Aquatic Biology Station (FAB) at Texas State University (TXSU). The study had three experimental groups, two treatments (19 ºC and 25 ºC) and one control (22 ºC). Each experimental group had a block design with three blocks of 7 to 10 individuals per experimental group for a total of 23 to 30 replicates per experimental group. Larvae in their last instar were moved from SMARC to FAB and used for experimental replicates. Replicates were housed individually in a static array following the methods of Nowlin et al. (2016). Once every week, replicates were checked for survival and possibly the presence of pupae. If larvae were found to have pupated, the next step was to document the duration pupation lasted prior to eclosing into adults, however this never occurred. Therefore, the only response variable analyzed was the survival rate of each block. No significant relationship was found between temperature and survival [$F = 0.01$, $df = 7$, $p = 0.922$, Figure 16].

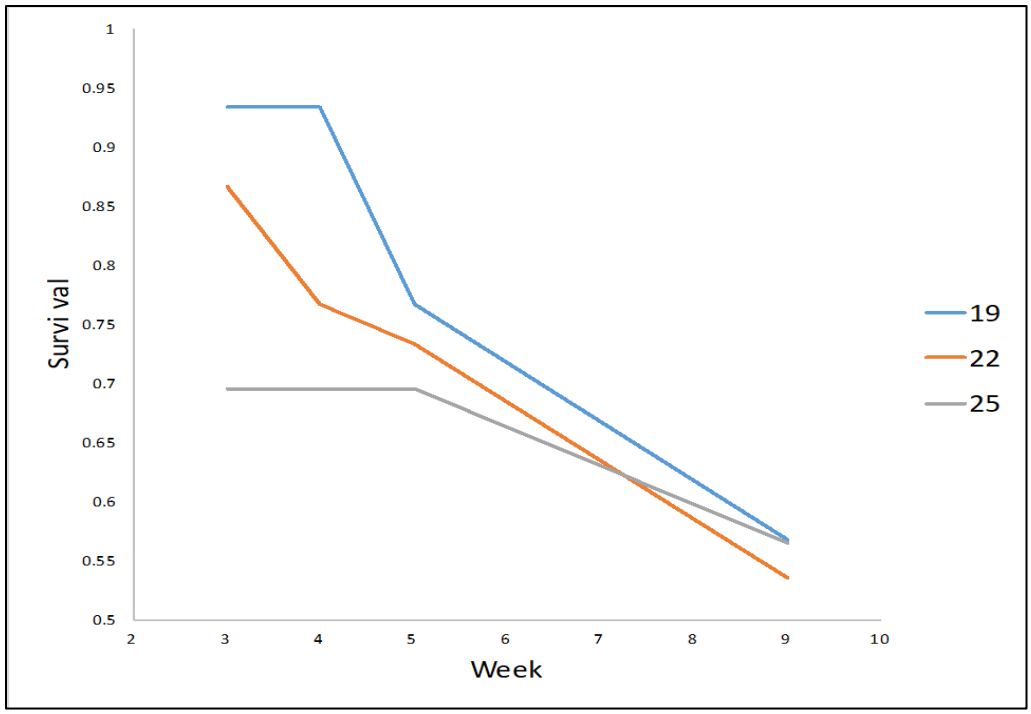

Figure 16. Survival of last instar larvae in different thermal conditions across nine weeks. Temperature is in degrees Celsius. Survival was not found to differ across different temperatures.

PUPATION

Larvae have been found by the present study to survive and grow on a diet of just leaves, just cloth, and cloth and leaves with the fastest growth rate being on a mixture of cloth and leaves. A combination of cloth and leaves has also been shown to lead to successful pupation (though in low numbers) suggesting that the synergy of these combined resources is important. The greatest insight into *H. comalensis* pupation was from a pilot study conducted at SMARC. The study strove to simulate the conditions that pupae have been found in situ, namely decaying tree branches submerged in spring openings. It was observed in the wild that pupae were often found under bark of decaying branches with substantial biofilm growth. Therefore, to simulate these structural requirements, a wooden dowel within a cylinder method was proposed. Between the dowel and cylinder was a mixture of leaves and poly-cotton lures. This substrate was placed in cylindrical flow-through tubes with a constant supply of flow through artesian water with upward flow direction (Figure 16). Flow-through tubes consisted of a 1” internal diameter clear PVC main chamber approximately 6” in length, threaded on each end. Each tube was capped with a threaded female coupling with a sheet of 100-µm mesh serving as a permeable barrier, allowing water to pass through the chamber while containing particular beetle life stages. The mesh is secured inside the coupling by inserting a reducer bushing into it with PVC cement. Inserted into each bushing is a threaded male adapter with a 1/4” nipple for tubing to be attached. One tube will allow water to enter and fill the chamber (influent) while the other tube carries away the water after its residence in the chamber (effluent). The chambers are secured perpendicular to the ground in an array. The influent line is attached to the bottom of the chamber and the effluent is attached at the top to allow trapped air to escape from the chamber. Each flow tube received 9 larvae and were left undisturbed for four months (Table 9). Variants of this methodology were found to have up to 56% of larvae successfully pupating (Table 9, Figure 17).

Figure 17. Flow through tubes with flow direction going upward from the bottom and out the top, then captured in the basin below. This system was designed to house cylindrical arrays of substrate intended to mimic *in situ* conditions likely to promote pupation.

Table 9. Results from pilot study. After four months, up to 71% of larvae in flow systems pupated.

Pupation device #	# adults	# pupae	Survival	Pupation
1	1	1	0.67	0.22
2	0	0	0.56	0.00
3	4	1	0.67	0.56
4	0	0	0.67	0.00
5	0	0	0.00	0.00
6	2	1	0.44	0.33
7	2	0	0.33	0.22
total=>	**9**	**3**		

Figure 18. Freshly produced pupa from flow through systems.

After the pilot study achieved very encouraging pupation rates, it was still unclear if this was due to the addition of a wooden dowel and if so, whether this was due to structural or nutritional effects. Therefore, we addressed these factors with a multifactorial design. The dowel factor was addressed as presence/absence with half of the replicates receiving a wooden dowel of poplar wood 1.25 cm in diameter, while the other half received a plastic rod of similar length and diameter. The ratio of leaf to cloth surface area was studied because cloth has been shown to promote biofilm growth and this biofilm has been observed to consume nutrients at variable rates depending on the availability of leaves. All treatments received a standardized surface area of substrate regardless of combination. It was hypothesized that some optimum ratio exists between these two factors. Each experimental replicate had either a wooden dowel or plastic rod wrapped in a combination of treatment factors (see Table 10) and inserted into a flow-through tube (Figure 17). A mixture of media was inserted into the chambers with the cloth sheet rolled up into a tube shape to allow water to freely move across all the substrate. Flow rates for the chamber were adjusted so that fresh artesian water was always flowing through the chamber, however the rate of flow did not disturb the media and substrate within the chamber. Each combination of treatment factors was replicated three times (Table 10) with each tube receiving five last instar larvae. Tubes were checked every two weeks for pupae and/or adults and the experiment was conducted for eight weeks.

Table 10. Combination of treatment factors and replication of multifactorial pupation study design.

	Proportion leaves				
	0	**0.25**	**0.5**	**0.75**	**1**
Wood	*n=3*	*n=3*	*n=3*	*n=3*	*n=3*
Plastic	*n=3*	*n=3*	*n=3*	*n=3*	*n=3*
Pro. cloth=>	**1**	**0.75**	**0.5**	**0.25**	**0**

The rate and proportion of successful pupation was quite low in this experiment. It is believed that typically four months in the last instar is necessary for pupation, therefore the two-month duration of this study may explain the low yield. Therefore, further pupation studies should follow this design but should be conducted for longer durations. Despite amounts of pupation being too low for statistical analysis of the contribution of substrate composition affecting pupation rates, we were able to confirm the long duration spent in the last instar is likely essential to successfully pupation. Importantly, not only were larvae observed successfully pupating, but pupae eclosing into adults were also observed (Figure 19).

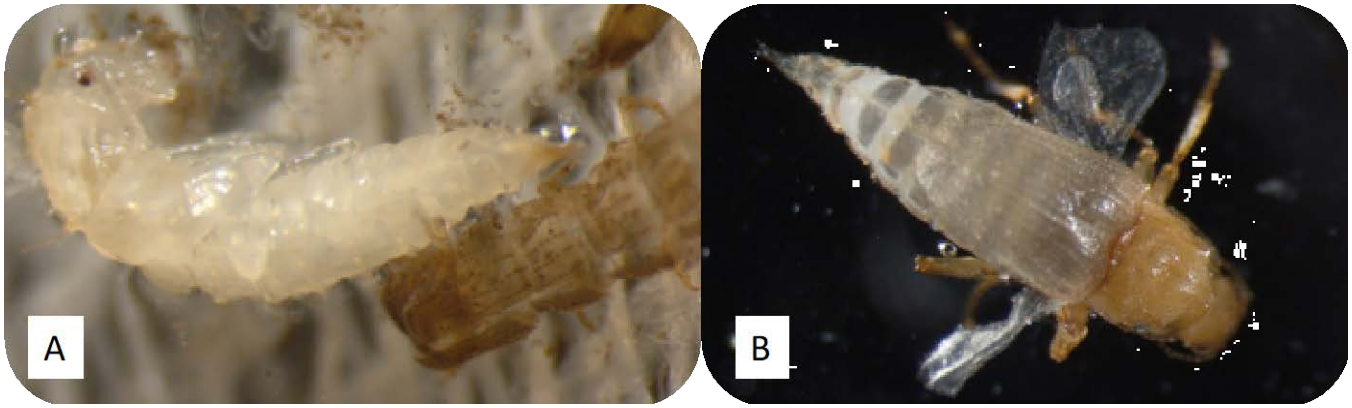

Figure 19. A) pupa recently eclosed from larvae. B) teneral adult recently eclosed from pupa.

SYNOPSIS OF LIFE CYCLE

Based on findings throughout the last two years the following life-cycle diagram has been produced (Figure 20). In general, eggs hatch about three weeks after they are laid. Thereafter, the larvae undergo six molts for a total of seven instars (Figure 13). The first six instars cumulatively span a duration of four months while the 7th instar lasts at least four months and is unknown how long the upper duration is (Figure 13). After some time in the 7th instar larvae molt into pupae (Figure 19A). After about a month, pupae molt into adults (Figure 19B). It is thought that the life span as an adult is approximately a year, therefore the average generation time for *H. comalensis* is two years.

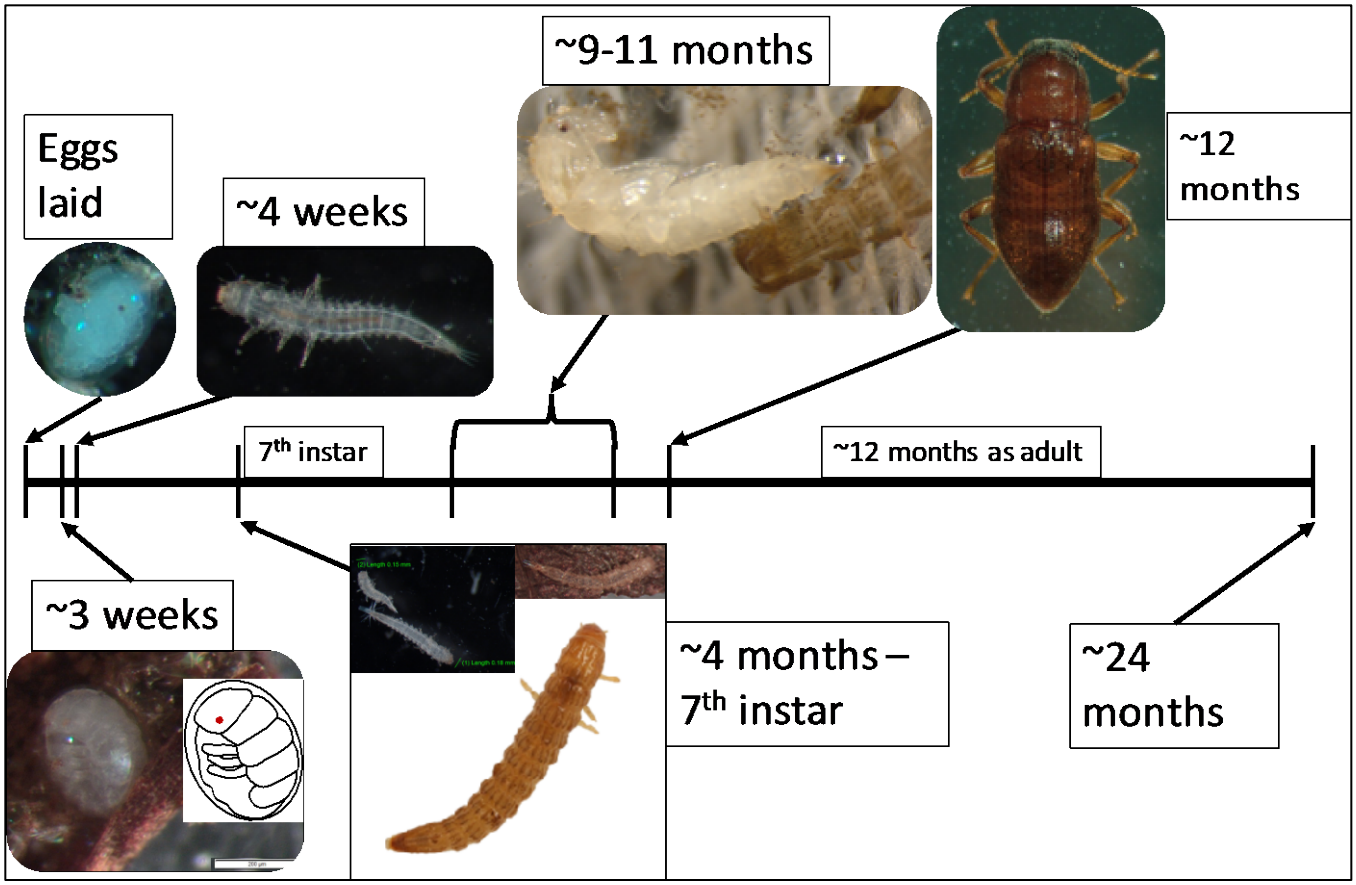

Figure 20. Life cycle timeline of *H. comalensis*.

CONDITION INDEX

The condition index was developed in two phases of study to determine if riffle beetle body condition can be estimated, and if length-weight metrics can be used to assess a response of beetles to different environmental conditions, such as food availability, temperature, substrate, etc. Body condition is indicated by the relationship between the body length and weight of individuals; within a population for example, the condition of individuals with shorter bodies and greater biomass is greater than in animals with long but thin bodies. Higher body condition is indicative of healthier, more robust individuals, and a greater potential for increased reproductive capacity (Stevenson and Wood 2006). Ultimately, the goal of this research was to compare the condition of captive-reared adult specimens (from here on referred to as captive) to wild stocks and infer whether or not captive individuals would be fit for a reintroduction event.

Surrogate proof of concept

The focus of the first phase was to develop sampling and analytical methods to effectively determine riffle beetle body condition. Methodology development began with surrogate species, *Heterelmis glabra* and *H. vulnerata*. Both species are congeners to *H. comalensis* and occur in Texas springs issuing from the Edwards Aquifer. Pilot studies found that body condition could be determined by measuring individual weights and measuring the length and width of different body parts. Some measurements of length and width appeared to be more informative than others, but individuals with longer, wider bodies weighed more in respect to body length than smaller animals, suggesting that body condition increases with length. Sexes were not segregated during this part of study. Seven *H. glabra* and nine *H. vulnerata*

were received at the Desert Research Institute (DRI) frozen, in good condition, and encased in individual aluminum foil packages.

During the preliminary investigation, length-weight relationships were examined for seven *H. glabra* and nine *H. vulnerata*. 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 ^{o}C. Each individual was weighed using a Mettler Toledo Model XP56 analytical balance that measures to the microgram. The balance was calibrated before each day's use.

After being weighed, each specimen was 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 these relationships were significant (Figures 21 – 28). There was a positive relationship between length and weight for all measurements. All regressions were statistically significant ($p < 0.05$); however, some relationships were stronger than others (pronotum length was not measured due to the inability to precisely determine its boundaries). 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). Reasons for differences between the two species are unknown. The total body length/biomass relationship for *H. glabra* was the strongest observed ($R^2 = 0.9271$, Figure 21). The elytron width/biomass relationship was strongest for *H. vulnerata* (Figure 27).

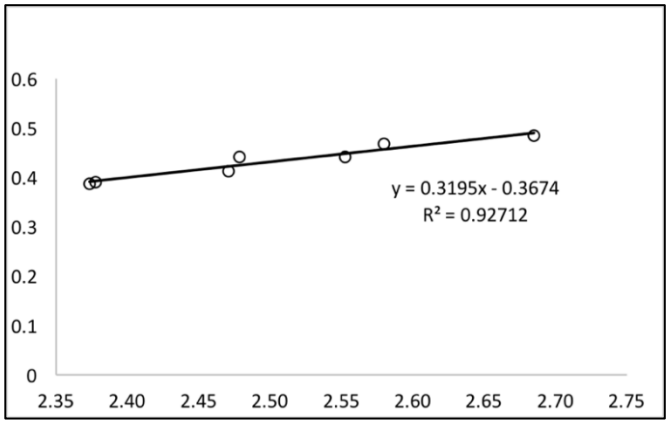

Figure 21. *H. glabra* total body length (mm, x axis)/biomass (micrograms, y axis) relationship.

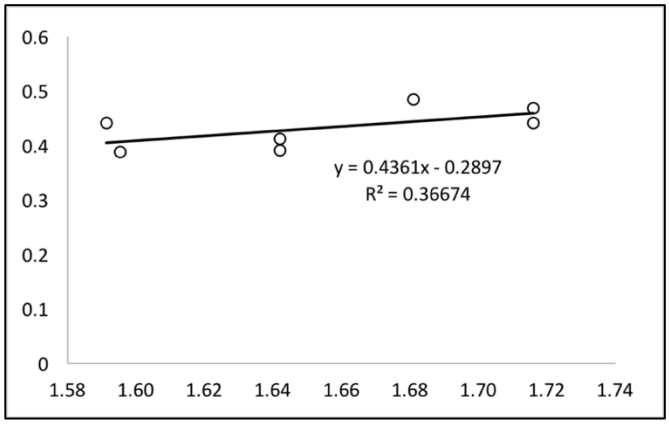

Figure 22. *H. glabra* eltyron length (mm) / biomass (micrograms) relationship.

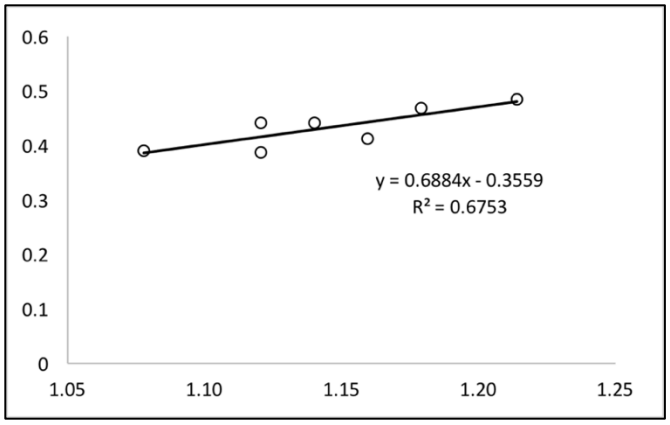

Figure 23. *H. glabra* eltyron width (mm)/biomass (micrograms) relationship.

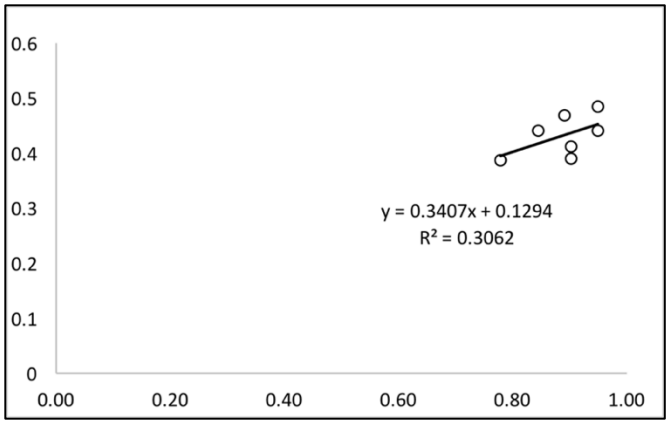

Figure 24. *H. glabra* pronotum width (mm) / biomass (micrograms) relationship.

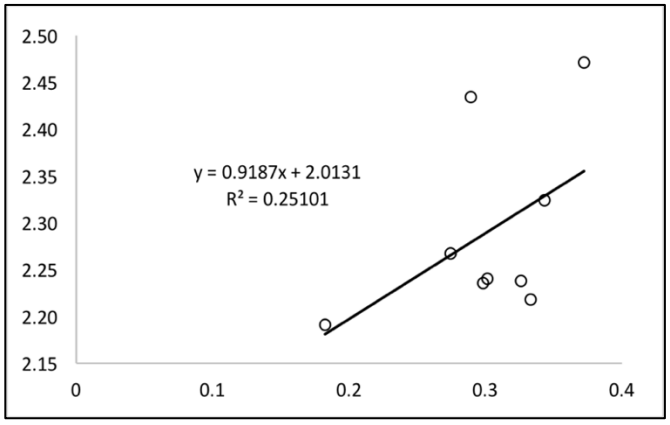

Figure 25. *H. vulnerata* total body length (mm) / biomass (micrograms) relationship.

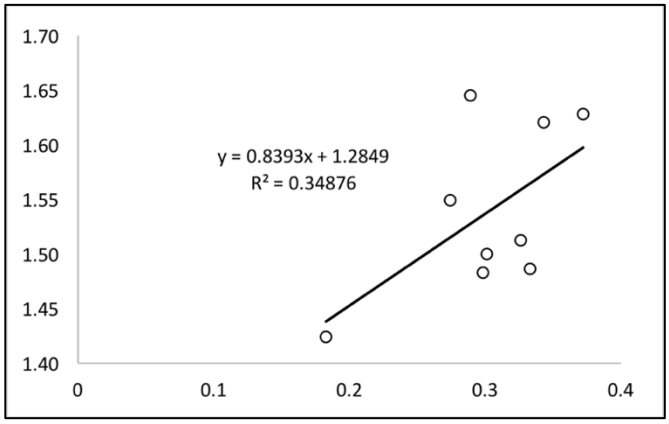

Figure 26. *H. vulnerata* elytron length (mm) / biomass (micrograms) relationship.

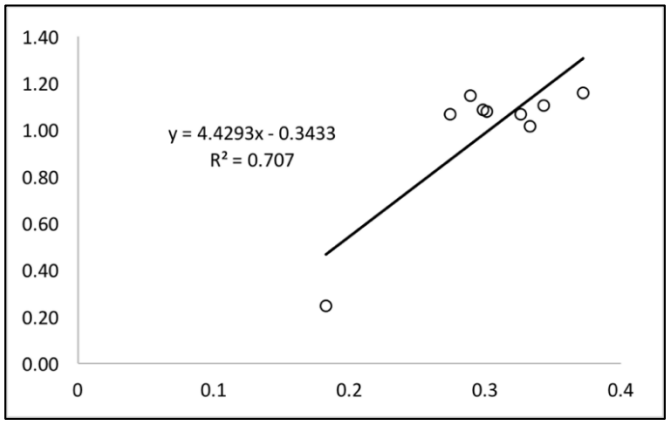

Figure 27. *H. vulnerata* elytron width (mm) / biomass (micrograms) relationship.

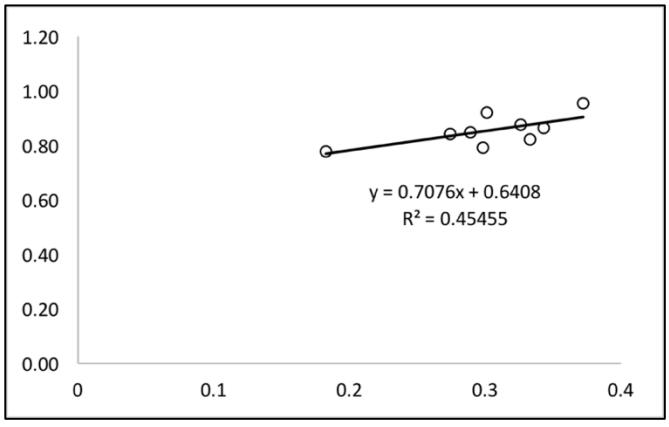

Figure 28. *H. vulnerata* pronotum width (mm) / biomass (micrograms) relationship.

Comal Springs riffle beetle

Heterelmis comalensis were received at DRI frozen and in aluminum foil packets. A total of 42 beetles were included in the analysis, comprising of 14 wild males, 13 wild females, six captive males, and nine captive females. Insight into length/weight relationships were made by weighing each individual and measuring its total length, and the length and width of its pronotum and elytron.

Methods and equipment used to weigh and measure beetles followed techniques developed during preliminary investigations. *H. comalensis* were considerably smaller than *H. glabra* and *H. vulnerata*. This required increasing the accuracy of measuring their weight. This accuracy was improved by calculating a linear regression model to account for weight discrepancy between displayed measured weights of *H. comalensis* and known standard weights (Figure 29). Three weights were taken for each standard weight (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 g) to develop a mean weight for each known standard weight. The mean weight was then graphed in relationship to the known standard weights. The R^2 value equals 1, indicating that there are no changes to the weights as the known standard weight changes. This regression was used to confirm the measured weights, which confirmed that no adjustments were needed to improve the accuracy of *H. comalensis* measured weights.

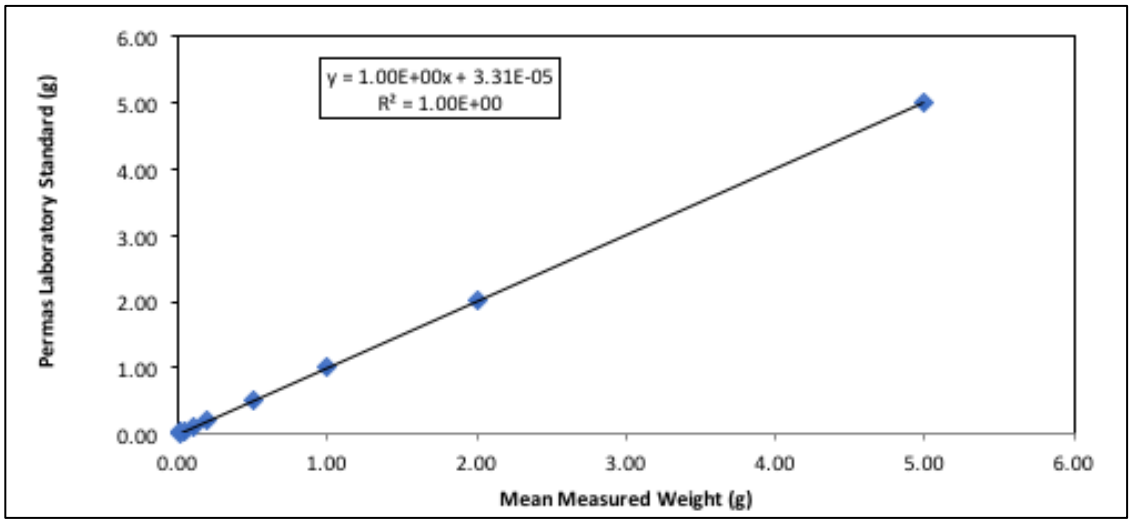

Figure 29. Relationship between Permas Laboratory Standard weights (0.01, 0.02, 0.05, 0.1, 0.2, 0. 1.0, 2.0, and 5.0g) and the mean of 3 measured weights for each known standard weight.

It was found that wild and captive bred *H. comalensis* used in this analysis ranged in length from 2.06 mm to 2.48 mm, and weighed from 0. 14 micrograms (mcg) to 0.26 mcg (Table 11). Differences in size were statistically significant only between the length of captive and wild males (Figure 30). Weight was not found to vary significantly between captive and wild caught individuals (Figure 30). Differences between all other comparisons were not statistically significant.

Table 11. Sample size (N), mean length (millimeters) and weight (micrograms), and range (in parenthesis) of wild and captive males and female *H. comalensis* examined for length/weight assessment. Differences between sexes were not significant ($p > 0.1$) comparing the length and weight of wild and captive samples, and between captive and wild females. Differences in length were significant ($p < 0.03$) between captive and wild males.

	N	Length (mm)	Weight (mg)
Wild Female	9	2.34 (2.06—2.38)	0.25 (0.17—0.25)
Wild Male	6	2.26 (2.12—2.48)	0.23 (0.17—0.26)
Captive Female	13	2.41 (2.06—2.38)	0.27 (0.17—0.25)
Captive Male	14	2.50 (2.26—2.42)	0.20 (0.14—0.25)

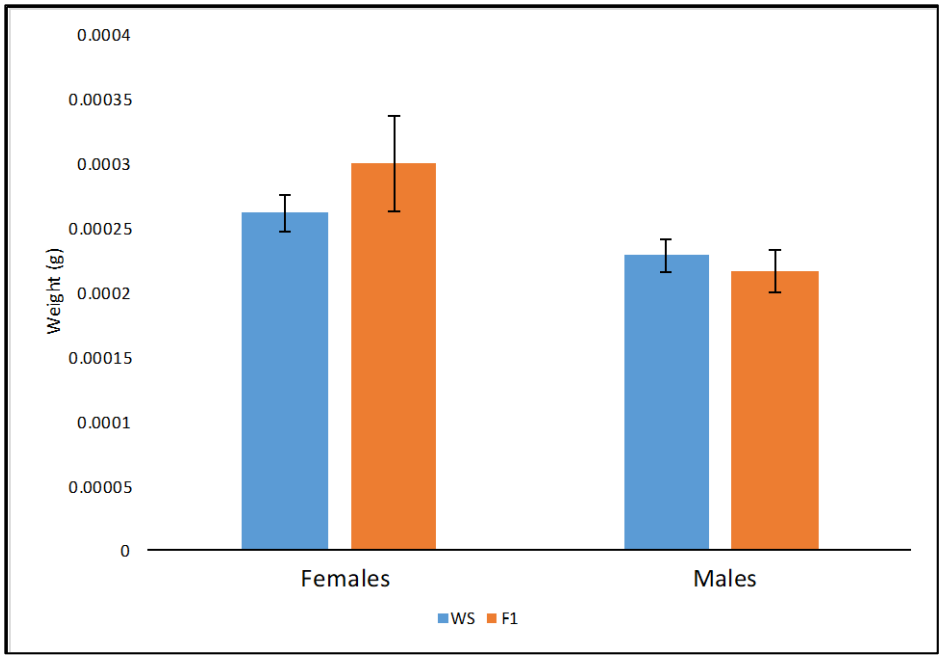

Figure 30. Comparison of mean weight of captive born and wild-caught beetles. Error bars represent 1 standard error. WS = wild stock; F1 = captive produced.

Analysis of Covariance found differences between length/weight regressions were not statistically significant for only the comparison of wild males and females and comparing the pronotum length of wild and captive males. Additionally, the slope of regressions for captive males and females were consistently greater than observed for wild beetles (see Figures 31 – 42). Reasons for this are unclear, but may be attributed to factors such as greater suitability of the laboratory environment (e.g., temperature, food quality and quantity) for more rapid growth, lower competition for food and space in the laboratory, etc. Further studies are needed to provide clarity to these differences.

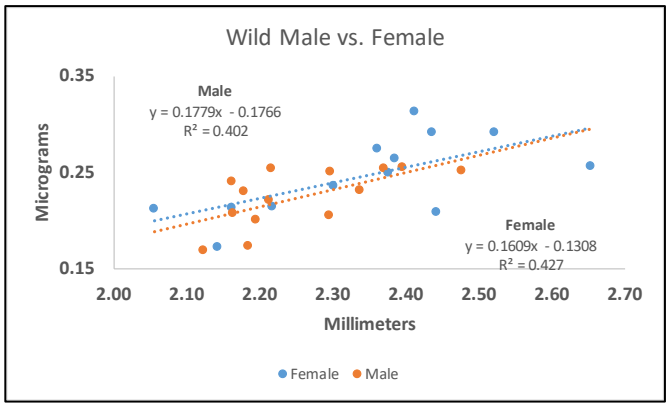

Figure 31. Total Length-weight regression comparing wild male and female *H. comalensis*. Male regression significant (p = 0.013), and Female regression significant (p = 0.015).

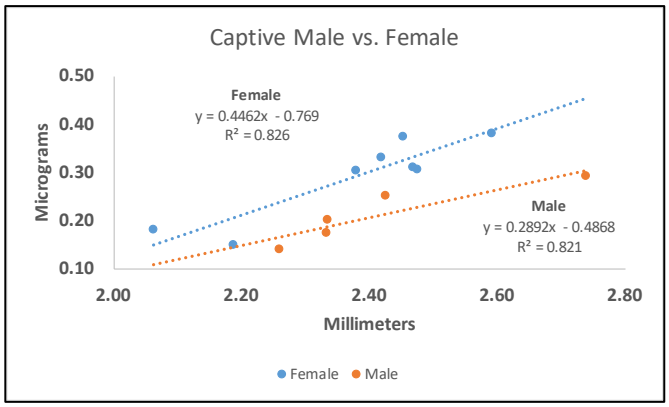

Figure 32. Total Length-weight regression comparing captively reared male and female *H. comalensis*. Female regression non-significant (p = 0.372), Male regression non-significant (p = 0.026). One male and 1 female outliers removed from analysis.

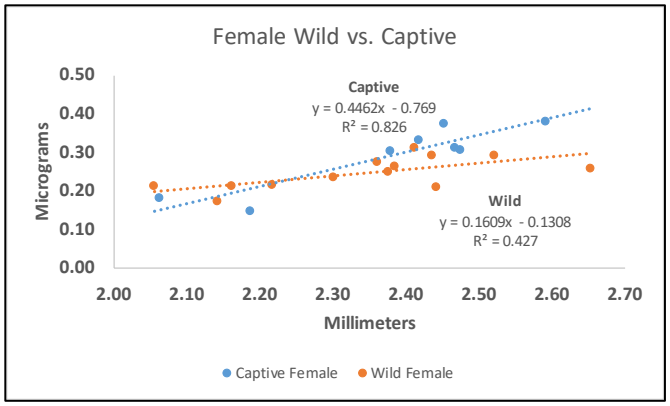

Figure 33. Total Length-weight regression comparing wild and captively reared female *H. comalensis*. Wild regression significant (p = 0.015), Captive regression non-significant (p = 0.372). One outlier removed from Captive sample.

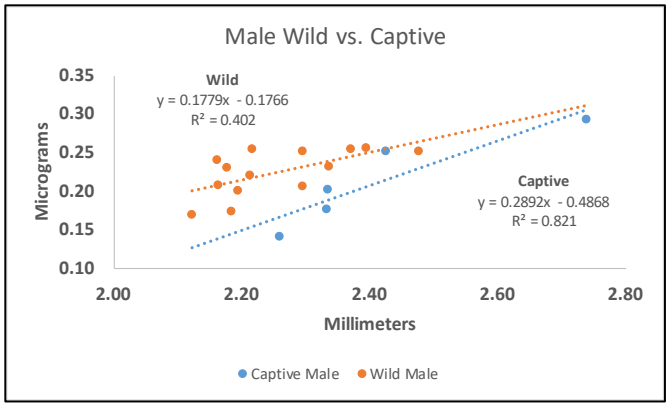

Figure 34. Total Length-weight regression comparing wild and captively reared male *H. comalensis*. One outlier removed from captive sample. Wild regression significant (p = 0.013), Captive regression significant (p = 0.026).

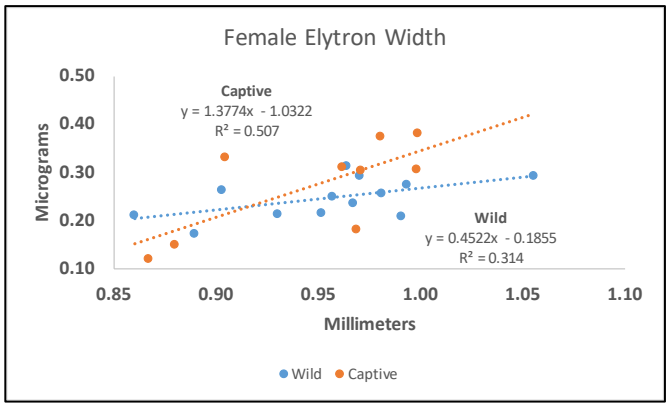

Figure 35. Female Captive vs. Wild *H. comalensis* elytron width/ total weight regressions. Wild regression non-significant (p = 0.054), Captive regression significant (p = 0.040).

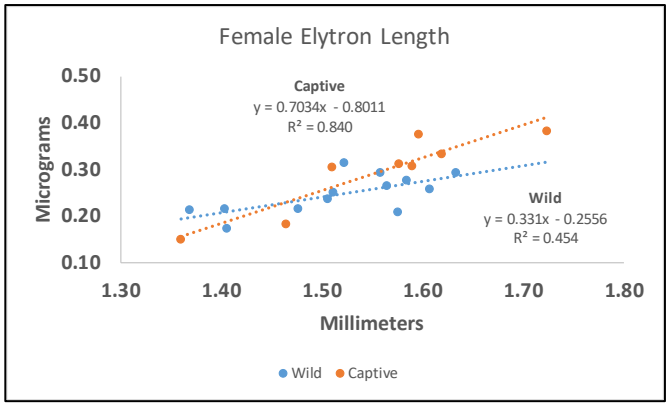

Figure 36. Female Captive vs. Wild *H. comalensis* elytron length/ total weight regressions. Wild regression significant (p = 0.012), Captive regression non-significant (p = 0.564). One outlier removed from Captive sample.

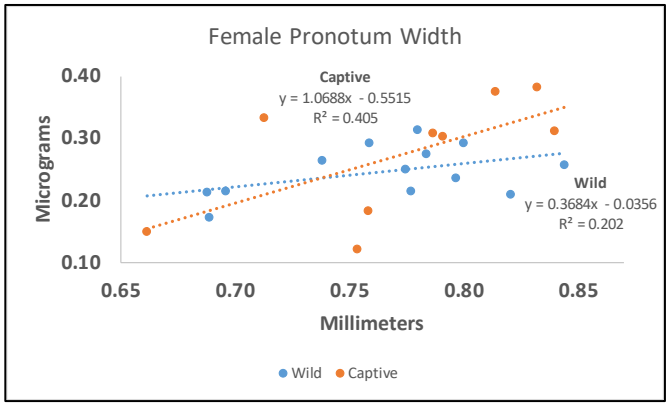

Figure 37. Female Captive vs. Wild *H. comalensis* pronotum width/ total weight regressions. Wild regression non-significant (p = 0.129), Captive regression non-significant (p = 0.069).

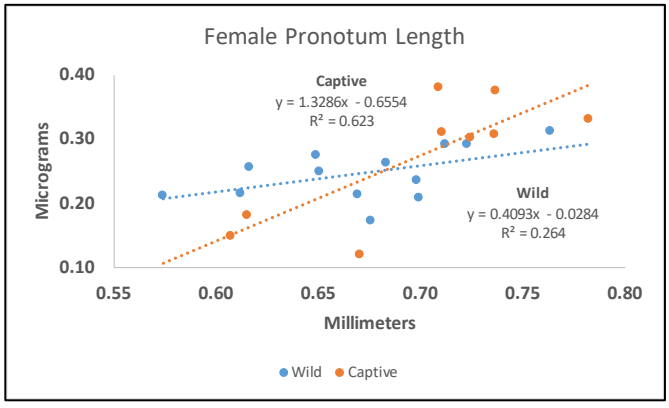

Figure 38. Female Captive vs. Wild *H. comalensis* pronotum length/ total weight regressions. Wild regression non-significant (p = 0.026), Captive regression significant (p = 0.010).

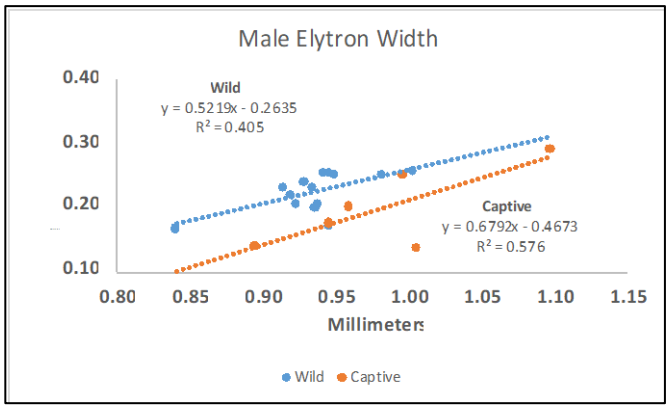

Figure 39. Male Captive vs. Wild *H. comalensis* elytron length/ total weight regressions. Wild regression significant (p = 0.020), Captive regression non-significant (p = 0.084).

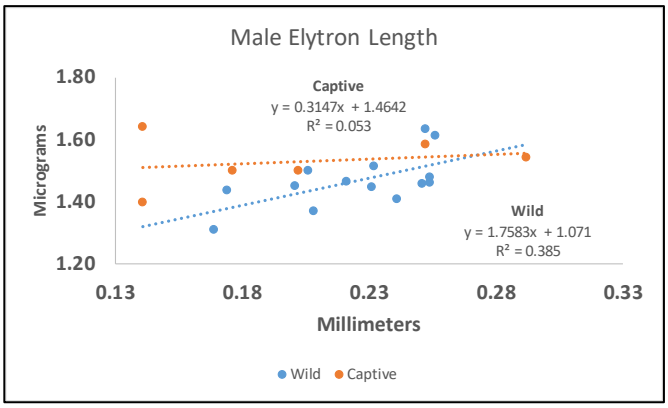

Figure 40. Male Captive vs. Wild *H. comalensis* elytron length/ total weight regressions. Wild regression significant (p = 0.018), Captive regression non-significant (p = 0.682).

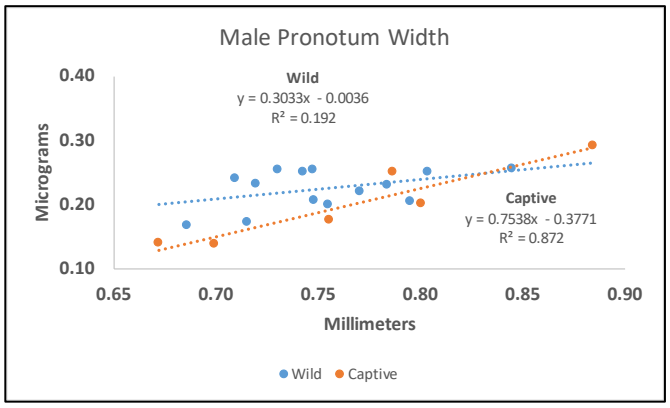

Figure 41. Male Captive vs. Wild *H. comalensis* pronotum width/ total weight regressions. Wild regression non-significant (p = 0.125), Captive regression significant (p = 0.006).

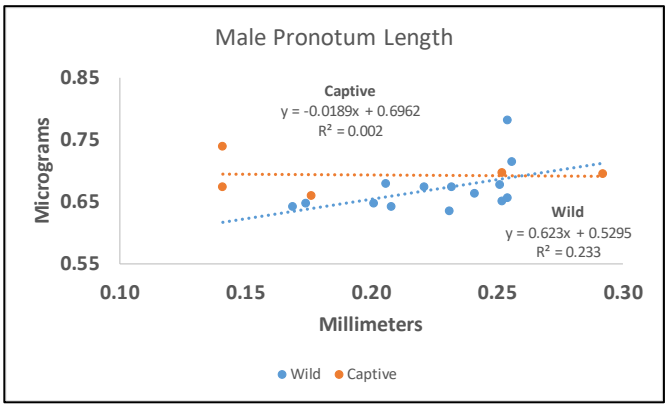

Figure 42. Male Captive vs. Wild *H. comalensis* pronotum length/total weight regressions. Wild regression non-significant (p = 0.084), Captive regression non-significant (p = 0.956).

Condition index work consistently documented that there are length-weight relationships for three species of riffle beetles in *Heterelmis*. Although the relationships were not consistently strong, weights typically increased more rapidly than lengths for most of the metrics examined. Comparing these relationships for wild and captive reared *H. comalensis* beetles yielded information providing insight into how these relationships may vary under different environments. This key observation indicates that beetles raised under different environmental conditions (such as diet, substrate, current velocity, etc.) may exhibit different growth, hence length/weight and body-condition characteristics. Even though regressions were not strong for wild individuals, the body condition of captive reared beetles increased more rapidly with increased length than observed in wild individuals. This suggests that laboratory conditions were more suitable to beetle growth than conditions in the wild.

CAPTIVE PROPAGATION

CURRENT METHODOLOGY

Egg production and mating

The following methodology assumes that adult beetles are available and collections are not necessary. In order to have controlled breeding of *H. comalensis*, it is important to know the sex of individual beetles. Therefore, following the methods of Subtask 1.2, adults should be sexed in a non-invasive and non-lethal way. After sexing, beetles should be placed in flow tubes following the methods described in Figure 17; each flow tube should receive *n*=5 pairs of beetles. Each flow tube should receive the same substrate of approximately equal amounts of leaf and cloth rolled around a wooden dowel; approximately 10-cm^2 of leaf and 10-cm^2 of cloth should be used (it is important to precondition cloth by soaking in artesian water for one to two weeks prior to use). Based on the ~3-week incubation duration of eggs, flow tubes should be checked after two weeks; this should ensure that no eggs have yet hatched during this check. At this time, all adults should be removed from the substrate and returned to the original flow tube with new substrate. This cycle should be repeated on two-week intervals. The substrate that was removed from the flow tubes is likely laden with eggs; the likelihood of none of the five females laying any eggs is low enough that it can be assumed that eggs are present on the substrate and searching for them is not necessary (see egg production subsection in Methods for further explanation). Throughout all procedures it is essential that beetles and substrate are not out of water longer than it takes to transfer them between treatments.

Larval development

The egg laden substrate should be placed into a 15-liter aquarium with flow through artesian water. This aquarium should receive additional cloth and leaf substrate every two weeks. The purpose of additional substrate is to replenish the food supply for developing larvae as early instar larvae appear to need more and possibly, higher quality food than later instar larvae or adults. If substrate is not continuously replenished it is likely that larvae will succumb to starvation. After four months, larvae will have reached or be near the final instar of development (see larval development subsection in Methods above for further explanation). At this time substrate should be checked for late instar larvae and larvae should be removed and placed in flow tubes for pupation.

Pupation

Later instar larvae should be placed in flow tubes; no more than five per tube. This is to ensure that late instar larvae do not deplete their food supplies before pupating. The flow tube should receive at least 20-cm^2 of leaf and 20-cm^2 of cloth (further experimentation is needed to determine how quickly late instar larvae will deplete their food supply). Flow tubes should be sealed and left undisturbed for at least four months before checking. After four months, tubes should be checked for the presence of larvae, pupae, or adults. Larvae should be returned to the tube with fresh substrate, adults should be moved into F1 adult aquaria, and pupae should be placed individually in reusable Keurig coffee filter cups (K cups) without any substrate and suspended in a static array following the methods of Nowlin et al. (2016). K cups should be checked daily until the pupae are found to have eclosed into adults, at which time adults can be moved into F1 adult aquaria.

FUTURE PROPAGATION APPLIED RESEARCH

Early instar larvae have been shown to have poor survival until reaching the fourth instar (Figure 13). An improvement in survival is correlated with a decrease in molt rate after reaching the fourth instar suggesting the rapid early development may be so energy expensive that offspring are failing to consume enough high-quality food to keep up with energy demands. This correlation is thought to be due to three possible factors: 1) not enough availability of food leading to starvation during periods of faster development, 2) inadequate quality of nutrition during early instars leading to malnutrition, 3) or this is simply the natural biology of the species. In other words, it is possible that certain nutritional requirements are not being met at these young ages because there is a missing component in their diet. Therefore, we propose that future propagation research focus on achieving higher rates of survival in early instars by experimenting with nutrition. It is impossible to say at this time what aspects of nutrition may be missing, if any at all, therefore we recommend a series of pilot studies. This will hopefully lead to higher quality and healthier larvae and greater survival of larvae. Healthier larvae will also increase the probability of producing adults as more late instar larvae increases the odds of entering pupation. Furthermore, good nutrition in early instars likely increase the probability that each larva will pupate.

Similarly, it is unknown if the long durations spent in the last instar and low pupation rates are natural or an artifact of conditions in captivity. If it is an artifact of captive propagation this is likely due to the quality of nutrition. Late instar larvae likely use the last instar as an opportunity to accumulate the necessary reserves to pupate and if nutritional quality requirements are not met, it is possible that late instar larvae spend extended durations in the last instar prior to pupation due to nutrient deficits. Once again, this deficit could be due to quality or abundance of nutrition. However, it is possible that the timing of developmental events and low pupation rates are natural artifacts of genetically depauperate wild populations.

During this life history study, it has been observed that *H. comalensis* moved toward flow when placed in flow-through tubes, therefore their behavior is presumed to be influenced by flow. It has also been demonstrated that *H. comalensis* is a non-selective grazer and that approximately 70 – 90% of its diet originates from terrestrial derived coarse particulate organic matter (CPOM) inoculated with bacterial biofilms (Nowlin et al. 2017a). Currently it is unknown if *H. comalensis* stratify near the surface because of the availability of preferred food sources and/or if this is affected by flow. It is conceivable that the individuals tested spent more time at the surface rather than deeper interstitial spaces that do not have large amounts of CPOM. Therefore, we are proposing to study how beetles respond when given the opportunity to colonize artificial habitats on the basis of moving towards flow or maintaining their position in food rich habitat. Will they move towards a food rich habitat if one is in the opposite direction to the path they would normally travel due to the direction of flow? To test these questions, the variable of flow needs to be studied independently of food availability with a two factor design. The factor of flow will be studied to determine what rate of flow attracts beetles at the greatest level and which if any rate of flow is high enough to dislodge beetles. Simultaneously, different levels of food availability should be studied to determine under which combinations of conditions flow or food sources to different degree. Presumably there are certain levels of flow that food sources are not primary considerations for where beetles distribute themselves and vice versus in respect to food sources.

All proposed nutritional and flow preference applied research would be designed to assist in streamlining and enhancing future captive propagation efforts, while also providing insight into wild populations. Finally, further evaluation and refinement of the *H. comalensis* condition index could be explored by comparing the length/weight relationship of beetles grown under different scenarios in

captivity in an effort to optimize propagation. Furthermore, comparing wild-caught *H. comalensis* from different microhabitats and across years of environmental fluctuation shows promise as a monitoring technique to evaluate riffle beetle health in response to environmental conditions. Insight form *in situ* studies of condition can also better inform captive propagation conditions. Overall, it is thought that a better understanding of *H. comalensis* condition could benefit both captive propagation and long-term monitoring components of the HCP.

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