2019 EAHCP Refugia Work Plan

Introduction

The U.S. Fish and Wildlife Service's (USFWS) San Marcos Aquatic Resources Center (SMARC) and Uvalde National Fish Hatchery (UNFH) will provide refugia, salvage, reintroduction, and monitoring services in fulfillment of the Refugia Contract (Contract # 16-822-HCP) between the Edwards Aquifer Authority (EAA) and the USFWS.

This annual work plan and associated cost estimate have been developed per the requirements of contract number 16-822-HCP for the Implementation of the Refugia Program under the EAHCP. The tasks and subtasks that follow provide the details for the services to be performed in 2019, which provide for the maintenance of a refugia population of the Covered Species (Table 1) including the salvage, propagation, and restocking of the species, if species-specific habitat triggers occur and species are extirpated, plus research conducted on the Covered Species.

Table 1: Eleven species identified in the EAHCP and listed for coverage under the ITP.

Common Name	Scientific Name	ESA Status
Fountain darter	*Etheostoma fonticola*	Endangered
Comal Springs riffle beetle	*Heterelmis comalensis*	Endangered
San Marcos gambusia	*Gambusia georgei*	Endangered*
Comal Springs dryopid beetle	*Stygoparnus comalensis*	Endangered
Peck's Cave amphipod	*Stygobromus pecki*	Endangered
Texas wild-rice	*Zizania texana*	Endangered
Texas blind salamander	*Eurycea rathbuni*	Endangered
San Marcos salamander	*Eurycea nana*	Threatened
Edwards Aquifer diving beetle	*Haideoporus texanus*	Petitioned
Comal Springs salamander	*Eurycea* sp.	Petitioned
Texas troglobitic water slater	*Lirceolus smithii*	Petitioned

*The San Marcos gambusia was last collected in the wild in 1983, and may already be extinct.

Long-term Objective

Background: Section 5.1.1 of the EAHCP requires the EAA to provide a series of refugia, with back-up populations, to preserve the capacity for these species to be re-established in the event of the loss of population due to a catastrophic event.

The concept of refugia is to house and protect adequate populations of the Covered Species and to conduct research activities to expand knowledge of their habitat requirements, biology, life histories, and effective reintroduction techniques. Actions and funding contained within this work plan will be limited to the Covered Species listed in the EAHCP and those associated species that have significant impact on the Covered Species such as predators, competitors, pathogens, parasites, food, cover, and shelter.

2019 Assumptions

As work plans are developed almost a year prior to implementation, it is possible that methods described herein may be contingent on the status of the current year's activities or authorization from the HCP process. If conditions change, this work plan may need to be amended to accommodate realized outcomes.

- Target numbers for the standing and refugia stocks to be housed at both the UNFH and SMARC are established by the USFWS-EAA Refugia Contract (Contract # 16-822-HCP).
- Species capture rates are expected to be similar to historic values.
- Mortality rates of specimens held in captivity are expected to be similar to historic values.
- Target species collection numbers from the 2018 work plan are expected to be reached.
- Construction and renovation will not be interrupted or unexpectedly delayed due to weather, equipment, procurement related delays, or other unforeseen issues.
- Staff members remain employed at the two Service facilities throughout the performance period.

Target for 2019 (Deliverables and Methods by Task):

Task 1. Refugia Operations

Standing Stocks The existing stocks at the SMARC and UNFH will be considered standing stocks under the executed contract (Contract # 16-822-HCP) and will be held in Service facilities until EAA specific Refugia and Quarantine facilities are complete and functional. USFWS staff will take all appropriate steps to collect and maintain standing/refugia stocks at their respective target captive population size in order to provide refugia for all the Covered Species. Table 2 displays the target species numbers.

Table 2. Species target refugia numbers and census.

Species	Standing Stock	Refugia Stock	Salvage Stock	SMARC census (Jan 2019)	Anticipated SMARC census (Dec 2019)	UNFH census (Jan 2019)	Anticipated UNFH census (Dec 2019)
Fountain Darter (Comal)	1000	1000 including specimens within the standing stock	2000	233	300	49	200
Fountain Darter (San Marcos)	1000	1000 including specimens within the standing stock	2500	504	500	444	500
Texas Wild-Rice	430	430 including specimens within the standing stock	1500	228	215	83	150
Texas Blind Salamander	500	500 including specimens within the standing stock	500	95	110	--	15[1]
San Marcos Salamander	500	500 including specimens within the standing stock	500	264	250	234	250
Comal Springs Salamander	500	500 including specimens within the standing stock	500	72	80	19	50
Peck's Cave Amphipod	500	500 including specimens within the standing stock	500	208	250	23	160
Comal Springs Riffle Beetle	500	500 including specimens within the standing stock	500	88	#	0	#
Comal Springs Dryopid Beetle	500	500 including specimens within the standing stock	500	6	*	0	*
Edwards Aquifer Diving Beetle	500	500 including specimens within the standing stock	500	0	*	0	*
Texas Troglobitic Water Slater	500	500 including specimens within the standing stock	500	509 (Fx)**	*	0	*

[1]transfer of Texas blind salamanders to UNFH is contingent upon completion of facilities construction and tank system set-up

for 2019 we plan on collecting Comal Springs riffle beetles mainly to support research purposes rather than standing stock, until we can increase survivability in captivity

*catch rates and hatchery survival are uncertain given the rarity of the species

***unable to distinguish wild stock from captive bred (Fx) generations*

Collection: In 2019, we will collect Covered Species as required to reach and maintain target standing and refugia stock numbers as shown in Table 2. Species collections will be coordinated with other ongoing HCP activities (e.g. Biological Monitoring Program) so that collections for refugia do not adversely impact other efforts. Species specific collections will be carried out through a variety of passive and active collection methods. Prior to collections, Hazard Analysis Critical Control Point (see Appendix A 2017 Work Plan) will be conducted to minimize aquatic invasive species transfer. Collection efforts will be documented and reported to EAA. Captured specimens will be divided between the SMARC and UNFH facilities in order to ensure redundancy and to expedite the obligation to establish and maintain two refugia populations at separate locations. All species will be held in respective quarantine areas until their health has been assessed. Once it is determined that specimens are free from pathogens, parasites, and invasive species they will be incorporated into the general refugia population. USFWS will share reports, including test results, produced as part of the quarantine process. Species-specific collection plans generally follow those detailed within the 2018 Work Plan; however, collection efforts vary based upon collection and knowledge gained during the previous year's collection efforts. The following sections briefly describe planned 2019 collection, maintenance, and propagation efforts for each species.

Fountain Darters:

Collection: Fountain darters in 2019 will be collected primarily in coordination with the Spring and Fall Biomonitoring events to create efficiencies and reduce habitat disturbance. After fountain darters are collected via drop nets for biomonitoring, USFWS staff will retain them for refugia purposes. Specimens will be collected along a longitudinal gradient. Approximately equal proportions of fish from upper and lower reaches in the Comal (upper = above Landa Lake dam; lower = below Landa Lake dam) and San Marcos (upper = Spring Lake, Middle = Spring Lake dam to Rio Vista dam, lower = below Rio Vista dam to Capes dam) rivers will be collected. Historically, approximately 20% of the fountain darters collected annually succumb to natural mortality. If unusual mortality events occur, they will be thoroughly investigated and summary reports will be conveyed to the EAA as part of the monthly reports. As a result, fish collections will target additional fish so that as individuals perish the remainder within the captive population should not decrease below the target number between collection events. Higher mortality rates of incoming Comal fountain darters have been seen in the past three collections. We are currently working with the Fish Health Unit to determine the cause(s). Due to this we will target fewer Comal fountain darters to collect and have in Standing Stock until survivability is improved.

Due to the detection of largemouth bass virus in Comal fountain darters throughout the Comal River habitat, all Comal fountain darters will be maintained in quarantine facilities in consideration of other species located on the two stations.

As part of quarantine procedures, a subset of fish (N = 60) will be sent to the southwest regional Fish Health Unit or equivalent facility for pathogen (bacteria, virus, and parasite) testing prior to specimen incorporation into the general refugia population following standardized methods outlined within USFWS and AFS-FHS (2016) and AFS-FHS (2005); reports will be provided to EAA.

Maintenance: Water quality (i.e., temperature, pH, dissolved oxygen, total dissolved gasses) will be monitored and recorded weekly. Fountain darters will be fed live foods reared or

purchased. Ponds will be utilized to produce zooplankton and amphipods. Ponds will be managed to maintain idealized zooplankton assemblages and densities. Amphipods will be collected from other managed ponds and raceways. Black worms will be purchased when necessary along with other food resources (i.e. blood worms, black worms, brine shrimp, etc.) if the need arises. Food items are not routinely examined for pathogens. However, if they are suspect and tested for pathogens all diagnostic results will be conveyed to the EAA within monthly reports.

Propagation: Standing and refugia stocks for each river will be maintained to discourage reproduction unless HCP triggers occur. Fish will be maintained by their geographical locations. If reintroduction is warranted, subsets from each geographical location will be communally spawned. Subset groups will be culled to an equal number of progeny prior to release.

Texas wild rice:

Collection: Texas wild rice tillers will be collected from San Marcos River reaches (Fig. 1), with a break during summer months when wild rice does not fare well due to heat stress. In 2019 collections for SMARC will target stands that are not already part of the refugia population or require supplementation. Collections for UNFH will continue to build their refugia numbers and representative locations. The refugia populations will reflect the wild populations in both their respective proportion and genetic diversity that was historically documented within San Marcos River (Wilson et al. 2016). During tiller collection, the GPS coordinates, area coverage, and depth of the stand or individual plant will be recorded so the exact location of the clone is known. For larger stands, tillers will be collected at the beginning, middle and end of the stand, or every 20% of the stand's total length for the largest stands. Tiller collection will be done by wading and SCUBA diving. Please note that during the 2018 Texas wild rice survey no plants were found in Section I. Sections J and K were not surveyed. Plants were found in sections E, G, and H. All sections will be re-evaluated during the 2019 Texas wild rice survey.

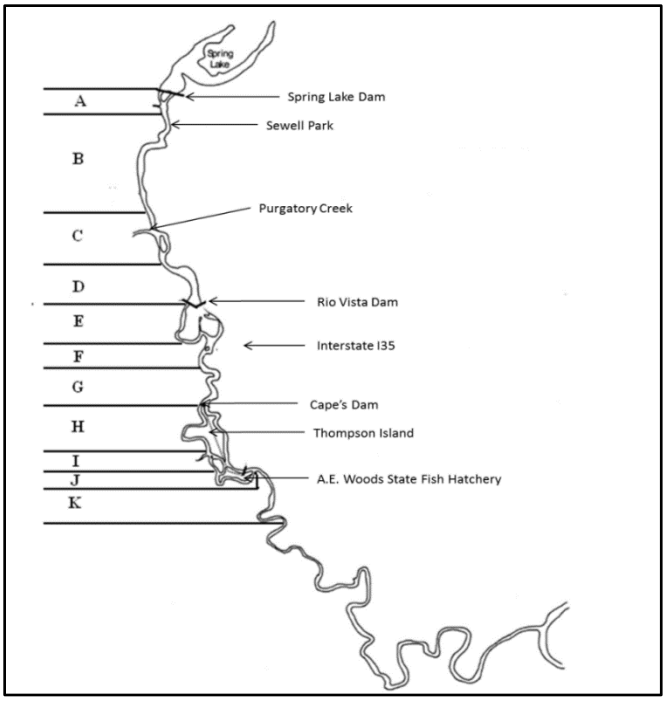

Figure 1 Letters define designated San Marcos River reaches where Texas wild rice is collected for refugia populations.

Maintenance: Once tillers have been successfully rooted they will be tagged and maintained so that their collection location is known.

Propagation: Plants will be maintained so sexual reproduction does not occur within the refugia population, unless HCP triggers occur. If reintroduction is warranted, seeds and tillers from each geographical location will be produced. Plants produced from seeds and tillers would be transplanted back within their original geographic location.

Texas blind salamanders:

Collection: Texas blind salamanders will be collected through the use of nets and traps. Traps will be deployed quarterly for approximately 12 consecutive days with traps checked every 2-4 days to collect Texas blind salamander individuals from Primers Fissure, Johnson's well, Rattlesnake cave, and Rattlesnake well (Table 5). To avoid oversampling these habitats, only 1/3 of salamanders observed from each of these locations will be collected during quarterly sampling events. Salamanders will also be collected from a driftnet on Diversion Springs in Spring Lake fished throughout the year during times when we are not actively trapping in caves and wells. Specimens from this site will all be kept, given the assumption that any Texas blind salamander leaving a spring orifice that enters a stream or lake environment will ultimately succumb to predation. These sites will be checked for specimens up to three times per week when applicable. All specimens will be transported live and maintained in the SMARC or UNFH refugia. Drift nets on Sessom Creek and Texas State Artesian Well are generally checked by Texas State staff, live Texas blind salamanders are transferred to SMARC according to their permits. USFWS staff may periodically check nets on these sites when they are not being

checked by Texas State staff.

Maintenance: Specimens will be marked by collection location. As part of quarantine procedures, all salamanders of each species will be non-lethally cotton swabbed. These samples will be sent to the southwest regional Fish Health Unit to screen for *Batrachochytrium dendrobatidis* (Bd, commonly referred to as chytrid fungus) and *Batrachochytrium salamandrivorans* (Bsal) prior to specimen incorporation into the general refugia population. Chytrid testing will occur in batches where groups of five swabs will be pooled for analysis. Duplicate individual swabs will be retained in case further testing is warranted. All salamanders will be held in quarantine for at least 30 days and until test results have returned. Chytrid (Bd) fungus has caused mortalities in amphibian species; however, some species appear to have innate immunity. Previous tests of wild caught salamanders at SMARC (both Texas Blind and San Marcos) have almost always tested positive for Bd. Clinically, the salamanders appear normal and do not have any lesions or signs of disease. Positive testing for Bsal will be treated more cautiously as it has not yet been documented in this area (or anywhere in North America); these salamanders would remain in quarantine until further study and recommendations from FWS Fish Health. Salamander tank and system maintenance such as acid washing and system sterilization will occur annually or as needed to ensure proper system function. Water quality will be monitored and recorded weekly. Salamanders will be fed live foods reared or purchased. Ponds will be utilized to produce amphipods. Amphipods will be collected from other managed ponds and raceways. Black worms will be purchased when necessary along with other food resources (i.e. blood worms, brine shrimp, etc.) if the need arises.

Propagation: Standing and refugia stocks will be maintained to encourage reproduction. Salamanders will be marked with visible elastomers, coded by their geographical locations. All progeny will be maintained separately by generations. If reintroduction is warranted, an attempt will be made to produce offspring from each geographical location.

San Marcos salamanders:

Collection: San Marcos salamanders will be collected up to quarterly from below Spring Lake dam and with SCUBA teams in Spring Lake (Table 5). The drift net on Diversion Springs will be checked routinely and specimens will be kept from this location. Collection efforts will be coordinated with the HCP Biological Monitoring Program. All specimens will be transported live and maintained in the SMARC and UNFH refugia. Historically, approximately 30% of the San Marcos salamanders collected annually succumb to natural mortality. As a result, salamander collections will target additional specimens so that as individuals perish the remainder within the captive population should not decrease below the target number between collection events.

Maintenance: As part of quarantine procedures, all salamanders of each species will be non-lethally cotton swabbed. These samples will be sent to the southwest regional Fish Health Unit to screen for *Batrachochytrium dendrobatidis* (Bd, commonly referred to as chytrid fungus) and *Batrachochytrium salamandrivorans* (Bsal) prior to specimen incorporation into the general refugia population. Chytrid testing will occur in batches where groups of five swabs will be pooled for analysis. Duplicate individual swabs will be retained in case further testing is

warranted. All salamanders will be held in quarantine for at least 30 days and until test results have returned. Chytrid (Bd) fungus has caused mortalities in amphibian species; however, some species appear to have innate immunity. Previous tests of wild caught salamanders at SMARC (both Texas Blind and San Marcos) have almost always tested positive for Bd. Clinically, the salamanders appear normal and do not have any lesions or signs of disease. Positive testing for Bsal will be treated more cautiously as it has not yet been documented in this area (or anywhere in North America); these salamanders would remain in quarantine until further study and recommendations from FWS Fish Health. Salamander tank and system maintenance such as acid washing and system sterilization will occur annually or as needed to ensure proper system function. Water quality will be monitored and recorded weekly. Salamanders will be fed live foods reared or purchased. Ponds will be utilized to produce amphipods. Amphipods will be collected from other managed ponds and raceways. Black worms will be purchased when necessary along with other food resources (i.e. blood worms, brine shrimp, etc.) if the need arises.

Propagation: Standing and refugia stocks will be maintained to discourage reproduction, unless specific needs arise for F1 generations. All progeny will be maintained separately by generation. If reintroduction is warranted, pair-wise and group mating will be employed to produce offspring. Stocking will occur once juveniles have reached 30 mm total length.

***Comal Springs salamanders*:**

Collection: Comal Springs salamanders will be collected up to quarterly from Comal Spring Runs 1-3 and Spring Island and surrounding areas (Table 5) by hand with dipnets using snorkelers. Close coordination with the HCP biological monitoring program will take place to ensure that to the degree practicable, refugia collections do not overlap with specific HCP long-term monitoring locales. In the event overlap of sampling areas is unavoidable, Comal salamanders for refugia will be collected at a rate of no more than 10% of salamanders observed in those specific locales per daily sampling trip. A SCUBA team will be used for a portion of these collection efforts if necessary. Annual natural mortality will be recorded.

Maintenance: As part of quarantine procedures, all salamanders of each species will be non-lethally cotton swabbed. These samples will be sent to the southwest regional Fish Health Unit to screen for *Batrachochytrium dendrobatidis* (Bd, commonly referred to as chytrid fungus) and *Batrachochytrium salamandrivorans* (Bsal) prior to specimen incorporation into the general refugia population. Chytrid testing will occur in batches where groups of five swabs will be pooled for analysis. Duplicate individual swabs will be retained in case further testing is warranted. All salamanders will be held in quarantine for at least 30 days and until test results have returned. Chytrid (Bd) fungus has caused mortalities in amphibian species; however, some species appear to have innate immunity. Previous tests of wild caught salamanders at SMARC (both Texas Blind and San Marcos) have almost always tested positive for Bd. Clinically, the salamanders appear normal and do not have any lesions or signs of disease. Positive testing for Bsal will be treated more cautiously as it has not yet been documented in this area (or anywhere in North America); these salamanders would remain in quarantine until further study and recommendations from FWS Fish Health. Salamander tank and system maintenance such as acid washing and system sterilization will occur annually or as needed to ensure proper system

function. Water quality will be monitored and recorded weekly. Salamanders will be fed live foods reared or purchased. Ponds will be utilized to produce amphipods. Amphipods will be collected from other managed ponds and raceways. Black worms will be purchased when necessary along with other food resources (i.e. blood worms, brine shrimp, etc.) if the need arises.

Propagation: Standing and refugia stocks will be maintained in gender-mixed set groups to allow for reproduction. All progeny will be maintained separately by generation. If reintroduction is warranted, pair-wise and group mating will be employed to produce offspring. Stocking will occur once juveniles have reached 30 mm total length.

Comal Springs riffle beetle:

Collection: Comal Spring riffle beetle collection in Spring Runs 1-3 and around Spring Island will be up primarily for research purposes with fewer numbers being held just for Standing Stock purposes as research into increasing survival rates is conducted (Table 5). Collections from the Spring and Fall Biomonitoring will be transferred to USFWS for refugia purposes. Riffle beetles will be collected with cotton lures. Cotton lures will be deployed in a variety of locations (Spring Runs 1, 2, 3, N = 5-15 lures per spring run; western shore of Landa Lake, N = 5 lures; Spring Island and associated Spring Lake habitats N = 15-20 lures) following EAHCP standard operating procedures (Hall 2016).

Maintenance: Specimens will not be maintained by collection location. Comal Springs riffle beetles will be maintained within custom built aquatic holding units and fed detrital matter and matured biofilms colonized on cotton lures.

Propagation: Propagation methods for this species are being developed.

Peck's Cave amphipod:

Collection: Peck's Cave amphipod collection will occur up to four times annually (Table 5). Adult Peck's cave amphipods will be collected with drift nets and by hand collection at variety of locations (drift nets: Spring Run 3, N = 2; Spring Island and associated Spring Lake habitats: hand collection). Collections will continue build up to target Standing Stock numbers.

Maintenance: Specimens will not be maintained by collection location. Peck's Cave amphipods will be maintained within custom built aquatic holding units and fed commercial flake fish feeds.

Propagation: Propagation methods for this species are being developed as part of standard refugia operations.

Comal Springs dryopid beetle:

Collection: Comal Springs dryopid beetles will be collected primarily through the use of wooden lures and hand picking from submerged wood found in the Comal Spring system. If dryopid beetles are found on cotton lures used for Comal Spring riffle beetles they will also be retained (Table 5). We will potentially conduct two events of trapping in Panther Canyon Well

during the year as access to the well and staff time allows. These will be bottle traps checked weekly for a month. We have ceased collection efforts of lures in Sessom Creek as these were not productive during 2017; a new design for Sessom Creek might be revisited at a later date.

Maintenance: Specimens will not be maintained by collection location. Comal Spring dryopid beetle will be maintained within custom built aquatic holding units and fed detrital matter and matured biofilms colonized on cotton lures.

Propagation: Propagation methods for this species are being developed as part of normal refugia operations and research projects.

Edwards Aquifer diving beetle:

Collection: Drift nets will be used to collect Edwards Aquifer diving beetle (Table 5). Drift nets will be set at a variety of locations where the species has been collected in the past (Texas State University Artesian Well N = 1; and Diversion Springs N = 1). Drift nets will be deployed and checked by USFWS staff when we are able to sample Texas State University Artesian Well (when not being used by Texas State staff).

Maintenance: Specimens will not be maintained by collection location. Captured specimens will be transferred to the SMARC and housed in custom made aquatic holding systems. Edwards Aquifer diving beetles are predators; they will be fed small invertebrates (e.g., ostracods).

Propagation: Propagation methods for this species are to be determined and will be conducted as part of normal refugia operations.

Texas troglobitic water slater:

Collection: Texas troglobitic water slater will primarily be collected using a drift net on Diversion Springs, but organisms found on lures in the Comal Springs system will also be retained (Table 5).

Maintenance: Captured specimens will be transferred to the SMARC and housed in custom made aquatic holding systems. Initially the species will be fed detrital matter and matured biofilms colonized on cotton lures. The species is also fed fish flake food to supplement their diet.

Propagation: Propagation methods for this species are to be determined and will be conducted as part of normal refugia operations.

Table 5. A tentative schedule for all species sampling during 2019. Collections listed here are subject to change with extenuating circumstances such as weather, coordination with external partners, and completion of construction projects. EEA and partners will be notified of sampling dates as they become known or changed.

Edward's Aquifer Species Collection Plan 2019			
Date (month)	**Interval**	**Location**	**Target Species**
Continuous	Check nets T and F every week; drift net collections suspended during Texas blind salamander trapping weeks	Diversion Springs	Texas Blind salamander, San Marcos salamander, Edward's Aquifer diving beetle, and troglobitic water slater
January	Check every T & F for 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
January	Check and reset lures	Spring Runs, Landa Lake	CSRB, CSDB, PCA, TTWS
February	Check every T & F for 2 consecutive weeks	Primer's Fissure & Johnson's Well	Texas blind salamander
February	Collect lures	Spring Runs, Landa Lake	CSRB, CSDB, PCA, TTWS
February	1-day sampling event	San Marcos River	Texas wild rice
March	1-2 day sampling event	Spring Lake and below dam	San Marcos salamander
March	1-day sampling event, hand pick	Landa Lake	Peck's Cave amphipod
April	Check every T & F for 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
April	1-day sampling event	San Marcos River	Texas wild rice
April	Throughout, coincide with bio-monitoring	San Marcos River, Comal River	Fountain darters, CSRB, CSDB

Edward's Aquifer Species Collection Plan 2019			
Date (month)	**Interval**	**Location**	**Target Species**
May	Check every T & F for 2 consecutive weeks	Primer's Fissure & Johnson's Well	Texas blind salamander
May	1-2 day sampling event	Comal Springs	Comal Springs salamander
May	1-day sampling event	San Marcos River	Texas wild rice
June	1-day sampling event, hand pick	Landa Lake	Peck's Cave amphipod
July	Check every T & F for 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
August	Check every T & F for 2 consecutive weeks	Primer's Fissure & Johnson's Well	Texas blind salamander
August	1-day sampling event, hand pick	Landa Lake	Peck's Cave amphipod
September	1-2 day sampling event	Spring Lake and below dam	San Marcos salamander
October	Check every T & F for 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
October	Throughout, coincide with bio-monitoring	San Marcos River, Comal River	Fountain darters, CSRB, CSDB
October	1-day sampling event	San Marcos River	Texas wild rice
November	Check every T & F for 2 consecutive weeks	Primer's Fissure & Johnson's Well	Texas blind salamander

Edward's Aquifer Species Collection Plan 2019			
Date (month)	**Interval**	**Location**	**Target Species**
November	Beginning of month set lures (if needed)	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle
November	1-day sampling event, hand pick	Landa Lake	Peck's Cave amphipod
November	1-2 day sampling event	Comal Springs	Comal Springs salamander
December	Check and reset lures (if needed)	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle
December	1-day sampling event	San Marcos River	Texas wild rice

Refugium Stocks:

Collection: Standing Stock numbers contribute to Refugium Stock numbers and collections will continue until Standing stock numbers are attained. In the event that Refugium Stock triggers, outlined in the contract, are reached and Standing Stock are not at full capacity, special targeted collections will be conducted to build up numbers.

Maintenance: Maintenance will be conducted in a similar manner described for standing stocks.

Propagation: Texas blind salamander, Comal Springs riffle beetle, Comal Springs dryopid beetle, Edwards Aquifer diving beetle, and Texas troglobitic water slater may be propagated to further advance culture techniques. Propagation for stocking is not anticipated during 2019.

Salvage Stocks:

Collection: If species-specific salvage triggers defined in the HCP are reached, the SMARC, in consultation with the EAA, will accommodate salvaged organisms no more than two times during the 12-year period. If triggers for multiple species are simultaneously reached, species collections during salvage operations will be prioritized based upon the perceived species-specific effect of reduced river and spring flow and habitat degradation (i.e. EAHCP triggers). Those species that are river obligate species (i.e., fountain darter and Texas wild rice) or that occupy spring orifice and interstitial ground water habitats (i.e., San Marcos and Comal Springs salamander, Peck's Cave amphipod, Comal Springs dryopid beetle) are presumed to be affected first as flows decrease. Those that reside solely within the aquifer (i.e., Edwards Aquifer diving beetle, Texas troglobitic water slater and Texas blind salamander) are presumed to be affected subsequently.

Maintenance: Organisms collected during salvage operations would be maintained at the SMARC for a limited duration (up to one-year) or until their disposition is determined. Research may be suspended or terminated if space is required for salvaged organisms. Research may also be suspended if personnel are directed to collection and maintain salvage stocks.

Propagation: Likewise, production of species would be limited to no more than two times during the 12 year period once species extirpation is determined. Species produced at the SMARC would be held for a limited time (up to one year) or less if stocking is required. Research activities may be suspended or terminated if space is required to house cultured species. Research may also be suspended if personnel are directed to reproduce, maintain, or stock salvage stocks or standing stock progeny.

Construction/Renovation/Infrastructure/Facility:

The renovations at UNFH are anticipated to be completed by January 2019, with the Quarantine area generator installed in April of 2019. Construction delays, however, are unpredictable. UNFH staff will install tanks upon the construction completion. After systems are set up, covered species will be moved into the renovated spaces.

After construction is complete (at both sites) the SMARC Center Director will develop and maintain a list of warranty problems during the 1-year warranty period, forwarding items, as they occur, to the Contracting Officer (CO) and the USFWS Project Manager (COR).

As detailed within the EAA contract with the USFWS (Contract No. 16-822-HCP) all invoices from the USFWS to the EAA for the construction services shall be billed on the last business day of the month, sent monthly, and shall provide an itemization of the expenses incurred including all supporting documentation.

All reasonable and practical security measures will be instituted by SMARC and UNFH staff to safeguard EAA refugia facilities, equipment, and species.

Staffing/Labor/Personnel:

The Supervisory Fish Biologists (SFBs) at both the SMARC and UNFH will continue in their duties including, but not limited to: supervising, mentoring, and training lower-graded employees, authorize purchases, oversee facility maintenance and repair, develop and implement budgets, and organize activities that relate to all contract activities. The SFBs will manage and coordinate research, propagation, culture, and field activities related to the refugia. The SFBs are expected to provide proper and efficient use of facilities and staff resources. The SFBs will work with the Center Director to ensure that contractual obligations are met in a timely manner. In coordination with the Center Director, they will prepare all the required written materials required for the reimbursable agreement reporting. Likewise, the SFBs will also prepare oral presentations to be used as briefing statements, outreach presentations, internal reports, work summaries, and technical presentations at professional meetings. The two SFBs will continue to work and communicate regularly with partners, Service personnel and other researchers to

effectively meet Service and reimbursable agreement goals.

Under the management of a lead supervisory biologist at both facilities, it is expected that six Biological Science Technicians, three at each station, will continue to assist with the collection, daily upkeep, maintenance, propagation, and research efforts for the ten species at the SMARC and UNFH. This includes maintaining experimental and culture production systems, keeping records along with entering, filing, and collating data. The technicians will also generate basic summary statistics and graphic analyses of data and document program accomplishments through the composition of Standard Operating Procedures (SOPs), reports, and manuscripts.

Permitting:
Both the UNFH and SMARC operate under the USFWS Southwest Region's Federal Fish and Wildlife Permit for Native, Endangered, and Threatened Species Recovery (number TE676811-3) and the Texas Parks and Wildlife Scientific Research Permits (UNFH SPR-1015-222, SMARC SPR-0616-153).

Biosecurity:
Both the UNFH and SMARC operate under the SMARC BioSecurity Plan (2014) (Exhibit E of 16-822-HCP). Specimen Collection, Hazard Analysis Critical Control Points, Quarantine, & Specimen Transfer: San Marcos Aquatic Resources Center Standard Operating Procedure.

Task 2. Research

The Research Plan for 2019 will involve a series of activities ranging from 1) methods to determine a better understanding of factors influencing pupation rates for Comal Springs riffle beetles; 2) conducting a salamander tagging/identification study; 3) investigating methodologies to reduce the loss of salamander eggs to fungus or refining salamander propagation techniques, and 4) evaluating the effect of different nutritional options on Comal Springs riffle beetle survivorship, larvae production and survival . The following section describes the basic components of each of these proposed 2019 activities.

Project 1:

Title: Increasing pupation success in the Comal Springs riffle beetle in a captive setting

Species: *Stygoparnus comalensis*

Principal/Co-PI: BIO-WEST, Inc., input by SMARC staff

Overview: The purpose of this project is to identify conditions that are optimum for incurring successful pupation and eclosion to Comal Springs riffle beetles. Different designs of holding larvae in flow-through tubes will be implemented. Researchers will also test if starvation may encourage pupation after a period of being well fed. Construct and test the efficacy of an apparatus that emulates bubble-stream conditions seen at Comal Springs. Finally, assess beetle fitness resulting for the different pupation methods.

Budget: $95,000

Benefit to the Refugia: A better understanding of factors influencing pupation will allow for increased offspring production in captivity, better estimation of production in captivity, more efficient husbandry practices, and better knowledge to create a reintroduction strategy for this species.

Expected Results: A report on the successes and failures of methodologies tested to increase pupation rates.

Project 2:

Title: Examination of the life history of the Comal Springs riffle beetle (*Heterelmis comalensis*) and assessment of factors which affect pupation rates

Species: *Stygoparnus comalensis*

Principal/Co-PI: Dr. Weston Nowlin, Texas State University, input by SMARC staff

Overview: Examine several factors which may contribute to successful pupation and emergence of adult Comal Springs riffle beetles in a captive setting. Specifically, examining three factors in captivity: 1) How does the origin (wild or lab-grown biofilms) and nutritional and microbial composition of biofilms utilized by riffle beetle larvae affect pupation and adult eclosion rate in captivity?; 2) Does the presence of conspecifics (Comal Springs riffle beetles) affect the quality (i.e., microbial composition and nutritional value) of biofilms utilized by Comal Springs riffle beetle larvae prior to pupation?; 3) How does the concentration of DO affect the survival and development of Comal Springs riffle beetle pupae and emergence of adult beetles in the lab?

Budget: $92,097

Benefit to the Refugia: A better understanding of factors influencing pupation will allow for increased offspring production in captivity, better estimation of production in captivity, more efficient husbandry practices, and better knowledge to create a reintroduction strategy for this species.

Expected Results: A report on the successes and failures of methodologies tested to increase pupation rates.

Project 3:

Title: Evaluating three different long-term tagging methods in aquatic salamander species

Species: *Eurycea nana, Eurycea rathbuni, Eurycea sp.8*

Principal: Dr. Lindsay Campbell, Linda Moon

Overview: The objective of the proposed study is to determine the efficacy of various tagging methodologies to best visually mark covered salamanders species for quick identification of captively held salamanders.

Budget: $23,723.94

Objectives and Methods: Salamanders will be tagged with three different methods to evaluate the utility of the method as a way to quickly visually identify salamanders. Techniques to be tested include Visible Implant Elastomer tags, Visual Implant Alpha tags, and small Passive Integrated Transponders. Tags and injection sites will be monitored overtime for health, retention, and clarity/readability. The ability to

individually mark salamanders would increase specificity of record keeping and allow us to follow information of an individual over its lifetime. Also, for genetic management purposes being able to identify individuals thus knowing the parentage of offsprings is key in many plans. Additionally with the ability to uniquely mark each individual would allow consolidation of specimens, increasing the probability of mating success while simultaneously affording efficiencies in refugia operations by reducing the number of systems needed to maintain the required number of captive salamanders.

Expected Results: The results of the study will be presented as a report to the EAA and potentially submitted to a peer reviewed journal. If a marking technique(s) is/are successful the Captive Propagation Manual for this species will be updated to include how marking can be effectively used in husbandry practices.

Project 4:

Title: San Marcos salamander Reproductive Dysfunction Research

Species: *Eurycea nana*

Principal: Dr. Lindsay Campbell, Kelsey Anderson

Overview: We will continue to investigate reproduction in San Marcos salamanders to try to find answers for low reproduction rates. A pilot reproduction trial will be conducted based on suggestions from the 2018 study. We will compare physiology of refugia females and freshly caught wild females. Lastly, we will test the water in our systems for potentially deleterious compounds.

Budget: $63,921.13

Objectives and Methods: A pilot reproduction trial will be conducted based on modifications suggested by the 2018 study, mainly: adding UV sterilization to take systems, observing for behavioral indicators of female reproductive readiness, and removing males after 48 hours. We will send off refugia population females and freshly caught wild females to be analyzed for fat soluble vitamins, trace mineral panels, and overall health and diseases. Results will be compared for differences. We will also test the water in our tank systems for total estrogenic compounds, PAHs, PVC by-products, and pharmaceuticals. We will also use Gore-sober modules and compare to results collected from our well-water source samples.

Expected Results: The results of the study will be presented as a report to the EAA.

Project 5:

Title: Captive population nutrition and longevity of the Comal Springs riffle beetle (*Heterelmis comalensis*)

Species: *Heterelmis comalensis*

Principal: Dr. Lindsay Campbell, Amelia Everett

Overview: The goal of this research project is to improve survival rates of adult Comal Springs riffle beetles in refugia through nutritional evaluation. This research has two

parts: 1) Compare the gut contents of refugia population wild stock adults to freshly collected wild adults, and 2) Compare survival rates and larval production in containers with different food item supplementation to that of standard food items offered.

Budget: $118,808.80

Objectives and Methods: We will compare the survival rates and larval production of containers with standard food items offered (leaves and biofilm on cotton cloth) to containers with the treatments of additional conditioned balsa wood and plant roots. Equal numbers of female and male riffle beetles will be added to the containers (with three replicates) and checked monthly for progress. We will also compare gut contents of adult riffle beetles from our refugia population and freshly collected wild adults. We will analyze whole meta-genome of their gut contents and bulk amino acids. The benefits to the refugia would be improved CSRB husbandry and production rates.

Expected Results: The results of the study will be presented as a report to the EAA and if warranted update the CSRB Culture Propagation Manual.

Task 3. Species Propagation and Husbandry

Development and refinement of SOPs for animal rearing and captive propagation: Continue to refine SOPs for all species as needed for updates to reflect new protocols that are instituted for each species throughout the year. As new information becomes available about genetic management, further develop draft Captive Propagation Plans for all species.

Task 4. Species Reintroduction

Reintroduction Plan for term of contract:
Continue to refine the Reintroduction Strategy as new information becomes available.

Reintroduction Plan for 2019: None

Any anticipated triggers being prepared for: Given current weather predictions, spring flows, and the Edwards Aquafer water level none are anticipated during the 2019 performance period.

Task 5. Reporting

5.1 Species specific Propagation plans (SOPs): Refine throughout year as needed
5.2 Species specific Genetic Management plans: None during 2019
5.3 Species specific Reintroduction plans: Refine as needed
5.4 2019 EAHCP Annual Program reporting– A year-end report of 2019 activities will be provided to the EAA no later than 1/31/2020.
5.5 Program reporting as required by ITP and TPWD. TPWD Scientific Research Permit Report will be conveyed to the EAA July 31, 2019.
5.6 Descriptions and photographs of procedures from collections to restocking – Photographs and documentation of collection and restocking will be included in the monthly report to

the EAA CSO along with the year-end report.

5.7 Summaries of any data analyses, research, or genetic analyses – Research projects and results of collection efforts will be provided to the EAA in the monthly reports, year-end documentation, and stand-alone documents (agreed upon by Center director and HCP CSO).

5.8 Description of terms and conditions of any permits received – As permits are received, their contents will be conveyed to the EAA.

5.9 Monthly electronic reports to HCP CSO: A monthly report of all activities will be provided to the HCP CSO. We anticipate providing the report by the 10th of each month for the previous month's activities.

Task 6. Meetings and Presentations

Planning or coordination meetings:
    - Yearly planning meeting with SMARC and UNFH staff
- Public meetings
    - EAA Board
        - End of year report
        - Present research results
    - Implementing Committee
        - End of year summary
    - Stakeholder Committee
        - End of year summary
    - Science Committee
        - Methods for research projects
        - Present research results

Monitoring:

Monitoring will be conducted through the use of progress reports and site visits to the refugia as well as through collaborative management by the EAHCP CSO.

Budget: Projected 2019 budget.

U.S. Fish and Wildlife Service 2019 Task		Task Budget Amount	Total Task Budget Amount
1	**Refugia Operations**		**$ 1,743,898.91**
	SMARC Refugia & Quarantine Bldgs.		
	Construction	$ 78,857.09	
	35 Ton Chiller (TBD)	$ 118,800.00	
	Building Maintenance	$ 10,000.00	
	Utilities	$ 80,000.00	
	Equipment	$ 119,223.60	
	UNFH Renovation Refugia & Quarantine Bldgs.		
	Construction	$ 40,109.90	
	AmeriVet	$ 239,967.55	
	Building Maintenance	$ 10,000.00	
	Utilities	$ 75,000.00	
	Equipment	$ 100,000.00	
	Water Quality Monitoring System	$ 70,000.00	
	SMARC Species Husbandry and Collection		
	Fish Biologist (GS-12, 590 hrs)	$ 31,024.65	
	Fish Biologist (GS-07, 1439 hrs)	$ 42,752.69	
	Fish Biologist (GS-07, 1443 hrs)	$ 42,871.53	
	Fish Biologist (GS-07, 1592 hrs)	$ 47,298.32	
	Diving	$ 2,500.00	
	Weekend Walk Thru	$ 7,000.00	
	Other Overtime	$ 2,500.00	
	UNFH Species Husbandry and Collection		
	Fish Biologist (GS-11, 1810 hrs)	$ 77,576.60	
	Fish Biologist (GS-07, 1904 hrs)	$ 54,720.96	
	Fish Biologist (GS-07, 1904 hrs)	$ 55,634.88	
	Fish Biologist (GS-07, 1546 hrs)	$ 45,174.12	
	Weekend Walk Thru	$ 7,000.00	
	Other Overtime	$ 2,500.00	
	Fish Health	$ 10,000.00	
	SMARC Reimbursibles	$ 60,000.00	
	UNFH Reimbursibles	$ 60,000.00	
	Subtotal	*$1,490,11.89*	
	Admin Cost Subtotal	*$ 253,387.02*	
2	**Research**		**$ 548,165.01**

	BIO-WEST: CSRB pupation			$	95,000.00	
	BIO-WEST: Peck's Cave amphipod life history			$	21,781.51	
	BIO-WEST: Dryopid life history			$	22,090.92	
	TxSt: CSRB pupation			$	92,097.00	
	USFWS salamander tagging			$	23,723.94	
	GS-12 (252 hrs)	*$*	*13,277.88*			
	GS-7 (302 hrs)	*$*	*8,972.42*			
	FWS Administrator (6 hrs)	*$*	*473.64*			
	Materials	*$*	*1,000.00*			
	USFWS salamander reproduction			$	63,921.13	
	GS-12 (248 hrs)	*$*	*13,067.12*			
	GS-7 (447 hrs)	*$*	*13,280.37*			
	FWS Administrator (6 hrs)	*$*	*473.64*			
	Materials	*$*	*1,600.00*			
	Water quailty instrament	*$*	*8,000.00*			
	Salamander analysis	*$*	*20,000.00*			
	Water analysis	*$*	*7,500.00*			
	USFWS CSRB nutrition			$	118,808.80	
	GS-12 (255 hrs)	*$*	*13,435.95*			
	GS-7 (451 hrs)	*$*	*13,399.21*			
	FWS Administrator (6 hrs)	*$*	*473.64*			
	Materials	*$*	*1,500.00*			
	Microbiome analysis	*$*	*15,000.00*			
	Metagenome	*$*	*5,000.00*			
	Metagenome analysis contingent on lab	*$*	*70,000.00*			
	New Research Development			$	31,093.80	
	GS-12 (251 hrs)	*$*	*13,225.19*			
	FWS Administrator (94 hrs)	*$*	*7,407.85*			
	USFWS Dryopid GS-7 (358 hrs)	*$*	*10,460.76*			
	Subtotal			*$*	*468,517.10*	
	Admin costs for Task 2			*$*	*79,647.91*	
3	**Species Propagation and Husbandry**			*$*	-	**$** **-**
	Subtotal			*$*	-	
4	**Species Reintroduction**			*$*	-	**$** **-**
	Subtotal			*$*	-	
5	**Reporting**					**$** **91,815.48**
	SMARC Staff			$	51,815.81	
	FWS Administrator (80 hrs)	*$*	*6,382.31*			
	Fish Biologist (GS-12, 422 hrs)	*$*	*22,762.08*			

	Fish Biologist (GS-07, 166 hrs)	*$*	*4,931.86*			
	Fish Biologist (GS-07, 166 hrs)	*$*	*4,931.86*			
	Fish Biologist (GS-07, 166 hrs)	*$*	*4,931.86*			
	SMARC Staff (GS-11, 168 hrs)	*$*	*7,875.84*			
	UNFH Staff			$	26,658.96	
	Fish Biologist (GS-11, 248 hrs)	*$*	*10,629.28*			
	Fish Biologist (GS-06/7, 156 hrs)	*$*	*4,483.44*			
	Fish Biologist (GS-07, 156 hrs)	*$*	*4,558.32*			
	Fish Biologist (GS-07, 156 hrs)	*$*	*4,558.32*			
	UNFH Staff (GS-07, 80 hrs)	*$*	*2,429.60*			
	Subtotal			*$*	*78,474.77*	
	Admin costs for Task 5			*$*	*13,340.71*	
6	**Meetings and Presentations**					**$ 14,310.00**
	SMARC staff			$	9,115.65	
	FWS Administrator (50 hrs)	*$*	*4,355.49*			
	Fish Biologist (GS-12, 52 hrs)	*$*	*2,739.88*			
	Fish Biologist (GS-07, 28 hrs)	*$*	*831.88*			
	Fish Biologist (GS-07, 20 hrs)	*$*	*594.20*			
	Fish Biologist (GS-07, 20 hrs)	*$*	*594.20*			
	UNFH Staff			$	3,115.12	
	Fish Biologist (GS-11, 32 hrs)	*$*	*1,371.52*			
	Fish Biologist (GS-7, 20 hrs)	*$*	*574.80*			
	Fish Biologist (GS-07, 20 hrs)	*$*	*584.40*			
	Fish Biologist (GS-07, 20 hrs)	*$*	*584.40*			
	Subtotal			*$*	*12,230.77*	
	Admin costs for Task 6			*$*	*2,079.23*	

TOTAL **$2,398,189.40**

Remainder of 2018 construction costs detailed within the 2018 work plan will be applied to 2019.

Projected (2019) Budget Summarized by Task:

Task 1: $1,743,972
Task 2: $548,165
Task 3: $0
Task 4: $0
Task 5: $91,815
Task 6: $14,310

Projected (2019) Subcontractor Expenses Summarized by Task

Task 1: Southwest Regional Fish Health Unit, Dexter NM $10,000 (Health Diagnostics)
Task 2: BIO-WEST, Inc, $95,000; Texas State University, Dr. Weston Nowlin, $92,097; Washington Animal Disease Diagnostic Laboratory $20,000; Dr. Camila Carlos-Shanley's Laboratory $15,000-$70,000 (depending on availability of NextGen sequencing lab)
Task 3: $0
Task 4: $0
Task 5: $0
Task 6: $0

Projected Equipment Purchases

U.S. Fish and Wildlife Service

	Task	Equipment	Total	Total Task Budget Amount
1	**Refugia Operations**			**$222,000**
	SMARC	35 ton chiller	$ 118,800	
		Tanks	$ 55,000	
		Display Tanks	$ 2,000	
		Water flow meters	$ 5,000	
	UNFH	Tanks	$ 90,000	
	Water Quality Monitoring Package		$ 70,000	

Timeline of 2019 Milestones (List major deliverables)

January	Continue with species collection
	Subcontract research awards executed
	2019 Specific Research Study Plans finalized
July	Submit and renew TPWD permit
November	2020 Research Proposal Review
December	Draft Annual report

Chad Furl, PhD Chief Science Officer Edwards Aquifer Authority

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Ken Ostrand, PhD Center Director SMARC, UNFH US Fish and Wildlife Service

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Captive population nutrition & longevity of the Comal Springs riffle beetle (*Heterelmis comalensis*)

2019 Interim Report

Edwards Aquifer Authority HCP Applied Research

2019

Prepared by:

Amelia Hunter, M.S.
and
Lindsay Campbell, Ph.D.

United States Fish & Wildlife Service
San Marcos Aquatic Resources Center

Table of Contents

Background & Significance

The Comal Springs riffle beetle (*Heterelmis comalensis*; CSRB) is a federally endangered (USFWS 1980) beetle (family Elmidae) endemic to the eucrenal habitats of Comal Springs, Comal County; and San Marcos Springs, Hays County, Texas, U.S.A. The Edwards Aquifer Authority (EAA), in fulfillment of the Edwards Aquifer Habitat Conservation Plan (EAHCP), established a contract to cover 10 species, of which the CSRB is one, in a refugia with the U.S. Fish and Wildlife Service (USFWS) at two locations in Texas: San Marcos Aquatic Resources Center (SMARC) in San Marcos, Texas, U.S.A. and Uvalde National Fish Hatchery (UNFH) in Uvalde, Texas, U.S.A,. Targeted research with captive standing stock of CSRB is needed to successfully maintain healthy individuals and for the overall success of the refugia.

In 2018, wild stock CSRB at SMARC tracked by groups and delimited on date collected, showed a sharp decrease in survival after 5 months in captivity. CSRB collected in the wild are of unknown age; therefore, this observed captive mortality could be due to natural senescence. Current food items offered in captivity may not be adequate in macro or micro nutrients that could be supplemented by other sources. CSRB likely graze on biofilms found on wood and leaf materials, receiving 70 to 92% of their diet from course particulate organic matter (Nowlin *et al.* 2017). Based on low long-term survival of captive wild stock CSRB—even with implementation of previous applied research studies on CSRB captive holding techniques (Nowlin et al. 2017; Worsham et al. 2017)—we sought to investigate if there were nutritional deficiencies in the food items offered to captive CSRB populations and test offering additional food items not currently listed in refugia protocols.

Dr. Camila Carlos-Shanley at Texas State University was contracted based on her experience and expertise in invertebrate microbial ecology, specifically gut microbiome analysis. Preliminary studies by Dr. Carlos-Shanley indicated that the microbiome of CSRB were not transient, in that the biome was specific and did not just pass-through as seen in caterpillars. Comparing the microbiome and bacterial biodiversity of the CSRB guts can show if there is a change or loss of diversity in captive versus wild organisms. We compared wild and refugia held CSRB gut bacterial diversity.

Methods

Non-Traditional Diet Experiment

An in-situ experiment at SMARC was conducted February through October 2019 in order to quantify and compare adult survival and larval production based on nutritional treatments of the addition of different, non-traditional diet items (i.e., wood or root treatments; Figure 1) and a control treatment of current diet items offered to CSRB in captivity at SMARC refugia (i.e., cloth and leaves).

Prior to the experiment, Amelia Hunter collected and conditioned wood and roots in flowing aquifer water for at least one month prior to adding to flow-through tubes. The conditioning process grows biofilms on the items for CSRB to graze/scrape off for nutrition. We originally proposed using conditioned balsa wood dowels as the wood, but this became impractical for the experiment because the development of a good amount of biofilm and decay was insufficient in the time frame to start the experiment. As an alternative, conditioned wood from Spring Island collected in 2017 were utilized in the experiment. We were unable to identify the type of tree the wood came from nor was our station botanist due to the state of decay. Before wood sections were added to flow-through tubes, they were inspected for harmful fungus or invertebrates. Plant roots were collected from the Comal Springs system around Spring Island in December 2018. Upon collection, roots were cleaned of dirt and other debris with well water and toothbrushes at SMARC. Staff inspected roots under a microscope after cleaning to examine and remove any potentially harmful organisms, like macrophytes and insect larvae. Roots were then isolated in a flow-through container for two months before the start of the experiment in February.

The control for this study consisted of traditional diet items (i.e., cloth and leaves), and wood or roots were added to the traditional diet items for two, non-traditional diet item treatments. Dried leaves of American sycamore, black walnut, and anacua trees were weighed (1.655g total weight of mix), ensuring the same weight of leaves were placed in each of the flow through tubes. Equivalent amounts of conditioned poly-cotton cloth (6.5 inch x 1.5 inch) were placed onto a mesh matrix (8 inch x 6.25 inch) to be rolled into each of the nine tubes (Figure 2). The wood treatment (Figure 1C) incorporated segments of

equal length and similar diameter from the same limb for all three tubes. The root treatment (Figure 1B) had an equal weight of roots (2.90g) among all three tubes. Food items were rolled in a plastic mesh matrix to give structure to the items, while allowing the beetles to crawl between the mesh weave. Conditioned wood or roots were placed at the center of the container, similar to the set up seen in Figure 2.

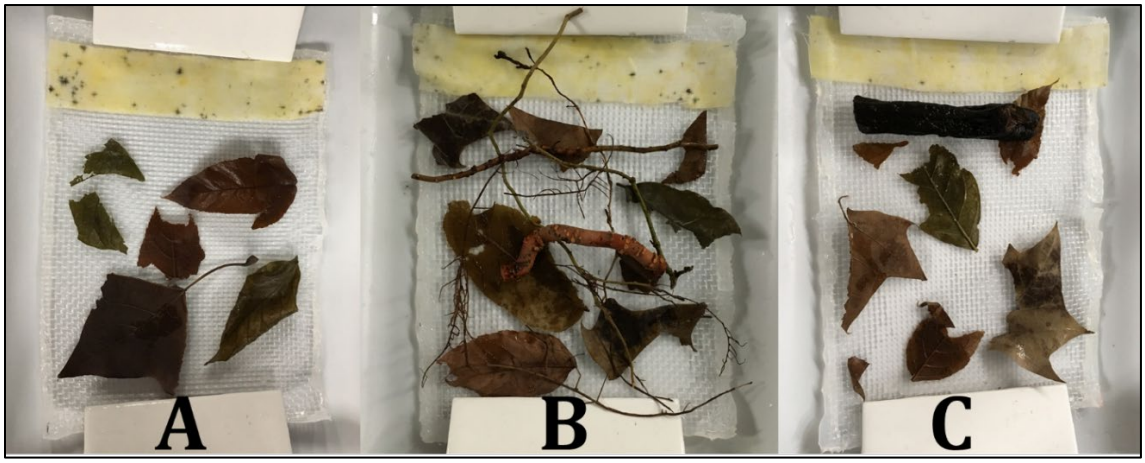

Figure 1. Flow through tube internal contents set up before rolling for insertion. Control (A), addition of roots (B), and addition of conditioned wood (C).

Figure 2 Flow-through tube design with cloth and leaves. The poplar wood dowels seen in this picture will be replaced with either balsa wood or roots. Pictured here is from a prototype in 2017. Current mesh is much larger in size and reinforced on the edges to reduce fraying.

Staff collected 180 adult CSRB using the cotton lure method on February 20, 2019 at Comal Springs, Spring Run 3 in Comal County, Texas, U.S.A. Amelia Hunter and Dr. Lindsay Campbell sexed the beetles at SMARC using the optics on an Olympus® SZX16 microscope to illuminate internal reproductive structures (Kosnicki, 2019). Adults were divided evenly

(20 adults; 10♀: 10♂) between nine (three tubes per treatment) 2 inch by 8 inch flow-through tubes (Figure 3).

Figure 3 Adult CSRB placement within flow through tube before assemblage of contents.

Tubes were visually monitored daily for water flow and development of harmful fungus. Amelia Hunter inventoried the tubes every 28-32 days and refreshed nutrient items. Any larva found during inventory were counted and removed to separate tubes labeled with corresponding treatment name to further track their growth and development. Additional leaves were added during inventory to refresh food items exhausted. Roots and wood were not refreshed because they did not decay or mature past palatability (larvae and adults found grazing or harbored within the items).

Count data collected each month for alive adults were compared by groups using logrank tests (Peto *et al*., 1977) with Kaplan-Meier estimates (Kaplan & Meier 1958; Goel *et al*., 2010). This type of test allows estimation of survival over time, even when individuals die or studied for different lengths of time. Additionally, Cox proportional-hazards model test was conducted to determine the effect of contributions of explanatory variables.

Beetle Gut Microbiome Comparison

Due to the lapse in funding with the 2018-2019 government shutdown, this project did not occur at the start of January as proposed. This delay pushed the start date back to April 2019.

Eight beetles from the refugia population (targeting those held over 4 months) and eight beetles from the wild were humanely euthanized for culturing of their gut bacteria. For a more comprehensive comparison, samples of water and conditioned wood pieces where adult CSRB were found were also collected. Beetles were washed and the wash was plated to remove anything on the outside of the beetles. The cleaned beetles were then homogenized using a sterile micropestle and plated on growth media consistent with the environmental samples. Environmental water samples were processed by diluting and plating onto growth media. Plates that had a reasonable amount of growth were used to select colonies for isolation. Wood samples were washed, and the wash was plated on the same type of growth media for consistency. Selective bacterial media for isolating *Enterococcus* was used to grow bacteria from one captive and one wild beetle. This genus is a commensal organism of many gut microbiomes, but some species can be pathogenic (Yuen et al., 2014). Both anaerobic and aerobic bacteria are growing on the selective media from the beetle microbiomes. Currently, the bacteria are being isolated for identification at the genus level.

During frozen stock preparation, 45 of the isolates were lost due to prolonged exposure to suboptimal temperatures. After inoculating the frozen stock cultures, 18 were found to be contaminated or not fully isolated, and 54 did not grow in liquid media. The genomic DNA of the remaining 223 isolates was isolated using a PureLink® Genomic DNA Mini Kit. The 16S rRNA gene was amplified and purified using the GFX™ PCR DNA and Gel Band Purification Kit by GE. DNA samples were quantified and adjusted to meet the specifications provided by the Center of Research Support at The University of Texas – Austin. Sequence reads were corrected using Chromas v. 2.6.6 and reads with an average quality score below 30 were disregarded. Contigs were formed using UGENE v. 1.3.7. The contigs were uploaded to BLAST, SILVA, and the Ribosomal Database Project (RDP) were taxonomically identified. Species names were taken from the BLAST results to form a list of 142 isolated bacterial samples.

Samples will be sent to the Joint Genome Institute (JGI) in late 2019 for full genome sequencing, alignment, and analysis (this will be an in-kind service provided by Dr. Carlos-Shanley through a separate grant she has for sequencing at the JGI). Dr. Carlos-Shanley will provide a report detailing the phylogenetic identification of the bacterial isolates and the comparison between the wild versus refugia CSRB.

For future sequencing and comparisons, we collected samples throughout the year of wild and refugia beetles. We sacrificed the oldest available CSRB held in refugia in March, May, September, and November for comparison of microbiome overtime as CSRB

are held in captivity for longer periods of time. Wild CSRB were collected in February, May, September, and December for comparisons of wild microbiome throughout the year. At each time point, staff collected eight to ten adults from both refugia and from the wild.

Results and Discussion Points

Non-Traditional Diet Experiment

No significant difference among food treatments was detected with the Hazard Ratio, which is the instantaneous probability of death (X^2 (2, N = sample size) = 2.9, p = 0.2, Figure 4). A smaller Hazard Ratio compared to the control treatment would suggest less a chance of death at any given time. A trend to a smaller Hazard Ratio was seen with roots and wood, which could be biologically important even if it is not statistically significant. A survival curve plot shows a trend of higher survival probability for the wood treatment (Figure 5).

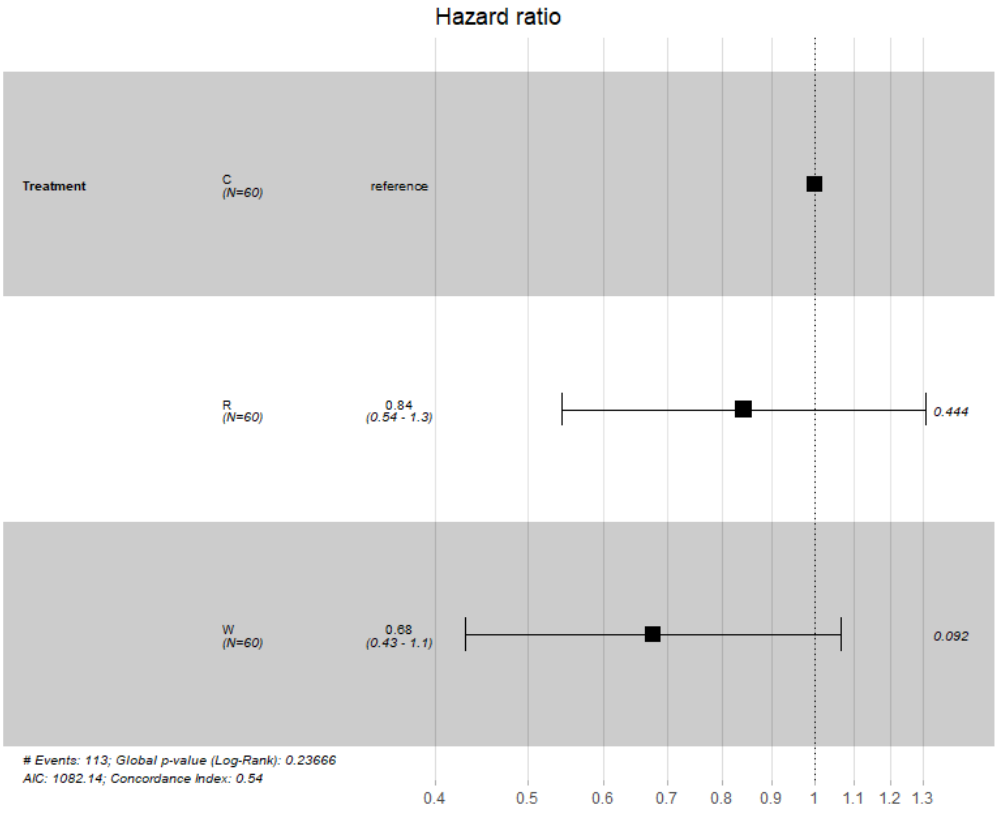

Figure 4 Hazard ration with the Control treatment as reference, R as Root Treatment, and W as Wood treatment.

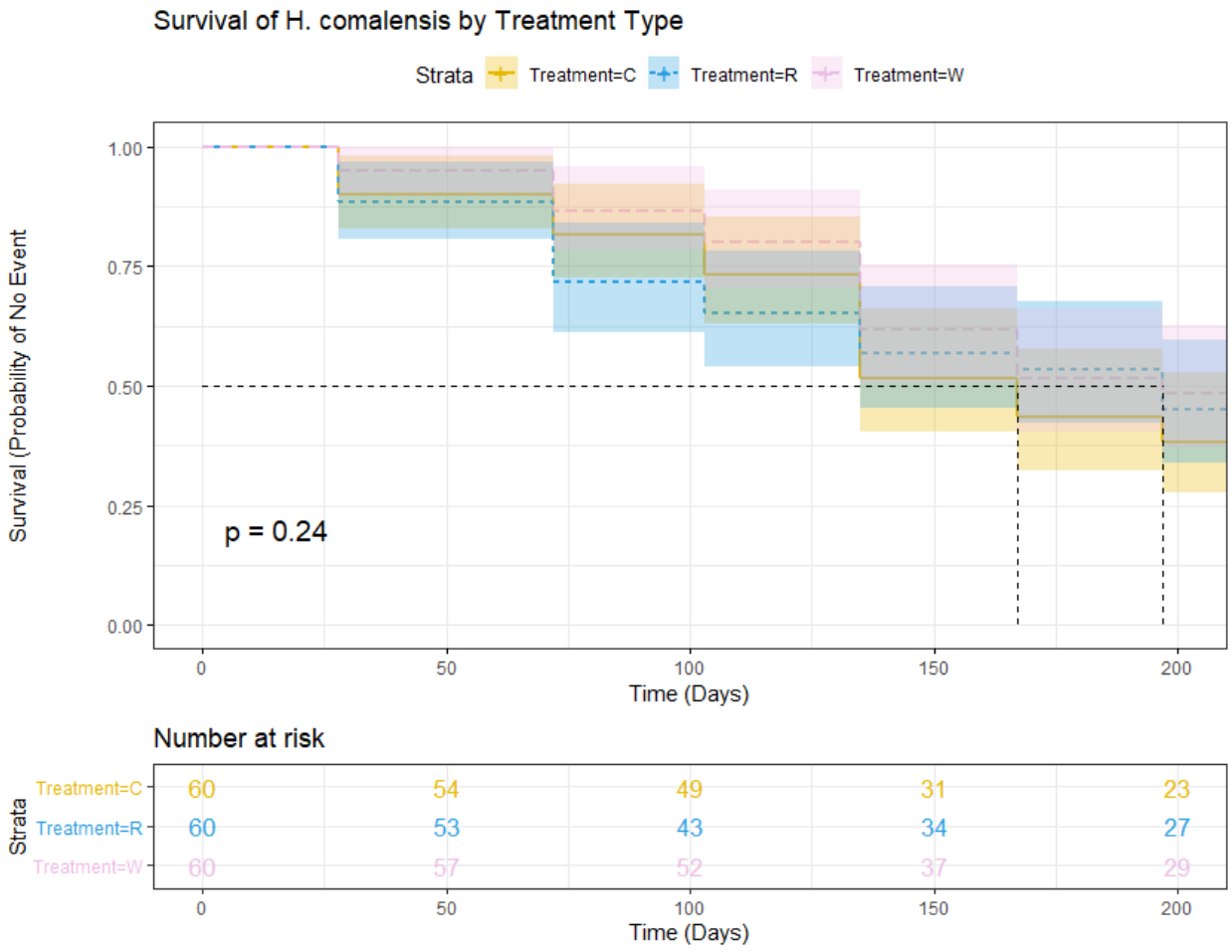

Figure 5 Survival curves grouped by treatment with confidence intervals shaded.

At any given time point, CSRB adults produced more larvae in the wood treatment than the control or root treatments (Figure 6). CSRB housed in the wood treatment produced double the amount of larvae cumulatively (552) than the Control (243) or Root (276) treatment. It should be noted that we perceive this as more production by adults, an alternative explanation could be that the production is the same, but more eggs and small larvae survive in the wood treatment.

In the future, we will be adding wood to our CSRB containers for both adults and larvae. The wood treatment did not reduce survival rates and showed increased larval production. Larvae were observed to trench through the wood, suggesting that wood might be important for their diet, and provided shelter habitat for the larvae.

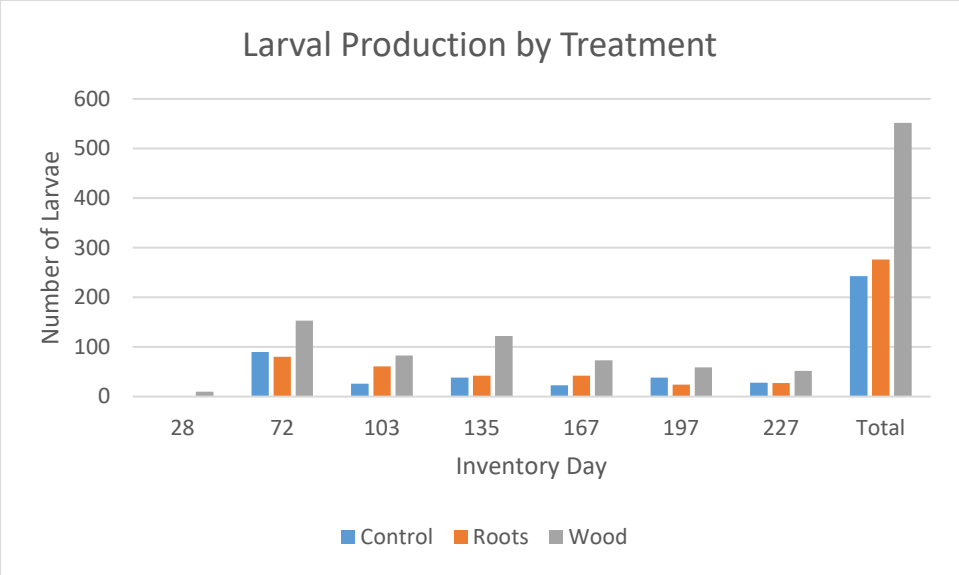

Figure 6 Larval production by treatment on each inventory day and cumulatively.

Beetle Gut Microbiome Culture-Dependent Results

A total of 344 isolates were grown from the environmental biofilm and riffle beetles collected from SMARC and the Comal Springs (Table 1). The isolates from each beetle, totaling 237, and their source were recorded (Table 2).

Table 1 Number of beetles, their isolates, and isolates from the environmental samples collected from the Comal Springs outflow and SMARC

	Beetles	Beetle Isolates	Wood Isolates	Water Isolates	Total
Comal Springs	8	81	27	52	**168**
SMARC	7	155	2	12	**176**

Table 2 Number of isolates per sample. Samples 1-7 were taken from SMARC and 8-15 were taken from near the Comal Springs outflow.

	SMARC							Comal Springs								Total
Beetle	Hc1	Hc2	Hc3	Hc4	Hc5	Hc6	Hc7	Hc8	Hc9	Hc10	Hc11	Hc12	Hc13	Hc14	Hc15	
# of Isolates	13	9	13	1	20	15	10	24	8	35	24	30	18	6	11	**237**

In total, 30 genera belonging to 4 phyla have been identified. Of these, 11 were found only in wild beetles, 23 only in captive beetles and 8 were found in both groups (Figure 7).

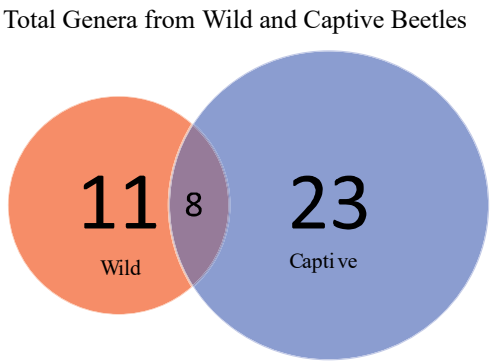

Figure 7 The numbers indicate the number of unique genera in beetles from each source and the number of genera present in beetles from both sources.

The diversity of culturable gut bacteria in wild beetle microbiomes was lower than their captive counterparts at the genus level (Table 3). However, the diversity in the wild water samples was higher than those from the refugium environment (Table 4). The percent relative abundance of each major genera (n >1) in wild and captive beetles demonstrates the difference of major and minor genera in each microbiome (Figure 8). The largest percentage of the captive microbiome is made of 14 low abundance genera that are not present in the wild microbiome. Alternatively, the wild microbiome contained 3 low abundance genera that are not present in the captive beetle microbiome. *Pseudomonas*, *Comamonas*, and *Acinetobacter* were identified from wild wood samples, but none were identified on wood from the refugia.

Researchers were concerned of the presence of *Chromobacterium sp.* as this genus has been reported to contribute to mortality in mosquito larvae and reduced levels of pupation and eclosion (Short *et al.* 2018). *Chromobacterium sp.* were found only in Refugia beetles and Refugia water. These isolates were sent for sequencing, so they can be identified to species and compared to databases of species found to be harmful and in some cases used as insecticides. Dr. Campbell plans to work with Dr. Carlos-Shanely in 2020 on exposure trials to CSRB larvae using the cultured isolates from the samples. Staff will also try to determine the source of these bacterium and/or ways to eliminate them from the water feeding the Refugia containers.

Table 3 Number of wild or captive beetles that contained isolates from a genus. Total number of beetles is shown in parentheses next to their respective source.

	Beetle	
Genus	Wild (7)	Captive (8)
Acidovorax	1	0
Acinetobacter	2	4
Aeromonas	6	1

Bacillus	3	4
Brevundimonas	1	5
Chromobacterium	0	2
Chryseobacterium	3	1
Comamonas	0	3
Dunganella	0	1
Enterococcus	1	0
Gordonia	0	1
Herbaspirillum	0	1
Mangrovibacter	0	1
Massilia	0	1
Mycobacterium	0	1
Novoaphingobium	0	1
Pelomonas	0	1
Pseudomonas	2	3
Rhizobium	0	1
Roseateles	1	0
Sphingomonas	0	1
Sporosarcina	0	1
Staphylococcus	1	2
Stenotrophomonas	0	1
Tsukamurella	2	3
Unclassified	0	1
Total	**23**	**41**

Table 4 Number of water samples from either SMARC or the wild environment that contained isolates from a specific genus. The total number of samples from each source is shown at the top in parentheses.

Water		
Genus	Wild (7)	SMARC (1)
Acidovorax	0	1
Acinetobacter	1	1
Aeromonas	3	0
Arcicella	1	0
Bacillus	1	0
Brevundimonas	0	1
Chitinimonas	1	0
Chromobacterium	0	1
Chryseobacterium	2	0
Comamonas	1	0
Flavobacterium	1	0

Mangrovibacter	1	0
Pelomonas	0	1
Pseudomonas	2	0
Streptomyces	1	0
Total	**15**	**5**

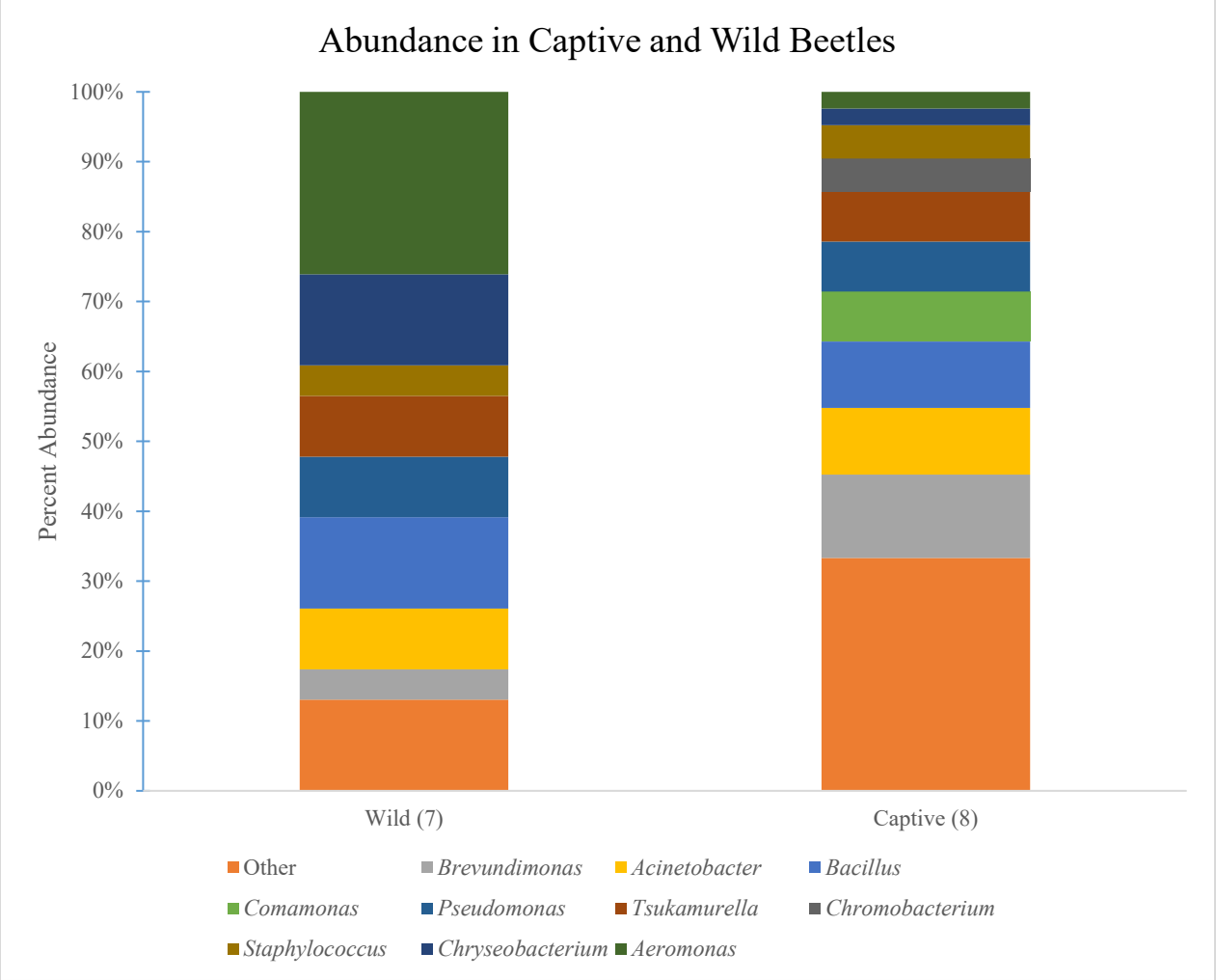

Figure 8 Percent relative abundance of the culturable bacteria within the wild and captive beetle microbiome. The number of beetles used are in parenthesis.

A total of 45 environmental samples, including wood samples, were sequenced. Since no wood samples were able to be sequenced from SMARC, only a table comparing the presence of bacteria in water samples was created to illustrate the difference between abundance of each genus from both sources (Table 5).

Table 5 The number of water samples that contain each genus are shown. Since there was only 1 plate from SMARC, the maximum number of sources that could contain a genus is 1.

Water		
Genus	**Wild (7 plates)**	**SMARC (1 plate)**
Acidovorax	0	1
Acinetobacter	1	1
Aeromonas	3	0
Arcicella	1	0
Bacillus	1	0
Brevundimonas	0	1
Chitinimonas	1	0
Chromobacterium	0	1
Chryseobacterium	2	0
Comamonas	1	0
Flavobacterium	1	0
Mangrovibacter	1	0
Pelomonas	0	1
Pseudomonas	2	0
Streptomyces	1	0
Total	**15**	**5**

A total of 97 bacterial samples were isolated and sequenced from beetles. Of these, 64 were unique samples that were not determined to be duplicates of other isolated samples (Table 6).

Table 6 The number of beetle samples that contain each genus are shown. The number in parentheses indicates the number of beetles used from each source.

Beetle		
Genus	**Wild (7)**	**Captive (8)**
Acidovorax	1	0
Enterococcus	1	0
Roseateles	1	0
Dunganella	0	1
Gordonia	0	1
Herbaspirillum	0	1
Mangrovibacter	0	1
Massilia	0	1
Mycobacterium	0	1
Novoaphingobium	0	1
Pelomonas	0	1

Rhizobium	0	1
Sphingomonas	0	1
Sporosarcina	0	1
Stenotrophomonas	0	1
Unclassified	0	1
Chryseobacterium	3	1
Aeromonas	6	1
Chromobacterium	0	2
Staphylococcus	1	2
Comamonas	0	3
Pseudomonas	2	3
Tsukamurella	2	3
Acinetobacter	2	4
Bacillus	3	4
Brevundimonas	1	5
Total	**23**	**41**

Next, 91 unique samples will be sent to the Joint Genome Institute (JGI) in late November for full genome sequencing, which will provide a more accurate identification of the bacterial sample (Table 7). In the case of some samples, the closest BLAST match was used to estimate the species so there is reason to believe that some of the samples of the same genus and source are not the same bacterial species. The list also contains all unique samples from riffle beetles and environmental samples. Samples that appear in wild beetles but not captive beetles and vice versa are of interest which is why all unique samples from each source were chosen. A phylogenetic tree of all sequenced samples was also considered when deciding which samples to submit. If the BLAST estimate, source, and position on the phylogenetic tree were the same, the bacterial samples were presumed to be the same species and only one was selected.

Future Work to be completed in 2020

The selected bacterial samples will be sent for whole genome sequencing, which may determine if the bacterial sample is a part of the wild beetle microbiome but is not present in captive beetles. Sequencing the whole genome can also determine if a bacterial sample is a known insect pathogen or produces metabolites that are known insect toxins. In addition to these culture-dependent studies, a culture-independent study of the wild and captive beetles will offer more information on their microbiome. All the DNA from the beetles will be isolated at once and universal primers for the bacterial 16S rRNA gene will amplify bacterial DNA.

Table 7 A list of all the samples being sent to JGI. This list contains the source from which the sample was isolated (Hc = Beetle; RefWater = Refugium water; CW = Comal water; Wood = wild wood), whether the sample came from SMARC or from the Comal Springs, an

Source	Comal vs SMARC	BLAST	BLAST Percentage
CW1	Wild	*Arcicella rosea*	98.26
CW1	Wild	*Streptomyces ferralitis*	98.26
CW2	Wild	*Acinetobacter johnsonii*	97.75
CW2	Wild	*Chryseobacterium lineare*	98.66
CW2	Wild	*Flavobacterium nitrogenifigens*	98
CW2	Wild	*Flavobacterium notoginsengisoli*	97.25
CW3	Wild	*Mangrovibacter yixingensis*	99.28
CW5	Wild	*Chitinimonas taiwanensis*	99.26
CW5	Wild	*Chitinimonas taiwanensis*	96.96
CW7	Wild	*Bacillus weidmannii*	99.5
CW9	Wild	*Pseudomonas peli*	98.48
Hc1	Wild	*Aeromonas veronii*	98.47
Hc1	Wild	*Elizabethkingia anophelis*	99.64
Hc1	Wild	*Tsukamurella ocularis*	99.36
Hc10	Captive	*Acinetobacter johnsonii*	98.45
Hc10	Captive	*Acinetobacter schindleri*	98.2
Hc10	Captive	*Bacillus aerius*	99.93
Hc10	Captive	*Bacillus arybhattai*	99.86
Hc10	Captive	*Chryseobacterium vietnamense*	93.84
Hc10	Captive	*Mycobacteroides chelonae*	99.64
Hc11	Captive	*Acidovorax konjaci*	96.71
Hc11	Captive	*Aeromonas hydrophilia*	99.65
Hc11	Captive	*Aeromonas veronii*	99.79
Hc11	Captive	*Bacillus aerius*	99.72
Hc11	Captive	*Brevundimonas bullata*	99.62
Hc11	Captive	*Comamonas terrigena*	96.72
Hc11	Captive	*Sporosarcina luteola*	98.02
Hc11	Captive	*Stenotrophomonas rhizophilia*	98.94
Hc12	Captive	*Bacillus velezensis*	99.79
Hc12	Captive	*Chromobacterium alkanivorans*	99.86
Hc12	Captive	*Chryseobacterium defluvii*	97.48
Hc12	Captive	*Comamonas odontotermitis*	97.63
Hc12	Captive	*Tsukamurella ocularis*	99.35
Hc13	Captive	*Brevundimonas bullata*	99.63
Hc13	Captive	*Chromobacterium alkanivorans*	99.14
Hc13	Captive	*Sphingomonas kyeonggiensis*	98.38
Hc13	Captive	*Tsukamurella ocularis*	99.35
Hc14	Captive	*Comamonas odontotermitis*	99.29

Hc14	Captive	*Pseudomonas alcaligenes*	98.71
Hc15	Captive	*Acinetobacter johnsonii*	98.73
Hc15	Captive	*Paucibacter oligotrophus*	98.57
Hc15	Captive	*Pseudomonas aeuginosa*	99.57
Hc2	Wild	*Aeromonas hydrophila*	99.29
Hc2	Wild	*Pseudomonas nitritireducens*	98.89
Hc3	Wild	*Aeromonas taiwanensis*	97.64
Hc3	Wild	*Chryseobacterium shigense*	96.2
Hc3	Wild	*Elizabethkingia anophelis*	99.05
Hc3	Wild	*Enterococcus rivorum*	99.59
Hc3	Wild	*Mitsuaria chitosanitabida*	98.25
Hc5	Wild	*Aeromonas caviae*	99.27
Hc5	Wild	*Bacillus velezensis*	99.65
Hc5	Wild	*Brevundimonas bullata*	99.63
Hc5	Wild	*Chryseobacterium sediminis*	99.5
Hc5	Wild	*Staphylococcus epidermidis*	99.65
Hc5	Wild	*Tsukamurella ocularis*	99.19
Hc6	Wild	*Acinetobacter johnsonii*	99.5
Hc6	Wild	*Aeromonas fluvialis*	98.15
Hc6	Wild	*Aeromonas veronii*	99.57
Hc6	Wild	*Bacillus toyonensis*	99.37
Hc7	Wild	*Acinetobacter johnsonii*	98.61
Hc7	Wild	*Aeromonas hydrophilia*	99.57
Hc7	Wild	*Aeromonas taiwanensis*	99.07
Hc7	Wild	*Chryseobacterium shingense*	96.99
Hc7	Wild	*Elizabethkingia anophelis*	99.25
Hc8	Captive	*Acinetobacter johnsonii*	99.13
Hc8	Captive	*Agrobacterium vitis*	98.79
Hc8	Captive	*Agrobacterium vitis*	99.27
Hc8	Captive	*Bacillus aerius*	99.79
Hc8	Captive	*Brevundimonas bullata*	98.91
Hc8	Captive	*Dunganella sacchari*	98.14
Hc8	Captive	*Massilia timonae*	98.79
Hc8	Captive	*Novosphingobium chloroacetimidivorans*	96.95
Hc8	Captive	*Pseudomonas fluvialis*	98.07
Hc8	Captive	*Pseudomonas oryzihabitans*	99.11
Hc8	Captive	*Pseudomonas rhodesiae*	99.49
Hc8	Captive	*Pseudomonas taiwanensis*	99.29
Hc8	Captive	*Pseudomonas viridiflavia*	97.68
Hc8	Captive	*Staphylococcus hominis*	99.3
Hc8 wash	Captive	*Bacillus safensis*	99.58

Hc9	Captive	*Acinetobacter johnsonii*	98.58
Hc9	Captive	*Brevundimonas vesicularis*	98.07
RefWater	Captive	*Acidovorax soli*	98.93
RefWater	Captive	*Acinetobacter lwoffii*	99.57
RefWater	Captive	*Acinetobacter proteolyticus*	97.16
RefWater	Captive	*Brevundimonas vesicularis*	99.4
RefWater	Captive	*Chromobacterium alkanivorans*	98.31
RefWater	Captive	*Mitsuaria chitosanitabida*	98.25
Wood1	Wild	*Comamonas odontotermitis*	99.29
Wood1	Wild	*Psedomonas rhodesiae*	99.36
Wood2	Wild	*Acinetobacter schindleri*	98.66
Wood3	Wild	*Acinetobacter johnsonii*	99.22

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EVALUATING THREE DIFFERENT LONG-TERM TAGGING METHODS IN AQUATIC SALAMANDER SPECIES

INTERIM REPORT

NOVEMBER 18, 2019

2019 EDWARDS AQUIFER REFUGIA PROGRAM RESEARCH

INTERIM REPORT

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BACKGROUND

Marking or tagging salamanders increases efficiencies in refugia operations and species management. Tags allow for data (e.g., collection date, age or estimated age, growth rate, sex, reproduction events, offspring produced, and health) to be tracked for each individual. Beyond refugia captive operations, long-term tags in individuals reintroduced to the wild could facilitate mark-recapture evaluations. For this study we have selected three tagging techniques used in monitoring other salamander species: visible implant elastomer (VIE), visible implant alphanumeric tags (VIA), and passive integrated transponders (PIT) (Davis and Ovaska 2001; Bailey 2004; Heemeyer *et al.* 2007; Osbourn *et al.* 2011; Appleby 2015; Whiteman 2016; Mitchell *et al.* 2017; Lunghi and Veith 2017). Of these, only VIE tagging has been evaluated in San Marcos salamanders (Phillips and Fries 2009); none have been evaluated for use in the other refugia salamanders; and comparison studies among these tags for these aquatic salamander species have not been performed. These tag types were selected for their effectiveness with other salamander species, ability to be quickly and easily identified tank-side, minimal negative effects on organisms, and perceived ease of implanting the tags. Analysis of these three different tagging methods with Texas blind, San Marcos, and Comal Springs salamanders provides valuable information for successful long term marking of individual animals.

OBJECTIVES

Our goalwas to evaluate the utility of three different, long-term tagging methods in Texas blind salamanders, San Marcos salamanders, and Comal Springs salamanders.

1. We tagged 20 salamanders of each of three species with each of the three tag types (20 salamanders x 3 species x 3 tag types = 180 salamanders total).
2. We compared tag retention and readability over a period of eight months.
3. We evaluated the use of each tag type for individual tagging or group tagging purposes.

METHODS

We tested three types of tags: Visible Implant Elastomer (VIE), Visible Implant Alphanumeric (VIA), and Passive Integrated Transponder (PIT) tags on three different species of salamanders held in refugia, including San Marcos salamanders (*Eurycea nana*), Texas blind salamanders (*Eurycea rathbuni*), and Comal Springs salamanders (*Eurycea spp. 8*). In each of the three species of salamanders, we selected 20 individuals to test VIE and VIA tags; 60 individuals across species received each tagging method (120 total for the two tag types).

We tested implanting PIT tags subcutaneously into the upper tail musculature rather than the body cavity of salamanders to prevent internal organ damage and to reduce the risk of the injection site compromising the body cavity. We first placed six PIT tags in the tails of F1 Texas blind salamanders that were 5.6 – 7.1 cm snout-vent length (SVL). After one month of evaluation the salamanders had no infection of insertion site and tolerated the PIT tags (movement and swimming ability were not compromised). We then implanted five PIT tags in F1 Texas blind salamanders that were 4.3 – 5.5 cm SVL. After this evaluation period, we decided to not proceed with injecting smaller individuals of Texas blind salamanders or test Comal Springs salamanders. Five of the 11 Texas blind salamanders shed their tags. Based on the observed skin tearing and rejection of VIA tags in Comal Springs salamanders, we concluded that further attempts with PIT tag injections should not be performed.

Salamanders were anesthetized using a low dosage of tricaine mesylate (MS-222) to reduce handling and tagging stress. Length (cm) and weight (g) were recorded and sex was identified by candling. Salamanders were kept wet with moist paper towels. The selected tag type was then inserted into the salamander.

- VIE tags were inserted via insulin-type needle subcutaneously posterior to the back hip of the salamander.
- VIA tags were implanted using the VIA injector needle subcutaneously posterior to the back hip of the salamander.
- PIT tags were implanted posterior to the back hip of the salamander, subcutaneously into the tail musculature using a tag injector.

After tag insertion (VIE/VIA), each salamander was photographed and then staff placed into a container with flowing water to fully recover from anesthesia (able to right itself, showed response to stimuli, and swam) before it was moved to its refugia tank. Equipment, including needles, injectors, scalpels, and forceps, was disinfected after each salamander was tagged. Half of the tagging was performed by Linda Moon (trained and proficient, but less experienced tagger) and half by Dr. Lindsay Campbell (more experienced tagger) with the tagger noted on the data sheets.

For consistency, Linda Moon checked tag retention and readability monthly and made any additional pertinent notes. Novel readers also read tags and scored them every three months (a new novel reader each time) to assess the utility of the tags to non-tag-experienced keepers. Tag readers scored VIE and VIA based on the following readability index patterned after Osborn *et al.* (2011):

Readability Index:

0: Tag not visible or not present

1: Tag visible but colors not distinguishable (VIE) / Tag visible but only color discernable (VIA)

2: Tag colors visible but incorrectly read (VIE) / Tag colors visible and partial code visible or incorrect code read (VIA)

3: Correct colors or code only read with use of blue LED light and amber filter glasses (VIE/VIA)

4: Correct colors or code visible without aid of amber filter glasses (VIE/VIA)

Every three months a new photograph was taken of the VIE and VIA tagged salamanders to evaluate potential tag movement. At this time, salamander length and weight were also recorded. Length and weight of Texas blind salamanders tagged with PIT tags were also recorded at the same three-month intervals.

PIT tags were evaluated based on whether or not they were retained, if the Radio Frequency Identification (RFID) could be read through air or through water, and the time required to read the RFID. We tested the distance tags could be read through water, both in the

tank and above the water surface. We also tested if the tag-reader could distinguish multiple RFID tagged individuals in a tank; we started with two individuals and added individuals until the tag-reader could not distinguish tags.

We continued to monitor the clarity of the tags in several of the Texas blind salamanders tagged prior to the study. Staff photographed these tags at two points during the year for comparisons, and tags were given a score from the Readability Index.

Current Results and Discussion Points

In general, individually marking salamanders with vertical VIE color combinations resulted in the highest readability and retention scores in all three species of aquatic salamanders verses VIA and PIT tags (Figure 1). Skin texture and thickness of each species affected the retention and readability scores of the three different tagging methods used. We will continue to monitor tags through a full year to more accurately evaluate their long term readability.

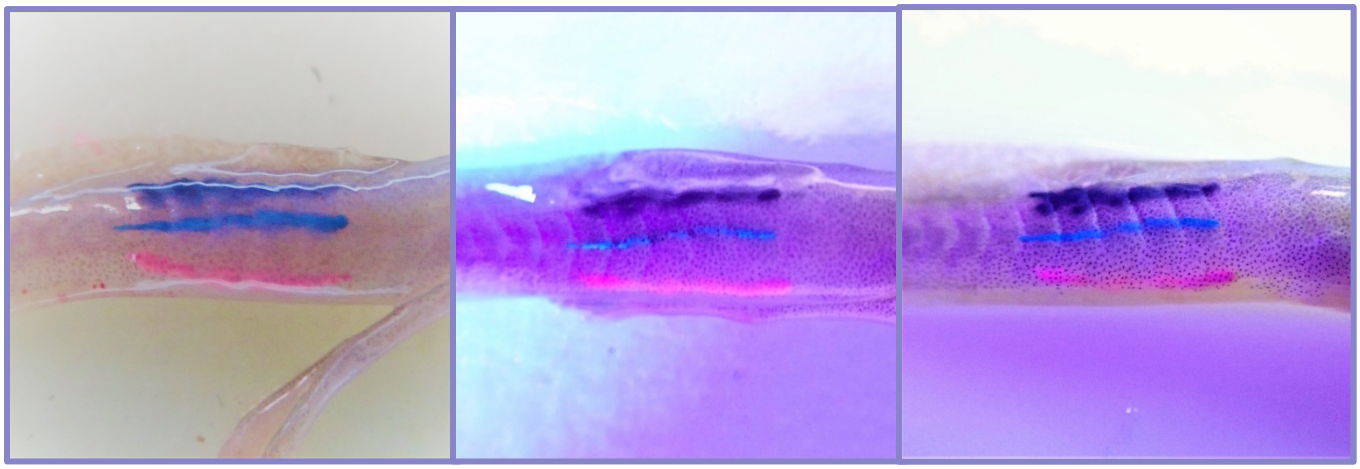

Figure 1 Texas blind salamander (designated "Minnie") with horizontal color combination black, blue, pink. Initial tag (right), 3 month (center), and 6 month photos (left). This time-sequence reveals what an ideal visible implant elastomer tag should look like with no breakage and straight lines.

Injection observations

We noted differences in skin between Texas blind and both the San Marcos and Comal Springs salamanders, especially evident when injecting the VIA tags: San Marcos and Comal Springs salamanders' skin easily tore (Figure 2). Both of these species' skin felt thin and fragile (like tissue paper) when using the VIA injection needle.

Figure 2 Photograph illustrating injection wound after injecting VIA tag in a Comal Springs salamander.

Researchers also found that the VIA tags themselves easily pierced the skin during injections, creating an exit wound in some salamanders. However, Texas blind salamanders have thicker skin that did not tear when the VIA needle was inserted. It was impossible to see or obtain a clear picture of the fresh tags in salamanders with injection sites closed with surgical glue, which formed an opaque layer over the site. The plunger of the flat VIA injection needle tended to stick to the tag or go over the top of the tag instead of pushing it out, forcing us to disassemble the needle before continuing. The alphanumeric code is only on one face of the tag (Figure 3). Thus, we could not adjust direction tag was loaded into the needle. For safety of the

Figure 3 Loading VIA tag into injector needle.

animal all injections were done with the needle facing away from the body cavity. For consistency of tag orientation, we adjusted salamander body orientation to correspond with the handedness of the person tagging; one tagger was left-handed, the other, right-handed (Figures 4 & 5).

Figure 4 Linda Moon inserting a VIA tag into a Texas blind salamander.

Figure 5 Dr. Lindsay Campbell inserting a VIA tag into a Comal Springs salamander.

When a researcher tagged away from the body cavity and the VIA tag was injected on the left hand side of the salamander, the alphanumeric code was right side up; when injected on the right side of the body, the alphanumeric code was upside-down (Figure 6).

When injecting the VIE polymer, researchers observed the polymer spreading into the lateral line and costal grooves, causing misshapen polymer lines or breaks at costal groove indents. Vertical line injection varied from horizontal line injections by adjusting the needle and salamander body position to account for the convex shape of their tails (Figure 7). PIT tags were more difficult to inject in salamanders that had slim tails compared to salamanders with wider tails (Figure 8).

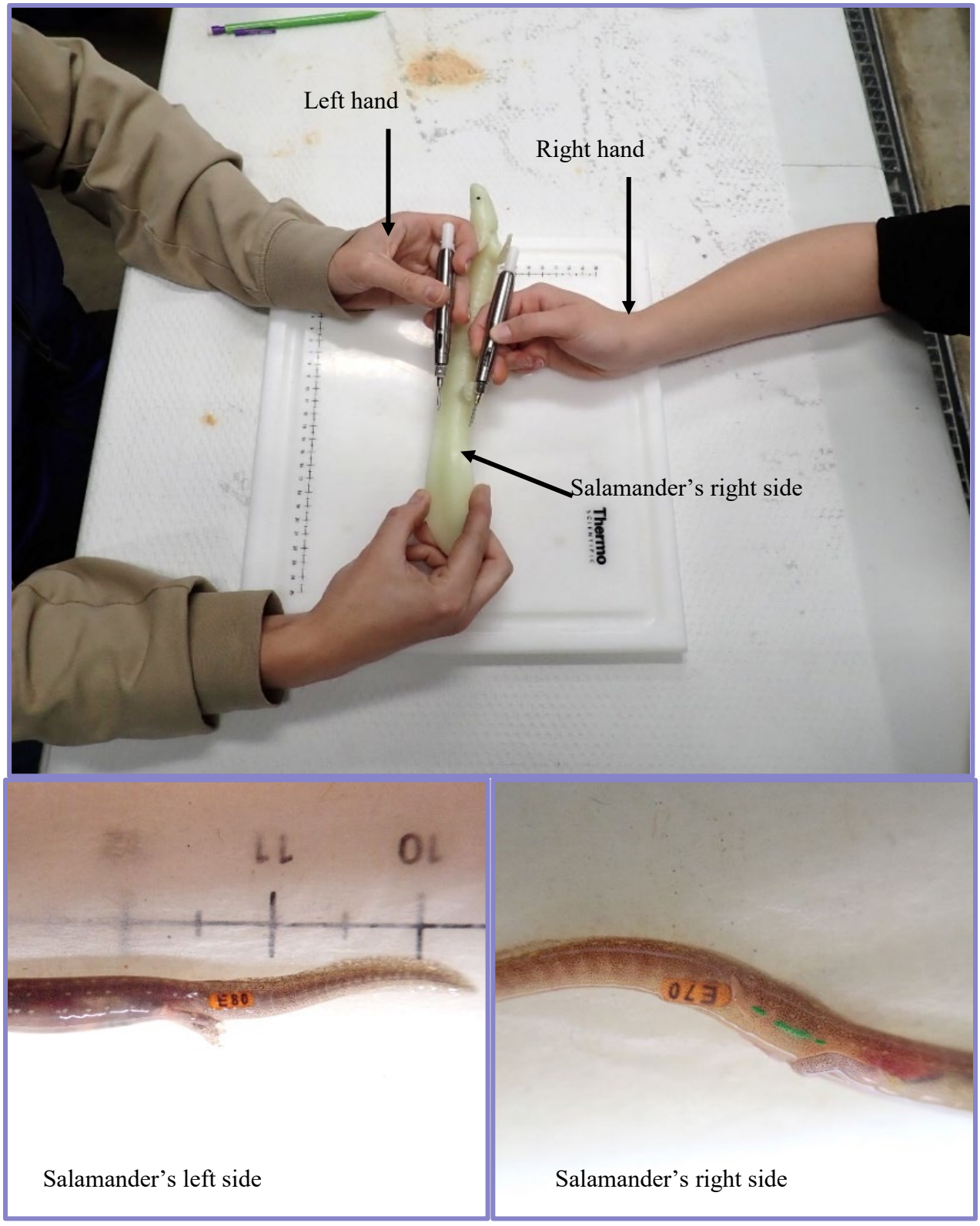

Figure 6 Top photograph illustrates right handed and left handed differences when tagging using a salamander model for demonstration purposes. Researchers wear gloves and have a surgery set up during actual tagging of live organisms. Bottom photographs showcasing the difference in tag orientation depending on which side of the body VIA tag was inserted.

Figure 7 Horizontal injection of triple VIE tags into a Texas blind salamander.

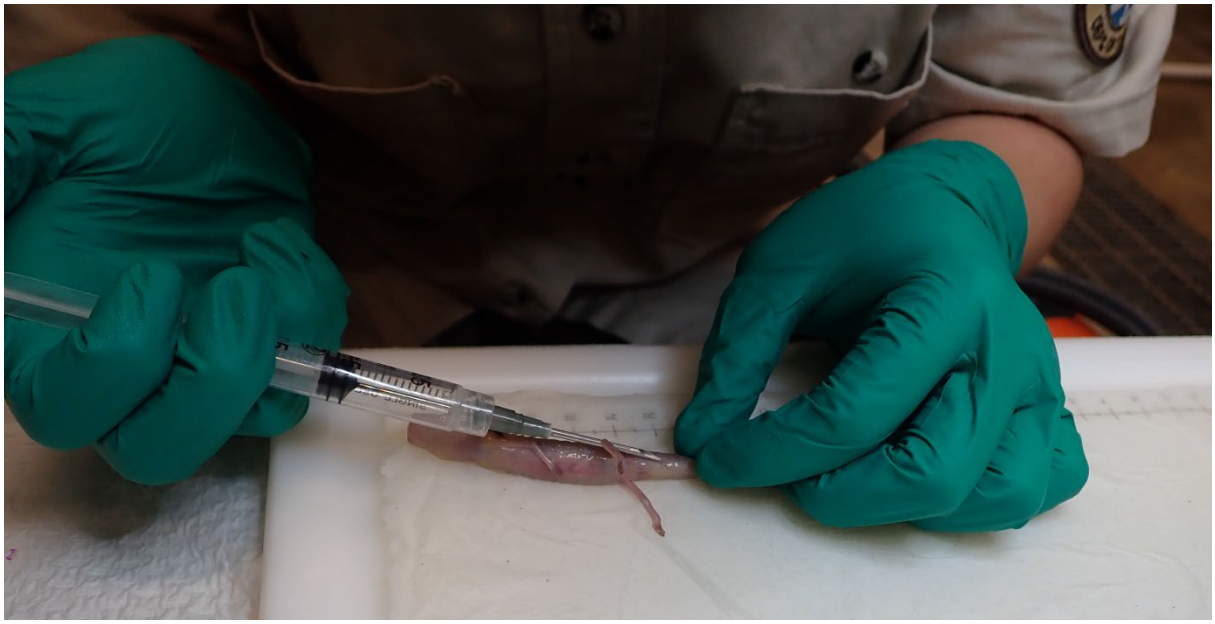

Figure 8 Injection of PIT tag into a Texas blind salamander.

Visible Implant Alpha Tags

Tag retention in both the San Marcos and Comal Springs salamanders was poor, with only one tag retained in a single San Marcos salamander and no tags retained in Comal Springs salamander individuals after two months. We first injected 20 Comal Springs salamanders with VIA tags and did not close the injection site with surgical glue because of concern that their back limb would become stuck in the glue. However, within two weeks, more than half of the salamanders shed their tags that were found on the bottom of the tank. Thus, when injecting the San Marcos salamanders, taggers closed the injection sites with surgical glue, taking care to keep the back leg away from the glue and dabbing off excess glue with a clean Kim wipe. Glue was given time (under 1 min needed) to dry to the touch before the salamander was placed in recovery. Because all but two of the Comal Springs salamanders shed their tags during the first month, we decided to tag a second group of 20 salamanders, this time closing the injection sites with glue. There was no difference in retention between the Comal Springs with non-glued injection sites versus salamanders with glued injection sites (Figure 9): In both Comal Springs salamander tests, all tags were shed by the second month. At the 6 month check point only one VIA tag (coded "E62") was retained by a San Marcos salamander. Thus, lack of retention prevented readability scores for VIA tags in these two species.

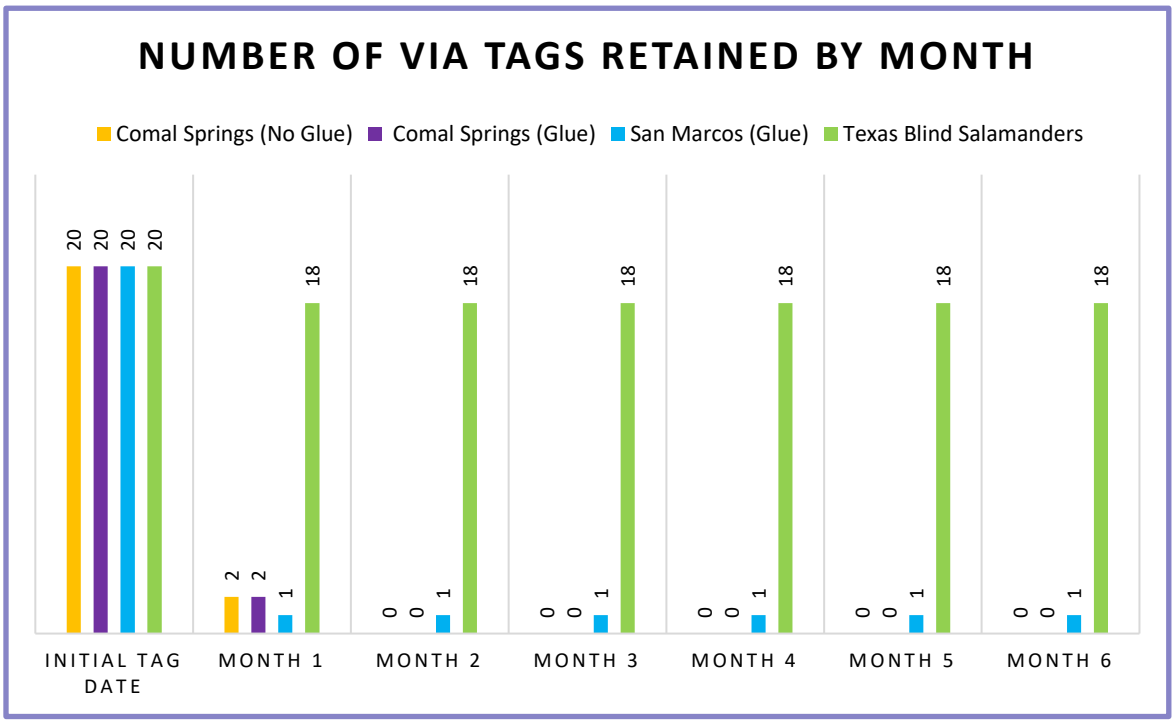

Figure 9 Tag retention numbers of VIA tags by species.

VIA retention in Texas blind salamanders was higher, with 90% of tags retained at the 6 month mark (Figure 9). Overall, average readability over 6 months was 3.275 out of 4 points on the readability index. Readability depends on the depth and angle of tag injection and the presence of melanophores, which can obscure the printed number (Figure 10). All the VIA tags started with the same letter ("E") so this letter was not assessed by the readers. We recommend future studies with VIA tags use multiple fluorescent colors along with codes that begin with varying letters. VIA tags come in different sizes, and\the larger sized VIA tags could be used on larger Texas blind salamanders to increase readability. Difficulties in injection and low retention rates discouraged us from recommending VIA tag use for individually marking San Marcos or Comal Springs salamanders. Individually tagging Texas blind salamanders with VIA tags is possible. However, this is not the best method because we found that readability was heavily dependent on skin clarity, and these tags had lower retention rates compared to VIE.

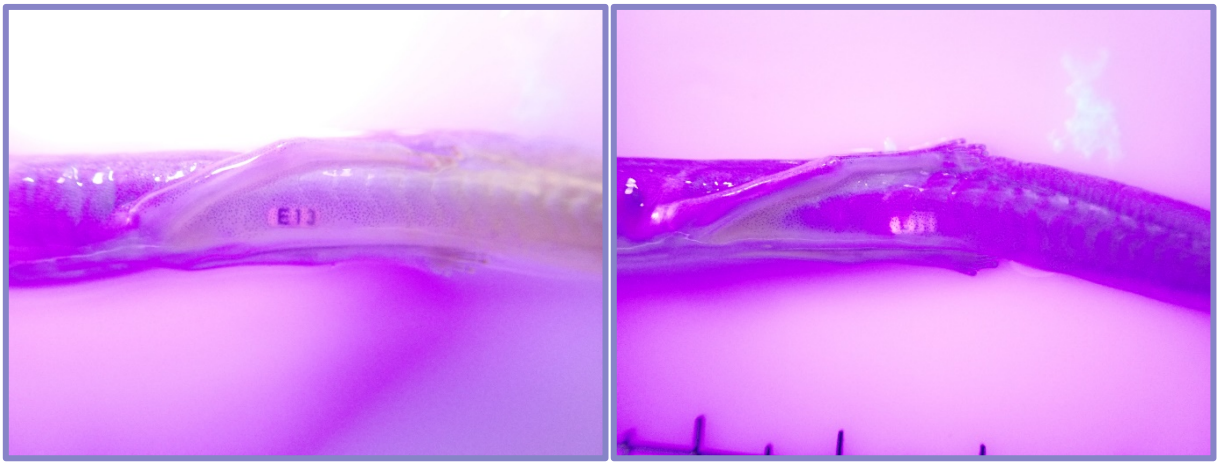

Figure 10 VIA tag "E13" (left) illustrates an ideal VIA tag under salamander skin. "E22" (right) illustrates a poor quality VIA tag, with the code being blurred beyond legibility.

Visible Implant Elastomer

All VIE tags were retained in all three aquatic salamander species; however, we noted several occurrences of breakage and migration of individual tag lines. VIE tag lines were injected vertically for San Marcos and Comal Springs salamander groups due to limited space on their tails for horizontal lines. Two groups of 20 Texas blind salamanders were given VIE tags: 20 sub-adult/adults were given horizontal tags and 29 younger, smaller juveniles were given vertical tags.

San Marcos and Comal Springs salamanders' VIE average readability scores were 3.71 and 3.65, respectively (Figure 11). In both species, position 1 had the least amount of breakage, position 2 was second, and position 3 (i.e., proximal to the head)had the highest amount of line

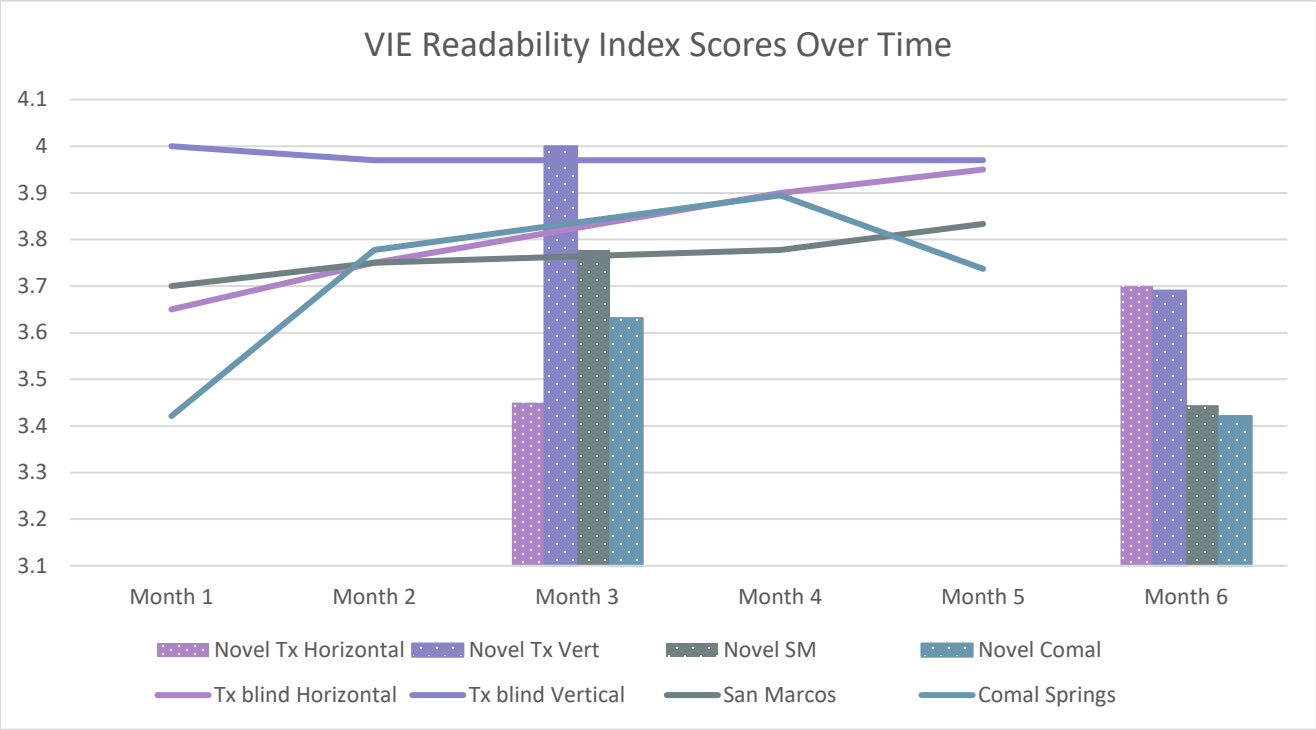

Figure 11 Readability index scores by Linda Moon (lines) and by novel readers (bars) over a six month period. Novel readers only read they tags at the three month and six month period, thus far.

breakage (see Figure 12 for position diagrams). Readability scores for horizontal tags in Texas blind salamanders averaged 3.73 out of 4 points (Figure 11). The average readability score for vertical tag lines in Texas blind salamanders was 3.93 out of 4 points. Horizontal VIE tags had

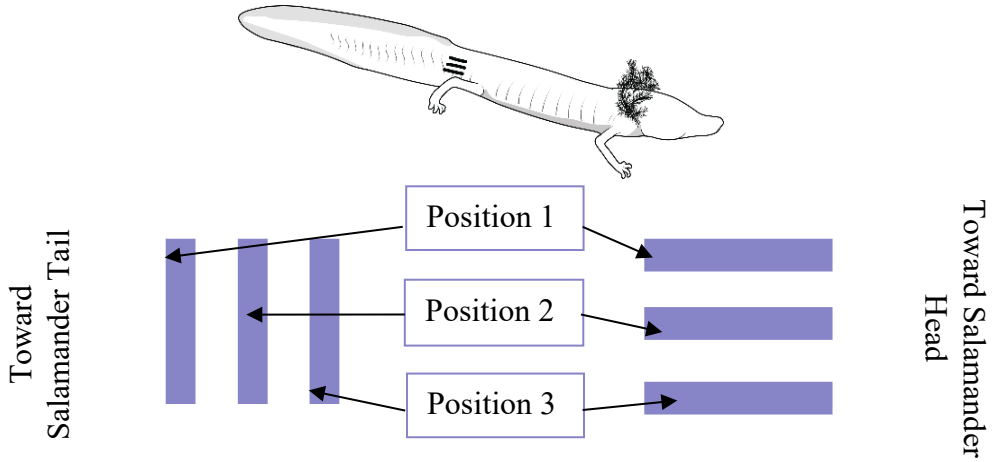

Figure 12 Vertical and horizontal lines depicting the two different visible implant elastomer tag compositions. Tags are read left to right or top to bottom.

higher numbers of breaks from dorsal to ventral, with position 1 being the most dorsal and position 3 being the most ventral. VIE tags tended to break at costal grooves, which was not surprising since pigments were observed separating at initial injection. (Figure 13). Breakage in VIE tags was initially noted at higher rates at the first month check than in later months.

- Vertical tags were more vibrant and easily photographed compared to horizontal tags in Texas blind salamanders. The vertical tags were placed in younger salamanders, which have thinner and less pigmented skin than the older salamanders.
- The lower lines in horizontal tags had a tendency to break apart/ diminish more readily causing readability difficulties.
- Vertical tags tended to retain central portion along lateral line allowing for sustained identification of tag colors.
- We plan to retouch broken tags from the group of Texas blind salamanders tagged prior to this study and take pictures after one and three months.

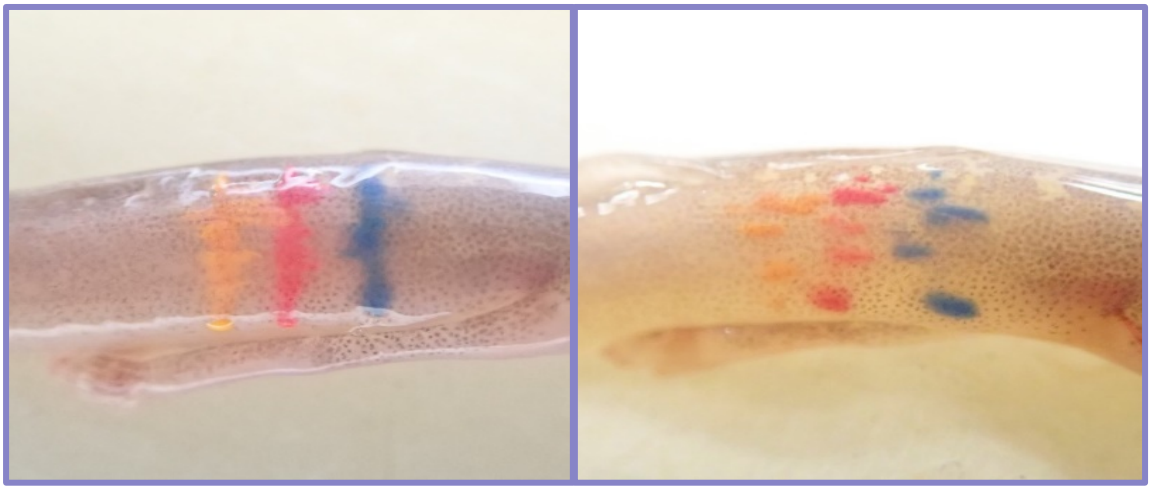

Figure 13 Texas blind salamander VIE color combo orange, red, blue "Ajax" initial tag data photo (right) and 3 month photo (left). Showcasing a striation breakage pattern that is a common observation with visible implant elastomer tags.

Novel Readers (VIA & VIE)

Novel readers were used at the three and six month tag checks, and will be used at the nine and twelve month checks. One novel reader, Amelia Hunter, conducted the 3 month tag

checks. There were three different novel readers, Taylor McCrary, KC Boren, and Dr. Amanda Haverland, for the sixth month tag checks. Average readability score for both VIA and VIE tags tended to be lower in novel readers compared to experienced readers (Figure 7).

Passive Integrated Transponders

Five of the eleven PIT tagged salamanders shed their tag during the first month, no tags were shed after the first month. Overall 55% of PIT tags were retained in the Texas blind salamander group. Salamanders that shed their tags had notable scars that diminished over time. No signs of infection occurred after injection or when tags were shed. In one case, an individual was found to have a PIT tag protruding from the opposite side of the initial injection site during first tag check (Figure 14). To reduce the chances of infection or further complications, the tag was removed by grasping the tag with forceps and pulling it free. The salamander was isolated in a holding tank for a week until the open wound healed and recovered without further complications.

Figure 14 Texas blind salamander with tag protruding from skin (left) and residual wound after tag was removed (right).

PIT tags were easily read using a Biomark reader. We were able to read all tags during monthly checks in under 15 seconds by waving the wand over the animal. Most PIT tags are not notable to the naked eye, but can been seen with close inspection (Figure 15).

Figure 15 Passive integrated transponder 6 month after initial injection in a Texas blind salamander. A light injection scar can be seen to the left of the PIT tag.

In order to test whether the Biomark reader could read the PIT tags above the water surface, a salamander was placed in a tank with water depth starting at 25 cm. The reading wand was held parallel to the water surface, just above the water. If the wand could not read the tag, we lowered the water level in 5 cm increments, and tried reading again until the tag was recognized. We found that a tag could be read at a water depth of 10 cm. We then slowly moved the wand further from the surface of the water until the tag could not be read. The wand could not read a tag farther than 2 cm above the water surface. When the wand was submerged in the tank it could read a tag when it was within 10 cm of the salamander.

We also tested how many tags the wand could accurately read at one time. One salamander was placed in a 20 gallon tank with a water depth of 10 cm. We then added tagged salamanders until the reader could not distinguish individual tags. The reader could not reliably read more than two tags at any given time. When moving the wand through the water in a larger tank, if more than two salamanders were close together, the wand only read two tags at any given time.

PIT tags were not injected on any of the smaller Texas blind salamanders and are not recommended for San Marcos or Comal springs salamanders due to their small size. Should a smaller PIT tag be developed in the future, additional studies are warranted. Potentially PIT tags could be used with submerged detection array in caves/wells for movement or population analysis studies; however, low retention rates and concerns about injection site infection in the wild should be considered.

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SALAMANDER REPRODUCTIVE DYSFUNCTION

INTERIM REPORT

NOVEMBER 18, 2019

2019 EDWARDS AQUIFER REFUGIA PROGRAM RESEARCH

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SUMMARY/ABSTRACT

San Marcos salamanders (*Eurycea nana*) are a federally threatened aquatic species endemic to San Marcos, TX and to the Edwards Aquifer. While this species has been held on station for years as part of a captive assurance colony, we cannot predictably produce offspring. We continue to investigate potential causes for this perceived disrupted reproductive behavior in captivity with three lines of investigation. Based on recommendations from the 2018 reproduction study, we conducted a pilot study in 2019 using only group mating and removal of males after 72 hours. Aside from this, we filmed one pair and one group from three angles to capture spermatophore deposition on video. Overall, engagement in courtship for this trial was low with animals engaging in more exploration than mating. Females were more engaged than males and courted without male participation. When males were involved, they walked the tank for over 45 minutes (an unusually long time) without deposition. This lack of courtship overall produced no ovipositions. Additionally, we tested our water quality for endocrine disrupting compounds and other deleterious compounds. For the third line of investigation, we sacrificed female individuals from wild and captive populations for toxology and histopathology to assess potential reproduction inhibitors, such as vitamin deficiencies, heavy metals, toxins, and/or disease. Initial findings show increasing levels of barium in captive individuals. Micropsoridial infection rates were much lower and less acute in wild populations. Micropsoridial infections tended to be concentrated in the ovaries and reproductive organs of the salamanders. Further studies are needed to investigate, or remedy problems discovered with these findings, and to improve the health of captive populations. Initial consultation with a salamander reproductive specialist, Dr. Ruth Marcec-Greaves, suggested hormonal studies and altered reproduction trials for the future.

OBJECTIVES FOR 2019

The main goal for San Marcos salamander research in 2019 was to further investigate reproduction dysfunction of San Marcos salamanders in captivity using information learned from our previous study of courtship behavior, combined with exploring additional avenues of physiology and water quality. Our goals are further defined below.

1) Conduct a modified reproduction pilot study using information learned from 2018's study.

2) Compare gravid San Marcos salamander females from the wild to captive populations via histopathology and toxicology tests conducted by Dr. Allan Pessier at Washington Disease and Diagnostic Laboratory.

3) Test water quality in salamander systems for potentially deleterious compounds that may lead to reproductive dysfunctions.

METHODS

Pilot Reproduction Trial

Based on behavioral observations of filmed courtship behavior in our 2018 reproduction trail, we determined that female salamanders in group tanks engaged and participated in courtship behavior at a significantly faster rate than in paired tanks, and that one third of female salamanders in paired tanks did not engage in courtship behavior at all. Thus, we used group tanks of four females and four males for this pilot study. The number of salamanders used in group tanks is based on a 1:1 sex ratio with the density of salamanders at or below an average of one salamander per gallon (20 gallon tank only half filled with water volume). This density has been recommended by USFWS biologists for this species. Review of the videoed courtship period in 2018 revealed that numerous instances of tail-straddle-walk occurred during the first 48 hours after the sexes were combined in experimental tanks. However, over time, females became less engaged in courtship behavior despite repeated attempts by males. As these repeated non-reciprocated advances could be a stressor on both females and males, we removed males from the tanks after 72 hours. Females were then either left in group tanks or separated into individual tanks while we monitored them for oviposition.

Phase I Total Isolation

Only sexually mature (having enlarged and apparent testes or ova) San Marcos salamanders collected in late 2017 to 2018 were selected for this study and moved to the new quarantine room in early February 2019. The individuals selected were of younger, healthier stock and had been housed together in mixed sex groups in the Research Pad for approximately 1 year before the trial. This group produced no eggs in their shared housing prior to their selection for the 2019 trial and, especially when compared to historic older populations of San Marcos, this group experienced much greater survival. None of the older salamanders used in the 2018 reproduction study were used in this trial.

Before the experiment began, we sexed each animal via candling and marked salamanders with a small Visible Implant Elastomer (VIE) tag on the back hip (Red = female, green = male) that had not already been marked; this mark gives researchers a quick way to identify the sex of the individual in the future with minimal to no handling. Salamanders were anesthetized with MS-222 prior to handling to reduce stress during sex identification and tagging and needles were disinfected between animals. Males and females were placed in separate recovery aquaria with high rates of flow-through until they recovered from anesthesia. After recovery, males and females (n = 45 of each sex, excess of what would be needed for trial, n = 24 of each sex, minimum) were housed by sex in isolated raceway systems that did not share water. Salamanders were monitored for any negative effects after tagging, such as infection of the tagging site. Excess salamanders were also stocked in these tanks to account for any mortalities that might occur before salamanders are introduced into group tanks for mating and so that only gravid females with visible eggs would be used in the experiment. Female salamanders are able to store sperm internally; though the duration of which the sperm is viable is unknown. Thus, females and males must be separated to encourage mated females to deposit

eggs. Additionally, separation of the sexes may facilitate heightened engagement in courtship and mating once they are reunited.

While in isolated raceways, animals were separated into three sections (15 salamanders per section) with ample rocks and artificial vegetation. The systems were set as flow-through, chilled-well water systems, with an ultra-violet (UV) sterilizer installed on the incoming chilled well-water line to the Quarantine building. During a routine inventory of the salamanders, we noted that individuals had been climbing over section dividers. To remedy this, we constructed "corrals" to contain salamanders in their respective sections consisting of 3 in PVC pipe cut into lengthwise sections and secured to the tops of the section dividers (Figure 1). If the salamanders climbed up the dividers, the curve would carry them back to their section. Movement between sections after the addition of these containment units was not observed.

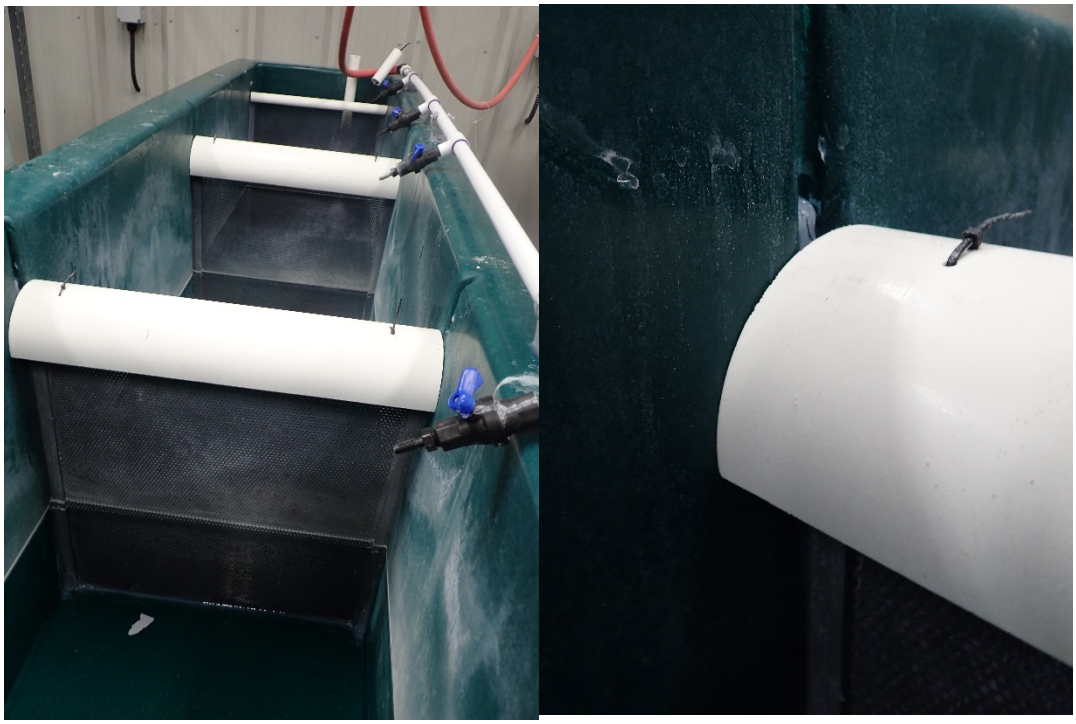

Figure 1 Corrals placed on top of section dividers to keep salamanders from moving between sections.

Phase II Partial Combination

Several factors delayed the progression of the trial; and salamanders remained separated by sex for longer than the planned 30 days. As the refugia program moved organisms into the new buildings, staff time was diverted to setting up new systems. Preparations for the April grand opening of the Refugia building further delayed progression of the experiment. At the same time, March and April presented the facility with a record number of juvenile Texas blind salamanders collected from the net at Diversion Springs in Spring Lake, San Marcos, Texas. Collecting, caring for, and housing this unusual influx of animals presented a challenge for the staff. After April's grand opening of the EA Refugia Building, and once we began collecting less

juvenile salamanders, the SMARC staff needed to prepare for potential salvage operations for San Marcos salamanders and fountain darters. The Spring Lake Dam renovation project was approved and quickly started. This necessity spurred the experiment forward into its second phase, as the tanks originally selected for use in the trial were needed for salvaged animals. While we have tanks set up in our quarantine area at all times for salvage preparedness, at time we utilize some, not all, for experiments; however, in the event of salvage these organisms can quickly be moved back to refugia tanks to free up the additional tanks for salvage.

Salamanders in the middle section of the female-only raceway were switched with the occupants of the middle section of the male-only raceway. The sexes remained segregated by perforated screens, which allow water and pheromone cues to pass through and animals to see each other. We originally planned for these systems to be on partial recirculation of water during this phase of the experiment to distribute any pheromones produced by the sexes throughout the system. However, with incoming animals quickly needing the space, we used flow-through only in raceways for this partial combination phase, instead of plumbing in recirculation lines that would have to be removed for quarantined organisms. We placed small submersible pumps in separated back sections of the tanks to provide water flow that would mix pheromones throughout the tank. During the day, when the water quality of the tanks could be monitored, incoming flow was turned off to the tanks to reduce pheromones from quickly washing out of the tanks.

Infrared cameras recorded female-only sections to monitor for potential indicators of receptivity, including female-on-female courtship. The video of female San Marcos during the partial combination phase was examined daily, but female-on-female courtship was not observed. We combined the sexes after two-weeks following the protocol of the 2018 trial, moving into the third phase of the experiment.

Phase III Combination

We expected that regular staff activity coupled with the anticipated movement of tanks and equipment and increased staff presence in the Quarantine area due to salvage activities would create suboptimal conditions for this sensitive and easily disturbed species. Therefore, for Phase III, we set up aquaria for the mixed-sex groups in the Refugia space. Aquaria for Phase III combination of the sexes was also flow-through, and a special shelving, drainage, and water system were constructed to house the experimental animals.

On June 4, 2019, 24 males and 24 females were relocated from quarantine raceways into six, 20 gallon glass tanks of four males and four females each in the refugia room. Only gravid females with visible eggs were selected. Aquaria were painted with texturized epoxy to allow for increased grip, while walking or courting, and to prevent animals from seeing into neighboring tanks. In our 2018 experiment, habitat was minimal to capture the majority of courtship on camera. For this study, the experimental tanks had ample amounts of varied habitat added, including large and small rocks as well as plastic plants to encourage exploration and increase cover for animals. Habitat items and placement were standardized across tanks. We set up

cameras to record the groups to confirm that the salamanders were engaging in courtship.

Males were removed from experimental tanks after 72 hours, on June 7, 2019, and moved to other refugia systems. Concurrent with the removal of males, all females from three of the group tanks (12 total) were moved to individual 2 gallon, round tanks to assess differences in egg deposition between groups and individuals. As with group tanks, circular plastic aquaria were painted with textured epoxy. More habitat items were added to all aquaria following the removal of males to increase potential oviposition sites. Females were kept in experimental aquaria until October 1, 2019. After we concluded the experiment, animals were relocated to general mixed-sex standing stock refugia tanks.

Two additional tanks, not included in the pilot reproduction experiment were set up to capture spermatophore deposition and transfer on video, making use of the excess salamanders prepared for the experiment. One pair and one group of the gravid females and males were selected for video purposes. A stand was used to hold two, 20 gallon aquaria and was covered with shade cloth to reduce disturbance from staff movement in the room. Black construction paper was taped to the shared side between tanks to prevent animals from seeing into the neighboring tank. During the 72 hour period of video collection, lights were dimmed in quarantine and measures were taken to reduce disturbance and stress on the animals. Unlike those used for the courtship experiment, these tanks were not painted with texturized epoxy to allow a camera view from underneath the tank. Three palm-sized rocks were placed centrally in the tanks for animals to court around or under. Tanks were videoed from two sides and below using infrared cameras.

Comparisons between wild and refugia salamanders

Live, gravid female San Marcos salamanders were sent on July 2, 2019 to Dr. Allan Pessier at the Washington Animal Disease Diagnostic Laboratory (WADDL) for health testing. Fifteen wild females and twenty total females from two different groups of captive stock were selected for analysis. Wild females were collected below Spring Lake Dam and held on station in Quarantine for 2-6 days. Captive subsets (ten each) included animals from the historic population used in our 2018 reproduction trial (which were older individuals) and a younger group of salamanders collected in late 2018 to 2019. The historic population were present on station for more than two years and experienced higher rates of mortality. We isolated this population from younger, more recently collected animals, which experience less mortality.

Whole body toxicological and histological analysis, as well as pathogen and virus testing, were conducted on wild and captive individuals. Dr. Pessier specifically looked for microsporidia and mycobacteria, and for abnormalities of the reproductive tract, such as inflammation. Trace mineral panels were assayed to provide levels of heavy metals, iron, zinc and copper. Another 30 of our preserved, deceased San Marcos salamanders were also sent to Dr. Pessier for examination and necropsies. Live amphipods, blackworms, and samples of VIE were also sent analyzed for heavy metal contents.

Water contamination testing

Four Amplified Geochemical Imaging (AGI) Universal Passive Samplers (GORE-SOBER modules) were placed by Dr. Campbell on July 16, 2019 and retrieved by Dr. Campbell on July 17, 2019. We tested incoming chilled well-water in the Quarantine building (after water goes through a UV sterilizer), water flowing out of the tank housing the heritage population of San Marcos salamanders in Quarantine, chilled well-water coming out of taps in Refugia, and non-chilled well-water coming out of taps in Refugia. Dr. Campbell packaged the samples as directed and shipped them to AGI for analytical analysis using the concentration method of PAHs, PCBs, and pesticides. AGI returned their results on August 8, 2019.

We purchased two Method 1694 water analyses from ALS Environmental, which included analysis for steroids and endocrine disrupting compounds. ALS shipped collection bottles to SMARC, where Dr. Campbell collected water from the chilled and non-chilled well water lines in the Refugia building on August 5, 2019, then packaged and shipped the samples as directed to ALS. We received a report from ALS on August 30, 2019.

RESULTS AND DISCUSSION

Pilot Reproduction Trial

We recorded female only groups during the partial separation phase to observe for female on female courtship. In general, when animals were moved from their known habitat to an unknown or different one, activity increased. This activity was characterized as explorative. Explorative behavior decreased after the first two days. Female-on-female courtship following exposure to shared water and male pheromones was not observed. It is possible that activity occurred undercover, as raceways were filled approximately 50% with varied habitat items. Ultimately not having recirculating water, may have impaired the experiment, as partial recirculation facilitates the spread of sexual pheromones in the aquaria, thus potentially exciting and encouraging more courtship and mating.

After observing infrequent and incomplete courtship in our videoed group the first night, we also recorded the experimental groups to monitor for courtship. Initially, we did not video the experimental group tanks to minimize disturbance. However, we became concerned after watching the video of the other group tank. Two cameras were placed above the group tanks. Cameras views were wide, as we were looking for general courtship behaviors—not detailed information. As with our videoed group, little courtship activity relative to the activity level of 2018 was observed from hours 18-72 before males were removed.

In the group tanks videoed for spermatophore deposition, males were not generally engaged in courtship; however, almost all females participated in tail-straddle walk. Animals engaged in multiple-animal walks, with one male leading several females. Females even redirected others from the line and courted each other. One male in the group actively engaged in courting, leading tail-straddle walking for over 45 minutes without stopping.

Deposition of a spermatophore was not observed from any angle in the filmed tanks. General behavior expected with depositing a spermatophore was also not observed in San Marcos for this trial. Females also did not exhibit typical behaviors associated with picking up a spermatophore packet. It is unlikely that the process is quick in this species. In related species, Barton Springs salamanders and Texas blind salamanders, deposition has been filmed to take 5-7 minutes. In these species, female pick-up generally takes the same amount of time. While the spermatophore is very small and may be difficult to see on camera, videoing from multiple

angles should have revealed the behavior in the salamanders and provide some visual evidence of spermatophore deposition if it occurred.

Females in single-animal tanks experienced greater mortality than those in group tanks with 8 of 12 versus 11 of 12 surviving until the experiment was ceased on October 1, 2019. The reason for the heightened mortality is unknown. Escape behavior was noted in 2 of 12 individual tanks, while no escapes occurred in group tanks. No clutches were deposited by females in either group or individual tank during the 116 days between June 7 and the trial's end.

Dr. Campbell consulted Dr. Ruth Marcec-Greaves, an amphibian reproduction specialist and director of the Detroit Zoo's National Amphibian Conservation Center. An initial conference call allowed discussion of steps we have taken and her perspective on what to investigate next. She is a colleague of Dr. Pessier's and has worked with him on similar cases in the past. We shared the lab results with her too. Dr. Marcec-Greaves tentatively recommends small trials of hormonal exposure to our salamanders. We are planning on inviting Dr. Marcec-Greaves to do an on-site evaluation and consultation in early 2020. Communication and planning will continue between the parties before her visit.

Comparisons between wild and refugia salamanders

Dr. Pessier relayed the initial results of histology on two of the captive population San Marcos salamanders, where he found ovarian inflammation with microsporidia. Further histology results are forthcoming. In one case, the inflammation extended to the adjacent body wall, providing a potential example for external ruptures. Molecular diagnostics of mycobacterium isolated from one of the salamanders positively identified it via sequencing as *Mycobacterium marinum*. Toxicological reports of trace mineral screens showed elevated levels of barium in Refugia salamanders compared to wild salamanders (approximately 3 ppm) and barium levels tended to increase with salamanders that were heldlonger in captivity (shorter term captives averaged about 9 ppm, heritage salamanders averaged about 22.5 ppm) (Table 1). The Refugia salamanders had 32% higher total body copper and 28% lower total body selenium than wild salamanders. As there are no reference ranges established for this species, Dr. Pessier is currently running further tests to determine the significance of these data. The vitamin panel analysis is currently on hold while we decide if it would be more productive to use those samples to gather more data on barium/trace minerals and microsporidia levels in the populations. Soluble forms of barium can be toxic especially to muscle tissue because of its effects on potassium ion channels. The Environmental Protection Agency has set the maximum contaminant level for barium ions in water at 2.0 ppm. However, the most common form is barium sulfate (occurring in the mineral form barite), which is insoluble in water. Barium sulfate is a typical component of oil and gas extraction fluids and paint, and is used as a plastics filler. Barium sulfate is not considered toxic to humans, but toxicity has not been assessed in amphibians. Our initial report did not distinguish the type of barium detected. If the detected barium is in a soluble form, this might explain the severe rhabdomyolysis Dr. Pessier has seen in microsporidia cases and at least one salamander (from previous submissions) that did not have microsporidia. He thought the muscle degeneration was more than what microsporidia could account for. The two could work in concert with one another to magnify the effects of microsporidia eating away the tissues. Studies in rats show reduced spermatogenesis in those exposed to high levels of barium gas.

Trace Mineral Results of San Marcos salamanders											
	Wild Salamander			Heritage Refugia				Younger Refugia			
	W1	W4	W5	C4	C5	C6	CX	C1	C2	C3	C15
	Results (ug/g)			Results (ug/g)				Results (ug/g)			
Calcium	8600	9800	8300	11000	10000	8800	8800	8700	11000	4600	10000
Phosphorus	7700	8000	7200	7300	7600	7200	7000	6900	7700	9200	7100
Chromium	0.06	0.052	0.073	0.057	0.054	0.052	0.052	0.051	0.055	0.046	0.21
Manganese	4.5	8.9	6.3	7.3	3.1	6.6	3.3	3.5	8	7.4	3.2
Iron	16	25	17	25	27	26	26	40	32	31	27
Cobalt	<0.03	0.038	<0.03	<0.03	0.027	0.022	0.022	0.044	0.051	0.046	<0.04
Copper	0.73	0.72	0.66	1.5	1.3	1.1	1	0.72	0.64	0.59	1.4
Zinc	25	29	26	34	30	29	29	30	23	41	28
Arsenic	0.031	0.035	0.032	<0.03	<0.02	0.024	0.02	0.035	0.041	<0.03	<0.04
Selenium	0.96	1.4	1.1	0.55	0.56	0.69	0.47	0.7	1	0.95	0.56
Molybdenum	<0.03	<0.03	<0.03	<0.33	<0.02	<0.02	<0.02	<0.03	<0.03	<0.03	<0.04
Cadmium	<0.03	<0.03	<0.03	<0.33	<0.02	<0.02	<0.02	<0.03	<0.03	<0.03	<0.04
Barium	2.2	4.8	2.1	23	25	17	25	18	4.9	4.6	9.1
Lead	<0.03	0.2	0.035	0.05	0.045	0.037	0.036	<0.03	0.036	0.068	<0.04

Table 1 Trace mineral results from whole body analysis of representative salamanders from three groups, wild, Heritage salamanders, and younger/held in captivity for less time.

After receiving the unexpected report of elevated barium levels in Refugia salamanders, we endeavored to find potential sources. We sent samples of the two main food items, amphipods and black worms, plus a sample of the VIE tag to Dr. Pessier for analysis. The elastomer results showed very low levels of barium, but all captive salamanders were tagged with the same amount of VIE, thus, if this were the source all the levels would be the same. The blackworms proved to be a rich source of barium. Our next step is to determine the forms of barium in the worms and if the barium molecules transform as they go through the worms and salamanders. We are also sourcing alternative foods for all our species, and plan for follow up testing to see if the removal of this barium source reduced levels in salamanders over time. Little is known about the pathways that would be used to eliminate barium from their systems, but based on its properties, Dr. Pessier suspects that levels will begin to fall if the constant source is removed.

We contacted the manufacturer of our tanks to see if the paint and gel coat contained barium. Barite (barium sulfate) is used in some paints. The manufacturer reported that the paint company ceased using barite in their paints over a decade ago.

The Edwards Aquifer Authority provided us with observed levels of barium in aquifer water from several locations, including samples taken from the two wells from which SMARC pumps water. All levels are similar to those of the natural habitat of the salamanders, measured in µg/L (parts per billion), suggesting that the well water was not a significant source of barium (Figure 2).

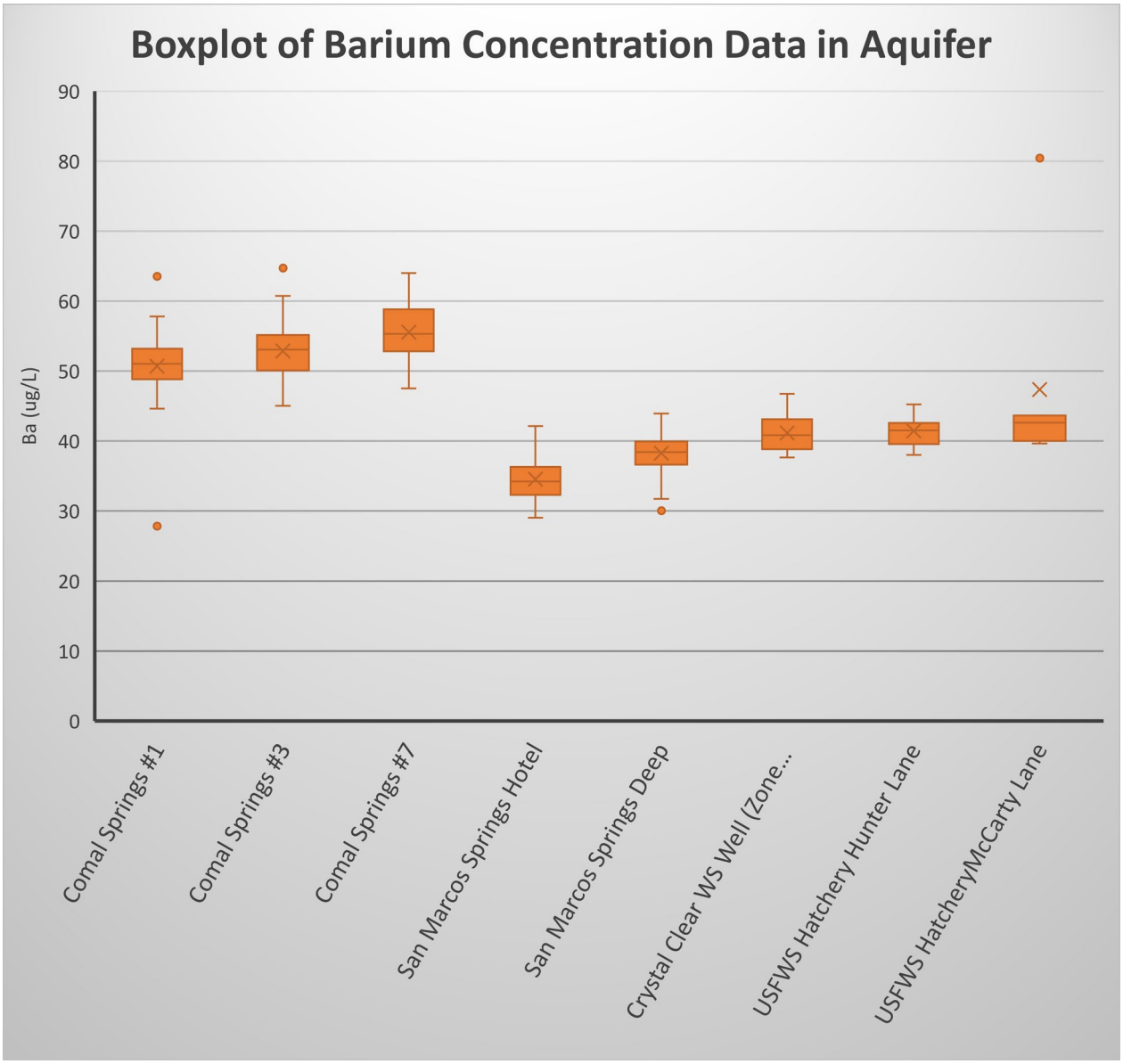

Figure 2 Boxplot of barium levels in aquifer water sampled at several locations an across the years. Data provided by Edwards Aquifer Authority. (Note: µg/L = parts per billion)

Water contamination testing

Levels of contaminates in the water tested in the Refugia Buildings did not yield any immediate levels of concern. Levels were similar to older analysis of water at our wells. See attached PDFs for values.

CONCLUSIONS AND FUTURE PLANS

- We will continue to investigate potential causes into the perceived reproductive dysfunction of San Marcos salamanders.
- This report is the first to establish trace mineral and toxicity levels for this species in the wild and can be used as a comparison in all future studies.
- We will bring in an outside expert in amphibian reproduction to evaluate our husbandry practices and salamander behavior.
- We will conduct pilot studies using amphibian derived hormones added to the water to encourage reproduction.
- We will investigate procedures to reduce mirosporidial spores in tanks. Microsporidial spores vary by species in their encapsulation and vulnerability to disinfectants and UV treatment.
- We will change food sources and then re-evaluate captive salamanders for barium levels after appropriate time has passed (based on expert opinion) to see if barium levels have decreased.

Comal Spring riffle beetle (*Heterelmis* comalensis) pupation enhancement interim report

Project Goals and Objectives

The goal of this project is to better understand the conditions that lead to successful pupation of the riffle beetle *Heterelmis comalensis*.

Study objectives:

a. Reexamine the utility of flow-through tubes specifically for enhancing successful metamorphosis.
b. Determine if starvation may encourage pupation after a period of being well fed.
c. Test the effectiveness of exposing mature larvae to terrestrial conditions.
d. Construct and test the efficacy of an apparatus that emulates bubble stream conditions seen at Comal Springs.
e. Assess beetle fitness resulting from different pupation methods.
f. Implement adaptive management considerations to enhance outcomes.

Methods

a. Flow-through tubes

Flow-through tubes were constructed out of 2" PVC pipe to accommodate 20 final instar larvae. The habitable space of the chamber, between the screens where resource/habitat materials and larvae would occur, had a volume of 525 mL (**Fig. 1**). Flow-through tubes were packed with various materials

Fig. 1. Example of a flow-through tube with resource/habitat packing materials.

for separate treatments, including rock, plastic mesh, and/or conditioned leaf, wood, and cotton. Plastic mesh was either placed around the circumference of the tube (termed "mesh outside") or fitted as an internal tube (termed "mid roll up"). Each treatment was based on the material and its packed position within the chamber. Each treatment was split into an upright vs. horizontal positioning of the flow-through tube. Upright flow-through tubes were suspended vertically with water intake flowing from the bottom to the top of the tube; pre-testing of this configuration was found to fill most of the chamber with water. The horizontal flow-through tubes were placed horizontally on top of a brick, allowing for water to flow laterally; pre-testing of this configuration was found to fill ca. 50-70% of the chamber, presumably leaving an upper layer of air within the chamber. Trials were initiated each month for a single treatment, run for one upright and one horizontal position in tandem with the same cohort of larvae. Larvae were retrieved from SMARC-F_1 stocks given as cohorts "Large", "Small", and "Mix" which

was a combination of “Large” and “Small” cohorts. Later in the year, additional larvae from the Uvalde-F_1 stocks (“Uvalde”) were utilized for trials (still running and not presented in this report). Twenty late instar larvae of the same cohort were placed into each flow-through tube tandem, respectively, to accommodate a trail. This design was implemented to allow for analysis via paired t-test within or among treatments, or for a split plot design once a suitable number of trials was performed for each treatment. However, for this report, each independent flow-through tube is considered a single group for tracking purposes. Other factors that were recorded included discharge (Q) and degree days (dd). Q was measured at the initiation, retrieval, and intervals in between trial runs. Dd were calculated by cumulating the average daily temperatures from loggers programed to capture temperature at 60 or 90 min intervals over the trial period for each flow-through tube, respectively.

Trials were run for 77 – 98 days in an attempt to approximate 2572 dd. Flow-through tubes remained relative untouched during the trial run. Upon retrieval, the contents of a flow-through tube were carefully inspected for adults, pupae, larvae, exuvia, and corpses. The total number of adults plus pupae (dead or alive) was considered a successful pupation event. Treatments that showed > 25% pupation success for the first trial were relaunched for a second and third trial (still running or not initiated). Remaining larvae were placed into starvation or terrestrial treatments (see below), or placed back into the flow-through tube or a smaller flow-through tube with replenished food supplies of the same type as their respective treatment (relaunch). Individuals that were relaunched were inspected ca. once a month for pupation events. Adults were sexed and photographed, then paired up for mating trials (see Assessment of beetle fitness below).

b. Starvation

A subset of larvae remaining in the flow-through treatments were transferred to a rearing chamber without food; the chambers were flow-through tubes consisting of plastic mesh and/or plastic dowels. Chambers were checked ca. once a month.

c. Terrestrial habitat

A subset of larvae remaining in the flow-through treatments were transferred to a rearing chamber that had humid conditions. Chambers consisted of a plastic sandwich box with a lid that had water flowing along the bottom so that humid conditions would be maintained. Large rocks and conditioned leaves were placed in the box and larvae were placed on top of these materials. Chambers were checked ca. once a month.

d. Tiny bubbles

An Artesian Spring Emulator (ASE) was constructed out of 2” PVC pipe and set up to take advantage of the degassing well water, suspected to emulate the environment this species inhabits. Because the well water naturally degasses as it comes out of pressure, it was decided that a simpler design was more favorable than constructing something that would create bubbles (advised by R. Gibson).

Twenty-five larvae in late stages of development were placed into the ASE rearing chamber. Trial 1 was run for 80 days from January to April 2019. Trial 2 was initiated in April and run until July for 87 days. Trials were inspected several times a week to make note of any conditional issues. Larvae that were found during inspection at the end of the trials were randomly placed into a modified post-treatment trial. At the time of the ASE2 retrieval, a different strategy for rearing pupae was implemented; the modified or “mod” rearing chamber was designed (see Adaptive management in Results and Discussion section).

e. Assessment of beetle fitness

F_1 generation reared adults have been photographed but have not yet been measured; measurements for quantifying fitness (BIO-WEST 2017) will be conducted at later stages of this project. F_1 females were paired with males into flow-through tubes with conditioned wood and leaf material. Eggs were initially counted; however, considering that not every egg hatches, larvae were counted and removed from the chamber ca. once a month and given to the refuge F_2 stock. The cumulative number of larvae for each female was recorded.

f. *Adaptive management*

The nature of these experiments was to undergo a reiterative process so that modifications or adaptations to the designs could be implemented as information was gathered and new observations were made.

Results and Discussion

a. Flow-through tubes

Treatment-trial results are listed in Table 1. Paired t-test results for the seven tandem runs for five treatments, indicated that there was a difference in percentage successful pupation between upright (2.9±5.2%) and horizontal position (24.3±19.4%) flow-through tubes (t-value = -2.585; p-value = 0.041). However, three of the treatments were found to be unsuccessful, due to explainable factors that probably led to unfavorable habitat conditions. The trial of the "rock, leaf, wood; vertical" treatment was probably too congested with material to allow for quality flow, and both groups suffered high losses. Treatment "mid roll up; leaf outside" most likely had an excessive Q as most of the larvae were found on the out-flow screen of the tube upon retrieval. This trial was useful as it helped determine an upper threshold for Q. Treatment "wood outside; mesh roll up inside" group 12 was allowed to decrease in flow without adjustment throughout the duration of the run; at retrieval the Q was measured at 5.3 mL/sec, resulting in only 2 pupation events with total mortality. This trial was useful in helping establish the lower threshold for Q. Throwing out these three trials, paired t-test results were much stronger (t-value = -6.301; p-value = 0.008) with horizontal position tubes (38.8±12.4%) having a higher success rate than upright positioned tubes (1.3±2.2%).

More trials are underway to help support the findings of two treatments that have shown success over two trials, respectively. These preliminary results imply that flow is important; it cannot be too fast or too slow. Furthermore, they also imply that increasing the amount of air pockets increases the pupation potential for larvae.

b. Starvation

A total of five individuals were selected for starvation post-treatments. Two individuals emerged into adults after eight and 48 days, respectively. During the inspections it was noted that some larvae were feeding on algae that had accumulated on the inflow and outflow screens of the tubes. Future investigations should reexamine these treatments with clean screens.

c. Terrestrial habitat

A total of nine individuals were placed on top of rocks with conditioned leaves inside. In all cases, larvae sought out water and usually ended up on the outflow screens. Only one of these individuals transformed into an adult female after 49 days.

d. Tiny bubbles

The mean daily temperature of ASE Trial 1 was 21.5 °C for a total of 1695.2 dd. The average daily discharge was 66.7 mL/sec. No living individuals were recovered from ASE Trial 1. ASE Trial 2 had a mean daily temperature of 23.1 °C for a total of 1987.9 dd. The average daily discharge was 86.9 mL/sec. Only four larvae were recovered from this trial and all were placed into a modified terrestrial or "mod" rearing chamber, filled with emergent rocks and submerged wood and cotton. The water depth of the mod post-treatment was ca. 2 mm and all larvae pupated to adult. Two pupated within 2 weeks and the others took over a month with the longest time equal to 98 days after placement into the mod rearing chamber. This experiment was largely unsuccessful; therefore, the third scheduled trial was not initiated.

In general, no conclusions could be drawn from these experiments. Flat rocks that were placed on top of the opening did accumulate air pockets, therefore it would stand to reason that air pockets formed within the chamber. Flow was increased from ASE Trial 1 to Trial 2, but perhaps even more flow was needed to maintain a healthy habitat.

e. Assessment of beetle fitness

Adult measurements still need to be taken; however, it should be noted that the size or “fitness” of individual adults may be related to their conditioning before initiation into the trials of this study, and therefore may reflect the cohort from which they were derived more than the treatment type that they were reared.

Seventeen females were paired with males and placed into smaller flow-through tubes with conditioned wood and leaf material. Some females maintained a continuous partner while other females had intervals of time where they did not have a partner, thus potentially limiting egg fertilization. The female that lived the longest came from the refuge stock and was found dead after 113 days and produced the most larvae at 70 (Table FIT). The average number of larvae produced by females that survived past the first inspection, but were deceased at the time this report was written was 27±22. Six females were still alive at the time this report was written, some with and some without mates and two of those were initiated post-treatment.

f. Adaptive management

The first treatment was mainly unsuccessful with high mortality from both tube positions; it was presumed that the tubes were packed with too much material which limited the flow throughout most of the occupiable habitat and therefore a point was made to pack tubes with less material. The third treatment, “mid roll up; leaf outside”, was mainly unsuccessful due to high flows (see above). The Q for these groups was > 33.0 mL/sec and so it was decided that future Q should be maintained under this amount. By the end of forth tandem run, there were two groups that had a > 25% pupation rate and it was noted that their Q averages were in the range of 12.3 – 15.6 mL/sec. From this point, Q ranges were considered more optimal close to this range. Also, as noted above, the “wood outside; mesh roll up inside” treatment, group 12 was allowed to decrease to a Q = 5.3 mL/sec. This confirmed that low-flow conditions were detrimental for survival.

It was apparent that some post-treatment trials were more successful than others. Although “terrestrial” trials resulted in few pupation events, it was noted that the larvae would seek out water, which led to the development of the “mod” post-treatment trials. Few starvation trials were implemented, but more should be considered in the future since these had a relatively high result in pupation (40%). Only one cohort was used for the “mod” post-treatment trials (individuals from ASE2); however, this resulted in 100% pupation rates. More investigation of this method should be pursued.

Other Notes

- Prepupal characters have not been observed to this point. These characters may not be easy to detect, or are only visible for brief periods of time, or both.
- Larvae weights were attempted to be taken pre- and post-trial run, but were not confidently reliable due to the difficulty in taking wet measurements of diminutive organisms.
- It is apparent that soon after pupation, beetles are in a delicate state for days or perhaps a week or more. Soon after eclosion to adult, individuals are light yellow in color and slowly darken to a orange-brown. During this early stage of adulthood, it is very difficult to see the internal abdominal structure for determining sex (Kosnicki 2019). Furthermore, individuals encountered in this condition should be handled as little as possible which means sexing them should be postponed until the individual reaches a more fully sclerotized condition.

Table 1. Treatment-trial tandems for upright and horizontal position flow-through tubes for completed intial runs for groups 1 - 14. Ave_Q = average discharge mL/sec; dd = degree days °C; Ave_temp = average temperature °C; %tans = percent of larvae out of 20 that transformed into a pupae or adult, dead or alive.

Treatment type	Group	Position	Initiate	Retrieve	Ave_Q	dd	ave_temp	%trans	larvae	Cohort
rock, leaf, wood; vertical	1	Upright	15-Jan-19	8-Apr-19	14.8	1745.2	21.3	0	0	large
rock, leaf, wood; vertical	2	Horizontal	15-Jan-19	8-Apr-19	8.6	1745.2	21.3	5	2	large
mid roll up; leaf, cotton, wood outside	3	Upright	19-Feb-19	17-May-19	12.4	1885.5	21.9	0	7	large
mid roll up; leaf, cotton, wood outside	4	Horizontal	19-Feb-19	20-May-19	15.6	1953.7	22.0	30	0	large
mid roll up; leaf outside	5	Upright	15-Mar-19	18-Jun-19	33.4	2110.2	22.5	0	5	large
mid roll up; leaf outside	6	Horizontal	15-Mar-19	20-Jun-19	38.9	2156.8	22.5	0	8	large
mesh outside; leaf rock mid	7	Upright	12-Apr-19	18-Jul-19	17.3	2205.0	23.0	5	2	small
mesh outside; leaf rock mid	8	Horizontal	12-Apr-19	19-Jul-19	12.3	2228.5	23.0	60	1	small
mid roll up; leaf, cotton, wood outside	9	Upright	21-May-19	6-Aug-19	18.5	1771.1	23.3	0	7	small
mid roll up; leaf, cotton, wood outside	10	Horizontal	21-May-19	8-Aug-19	11.0	1817.8	23.3	30	5	small
wood outside; mesh roll up inside	11	Upright	24-Jun-19	16-Sep-19	15.8	1943.4	23.4	15	4	Mix
wood outside; mesh roll up inside	12	Horizontal	24-Jun-19	18-Sep-19	11.5	1989.9	23.4	10	0	Mix
mesh outside; leaf rock mid	13	Upright	24-Jul-19	23-Oct-19	14.9	2105.4	23.4	0	2	Mix
mesh outside; leaf rock mid	14	Horizontal	24-Jul-19	25-Oct-19	8.9	2150.7	23.4	35	0	Mix

Table 2. Post-treatment results. Date is the final day of inspection; Group = the original groups from which the individual belonged; Post = post-treatment type; Beetle code = individual tracking code; Transform = a successful pupation event; Sex = includes sex of the adult or reason sex could not be detected; Days = number of days from the initiation of the post-treatment to the final inspection; Dead = individual was found dead or was not found at all following 2 consecutive inspections.

Date	Group	Treatment	Beetle code	Transform	Sex	Days	Dead
16-May-19	2	terr	2.t.ind1			38	x
16-May-19	2	terr	2.t.ind2			38	x
23-May-19	3	terr	3.t.ind4			6	x
23-May-19	3	terr	3.t.ind5			6	x
12-Jun-19	3	terr	3.t.ind2			26	x
5-Jul-19	3	terr	3.t.ind1	x	Female	49	
5-Jul-19	3	terr	3.t.ind3			49	x
5-Jul-19	3	starve	3.s.ind6	x	Female	49	
10-Jul-19	6	relaunch	6.r.ind2	x	Dead	34	
10-Jul-19	6	relaunch	6.r.ind3			34	x
10-Jul-19	6	relaunch	6.r.ind4			34	x
10-Jul-19	6	relaunch	6.r.ind5			34	x
10-Jul-19	6	relaunch	6.r.ind6			34	x
10-Jul-19	6	relaunch	6.r.ind7			34	x
10-Jul-19	6	relaunch	6.r.ind8			34	x
9-Jul-19	5	relaunch	5.r.ind8			21	x
9-Jul-19	5	relaunch	5.r.ind9			21	x
9-Jul-19	5	relaunch	5.r.ind10			21	x
9-Jul-19	5	terr	5.t.ind11			21	x
18-Jul-19	6	relaunch	6.r.ind1	x	Missing	8	
6-Aug-19	7	relaunch	7.r.ind2			19	x
6-Aug-19	3	starve	3.s.ind7			81	x
6-Aug-19	5	terr	5.t.ind12			49	x
14-Aug-19	ASE2	mod	A2.r.ind1	x	Male	14	x
14-Aug-19	ASE2	mod	A2.r.ind2	x	Female	14	
14-Aug-19	9	starve	9.s.ind1	x	Dead	8	x
3-Sep-19	7	relaunch	7.r.ind1	x	Female	47	
9-Sep-19	9	starve	9.s.ind2			34	x
9-Sep-19	9	starve	9.s.ind3			34	x
9-Sep-19	10	relaunch	10.r.ind1			32	x
9-Sep-19	10	relaunch	10.r.ind2			32	x
9-Sep-19	10	relaunch	10.r.ind3			32	x
9-Sep-19	10	relaunch	10.r.ind4	x	Female	32	
9-Sep-19	10	relaunch	10.r.ind5	x	Female	32	
28-Oct-19	ASE2	mod	A2.r.ind3	x	Male	89	
28-Oct-19	11	relaunch	11.r.ind1			42	x
28-Oct-19	11	relaunch	11.r.ind2			42	x
28-Oct-19	11	relaunch	11.r.ind3			42	
28-Oct-19	11	relaunch	11.r.ind4			42	
6-Nov-19	ASE2	mod	A2.r.ind4	x	Male	98	

Table 3. Total number of larvae produced per female after treatment or post-treatment transformation to adult. Date = last date checked; Female tracked = tracking code; Larvae = total number of larvae produced at that date; Days = number of days from initiation to Date; fem_dead = female was found dead on Date.

Date	Female tracked	Larvae	Days	fem_dead
9-Jul-19	treat4.3	6	50	x
19-Aug-19	treat8.2	10	31	x
2-May-19	Refuge1	12	50	x
13-Sep-19	treat10.2	13	36	x
13-Sep-19	treat8.4	18	56	x
8-Jul-19	treat4.2	42	49	x
5-Jul-19	treat4.1	47	46	x
13-Sep-19	Refuge3	70	113	x
19-Aug-19	treat8.3	0	31	x
13-Sep-19	treat10.1	0	36	x
18-Sep-19	fem1.T3.terrstar	0	30	x
25-Oct-19	treat14.1	2	0	
16-Sep-19	treat11.1	5	0	
28-Oct-19	ASE2.1	8	59	
28-Oct-19	treat7.r.1	16	40	
23-May-19	Refuge2	23	71	
13-Sep-19	treat8.1	61	56	

Interim Annual Report

For USFWS

and

Edwards Aquifer Authority

Factors Affecting Pupation in the Endangered Comal Springs Riffle Beetle

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Background and Objective(s):

As a part of the Edwards Aquifer Habitat Conservation Plan (EAHCP), the USFWS is tasked with maintaining off-site populations of endangered and imperiled species covered under the HCP. These populations primarily serve as “refuge” populations, but the USFWS and collaborators have also performed research in the lab examining the life history and ecology of these organisms. Despite these efforts, there are still substantial questions and issues associated with many of these taxa which currently impede the ability to maintain captive populations. In particular, the USFWS can successfully hold the federally endangered Comal Springs riffle beetles (CSRB; *Heterelmis comalensis*) in captivity but has experienced difficulties in the refugium with low numbers of beetle larvae successfully pupating into adults. Given the requirement to maintain sustainable captive populations, there is clearly a need to examine factors which may contribute to the successful pupation of CSRBs in captivity.

The number of studies examining the CSRB has intensified over the last several years and have provided insights regarding this organism’s life cycle and trophic ecology. Recent experiments conducted by the Nowlin Lab at Texas State University and collaborators (USFWS personnel and BIO-West, Inc.) have provided information on the life history, trophic ecology, and environmental tolerances of the CSRB. These research efforts identified quantitative and non-invasive methods for sexing adult beetles, quantified the number and head capsule widths of CSRB larval instars, determined egg laying preferences, adult and larval food preferences, and conducted preliminary experiments on factors affecting pupation. Those research efforts found that CSRB are particularly sensitive to changes in water temperature and dissolved oxygen (DO) concentrations and that there were likely substantial nutritional constraints to larval growth and development that may also affect pupation rates in captivity.

We proposed to examine several factors which may contribute to successful pupation and emergence of adult CSRB in a captive setting. Specifically, we propose to examine several factors in captivity and has two main research goals:

1) How does the origin (wild or lab-grown biofilms) and nutritional and microbial composition of biofilms utilized by CSRB larvae affect pupation and adult eclosion rates in captivity?

2) Does the presence of conspecifics (CSRBs) affect the quality (i.e, microbial composition and nutritional value) of biofilms utilized by CSRB larvae prior to pupation?

Significant Deviation(s):

Suspension of federal work (furlough) led to substantial delays in the start date of the project due to hiring of full-time personnel. However, work is now ongoing and will be completed within the next year.

Progress Summary

(1) Examination of the role of microbial composition and origin of OM types on CSRB pupation rates

Growth chambers and experimental set-ups (including the "conditioning" of food materials) were constructed starting in February 2019. Experiments were conducted starting in April 2019 and are now concluding (December 2019). At this point, the last group of larvae are in housing chambers and we are tracking their development.

All samples for biofilm have been collected and sent off for sequencing and we have refined methods for nutritional quality analysis.

Preliminary results indicate that we have had considerable success rearing larvae and getting them to pupate (~40 pupation events); however, there is substantial mortality associated with pupae in captivity. At this point only 4 adults have eclosed from pupal cases. We are in the process of conducting data analysis on rates of larval mortality, pupation, pupal mortality, and successful adult emergence for both "wild" and "lab" grown biofilms. The last group of larvae are being tracked, but we expect to conclude these experiments in the next ~2 months. Biofilm analysis and data analyses are expected to continue for the remainder of the project.

(2) Determination of the role of presence CSRB grazing on the microbial composition and nutritional composition of OM biofilms

Experimental set-ups and "conditioning" of organic matter sources started in October 2019. Will begin larval experiments in December 2019. Expect the experiments to conclude in April or May 2020, depending on rates of larval development and pupation.

STANDARD OPERATING PROCEDURES:

Collection and Quarantine for

Etheostoma fonticola

Fountain Darter

(San Marcos and Comal)

Field Collections

Fountain darters are collected through a variety of methods including large dip nets, seines, and drop nets. In recent years, we have also used divers with large dip nets to collect fountain darters from habitat locations that are deeper than can be safely waded. At times we will also partner with the bio-monitoring program to take possession of the fish they collect via drop nets.

When collecting fountain darters be sure to record the number collected, the number returned (i.e. too small), and the GPS location data (see collection data sheets). All equipment should be thoroughly cleaned and disinfected between use in the San Marcos River and the Comal River. All equipment should be thoroughly cleaned and disinfected at the end of collections events before it is put away in storage.

Transporting from the Field

Fill clean, dry coolers with fresh water from the river. Choose appropriately sized coolers for the number of fish collected. Small coolers (~4.5 gals or 17 liters) can hold up to 25 darters. If you are transporting for longer distances/time than from rivers back to SMARC larger coolers might be more appropriate.

Steps after collection, for transport:

1) Refresh water in the coolers with clean river water
2) Take temperature of water in coolers
3) Label the coolers with date, number of fish, location collected from
4) If needed for longer transport secure an air bubbler to cooler
5) Secure coolers in vehicle to reduce any movement, sliding, or tipping/spilling
6) If needed fill and secure an additional cooler with fresh river water

If transport is extended be sure to check on coolers and water temperatures periodically throughout transport. Refresh water with water from extra cooler. Have ice packs on hand contained in clean plastic zip top bags to float in coolers if temperatures rise to critical levels.

Quarantine Procedures

All fish must be quarantined 30 days and have clearance from the Fish Health Unit prior to incorporation into refugia stock. During this time, fish will remain in isolation from the rest of the refugia and will undergo treatment for external parasites. Ahead of time tanks should be prepared (cleaned and disinfected) for the incoming fountain darters. Stand pipes with mesh screen covers should be placed in tanks along with habitat materials (clean PVC half rounds, clean plastic plants). Fill tanks and let run for at least 24-hours prior to collections.

The following is an approximate timeline of post-arrival activities.

After arrival at the station's quarantine area:

1) Take temperature of water in the coolers. Compare to the temperature of the water in the tanks that the fish are going into. Acclimate the fish to the water temperature of the receiving tanks at a rate of 1 °C per hour. If needed, add bubblers to tanks during acclimation.

2) After the fish have been temperature acclimated separate out any fish that are designated to send to Fish Health (60 from each river) and place in assigned tanks. This fish will not be treated.
3) If you have not already done so, take measurements of the water volume in the coolers to calculate the amount of formalin needed for treatment. Fish are treated in their transport cooler and then transferred into prepared tanks
    a. Formalin is 37% strength formaldehyde, i.e. Formalin is 37g (ml) of formaldehyde in 100g (ml) of water.
    b. Formalin is applied at a dosage of 170 microliters/liter for up to an hour.
    c. Have a secondary person independently check calculations. We have an Excel file called "Treatment Calculator" on the Refugia and Local drives that has the formulas for calculating the dosages of various treatments if needed.
    d. Example calculations:

        Cooler dimensions: 24 in L x 12 in W x 6 in D = 1728 in^3
        Volume conversion 1872/61.0237 = 28.317 Liters
        Formalin in microliters 28.317 x 170 = 4813.8674 microliters
        Formalin in milliliters 4813.8674/1000 = 4.81 mL

**It is standard and approved by Fish Health to treat our incoming fish to a static formalin bath dip to help control for external parasites. We give treatment as per the guidelines from the Approved Drugs for Use in Aquaculture developed by U.S. Fish & Wildlife Service's Aquatic Animal Drug Approval Partnership Program. Blue booklet or saved as a PDF on Refugia and Local drives---can also be found on the internet for download. See figure below:

FORMALIN - EXTERNAL PARASITES

Product Name & Supplier	Species	Indication	Dosing	Limitations & Comments

PARASITE-S Western Chemical, Inc. 1-800-283-5292
FORMALIN-F Natchez Animal Supply Co. 1-800-647-6760
FORMACIDE-B B.L. Mitchell, Inc. 1-800-817-5808
	All finfish	Control of external protozoa (species of the genera *Chilodonella, Costia, Epistylis, Scyphidia, Ichthyophthirius,* and *Trichodina*)
		and
		monogenetic trematodes (species of the genera *Cleidodiscus, Dactylogyrus,* and *Gyrodactylus*)
			• Salmonids (salmon & trout) in tanks and raceways:
			  • Above 50°F: up to 170 µL/L for up to 1 hr
			  • Below 50°F: up to 250 µL/L for up to 1 hr
			• All other finfish up to 250 µL/L for up to 1 hr
			• Earthen ponds: 15 - 25 µL/L indefinitely
				• Do not subject to temperatures below 40°F (4.4°C)
				• Do not apply when 1) water is warmer than 80°F (27°C), 2) there is a heavy phytoplankton bloom, or 3) dissolved oxygen is less than 5 mg/L
				• Ponds may be retreated in 5 to 10 days if needed
				• Do not treat ponds containing striped bass
				• Test on a small number of fish from each lot to check for any unusual sensitivity to formalin before proceeding
				• 0-day withdrawal time

4) Add air stones or bubblers to cooler prior to adding formalin. Formalin decreases the availability of oxygen in water for the fish, so air stones must be added during formalin treatments.
5) Set and start timer as you are adding formalin
6) Add formalin dosage to water and stir water with net to make sure it is thoroughly mixed (no stratification or pockets of concentrated formalin).
7) Watch fish closely over the treatment period. Fish may appear irritated in general, however remove to fresh water if fish are seen piping for air, flashing, or sudden mortalities occur.
8) At the 50-minute mark you may start removing fish to fresh tank (especially if re-counting fish during transfer) to ensure that all fish are moved by the hour mark.
9) New tanks should be labels with species, river collected from, location/section collected from, number of fish, and intake day.
10) Create/add daily log for fish with appropriate information
11) Do not feed fish on first day
12) If fish are in glass aquaria, shade cloth or screening may be placed around the outside of tanks during the first week of quarantine as fish spook very easily at any movement around their tanks.

***It is suggested that using doing a salt treatment or adding salt concurrently during a formalin treatment can help by helping reduce the mucus coating on the fish, thus allowing the formalin treatment to better target external parasites. If adding salt we would recommend at a 0.5% level.

Day 2

- General behavior is observed:
  - Do all fish look healthy?
  - Sick can be recognized by any number of symptoms (discoloration, air bubbles, piping for air, flashing, necrosis)
- Mortalities and sick fish are removed
- Do not overly disturb fish (this applies to the whole quarantine period, but especially important in the beginning when fish spook and startle easily creating excess stress)
- Fill out daily logs

Day 3

- General behavior is observed:
- Mortalities and sick fish are removed
- Light feed of fish
- Fill out daily logs

Day 4 through end of quarantine period

- General behavior is observed:
- Mortalities and sick fish are removed
- Feed fish on normal routine as refugia population fish. If there are small fish artemia can be added to food

- Siphon and clean tank on regular basis
- Fill out daily logs

Notes

*Fountain darters in quarantine at SMARC are held on purely flow-through water.

*Try to reduce the amount of debris transferred to tanks from the transport coolers. If needed for water quality purposes this debris can be gently siphoned out before day 4.

*Keep densities as low as possible in tanks and add habitat enrichment items to reduce fish stress.

*If large mortality events are seen consult the Fish Health Unit.

*Comal fountain darters have a history of doing well during the first two weeks of the quarantine period and then having large numbers of mortalities.

*One suggestion by biologists at Tishomingo was to keep fish at 0.5-1.0% salinity for 5-7 days after they are initially brought in and formalin treated. We are testing this on the Spring 2019 Comal fountain darters to see if this reduces mortalities, but this is not yet part of the SOP.

Standard Operation Procedures

Collection, Transport, Husbandry of Juvenile and Larval *Eurycea* salamanders at the SMARC

Updated: 6/04/19

Collection and transport

Larval (having a complete or partial yolk sac) and juvenile San Marcos and Texas blind salamanders are occasionally collected from Diversion Spring in Spring Lake. These small individuals should be treated with utmost care to increase chances of survival as they are fragile and have been whirled around in the collection cup depleting their energy. What is reported in this SOP has been learned through trial and error at the SMARC.

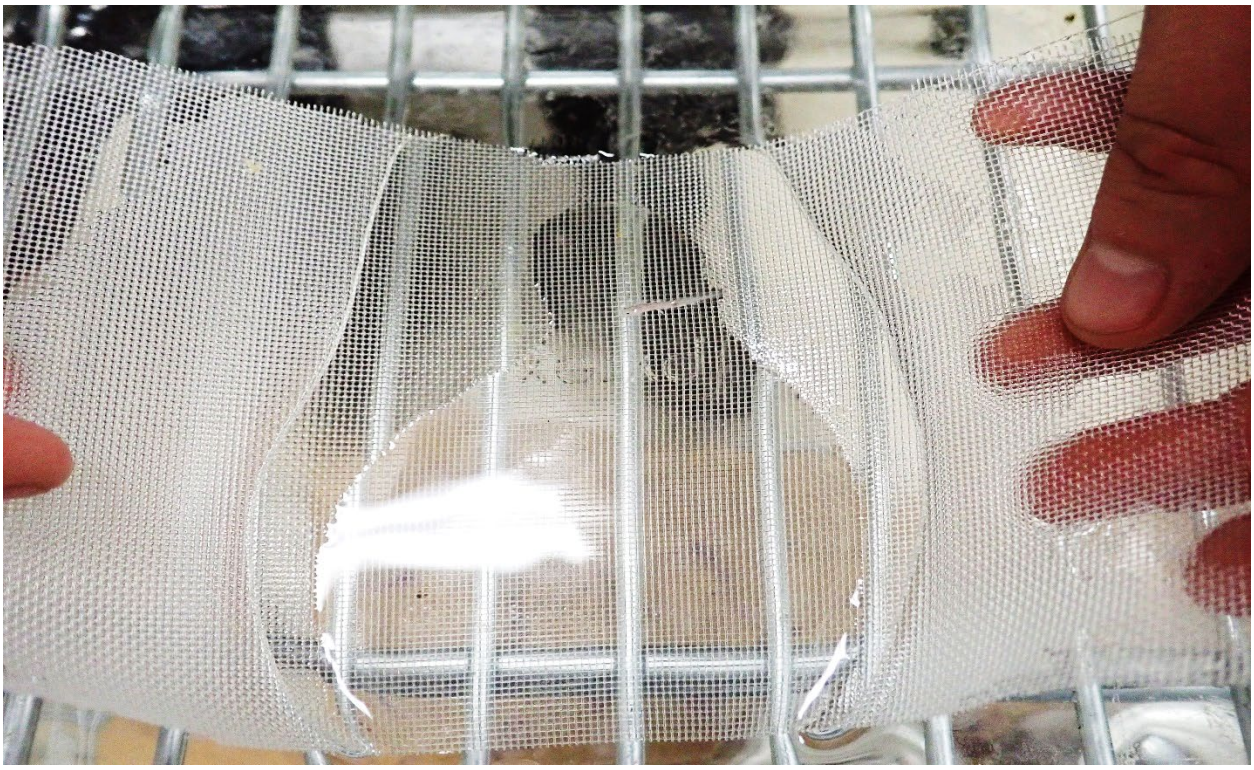
Checking the cup (2-3 people):

1. Pull up to the double orange buoys and begin pulling onto boat. Pull the collection cup onboard WITHOUT tilting or the contents will flush back into the net. Unscrew the cup from its adapter.
2. Carefully remove the baffling and mesh pieces and place in a cooler filled with a few inches of lake water.
3. Have one person lift and hold the cup upside down while the other uses nalgenes and spray bottles to first wash and then spray out all arms of the cup flushing from the outside to the inside. Check for salamanders inside and if you still see some, keep rinsing until they come out or you can try to gently get one to crawl onto a small piece of mesh. Salamanders like to cling to silicone edges and can get stuck in small grooves. Gently sort through the cooler either on boat at net or back at shore. Usually one person checks the cooler while the other scrubs the adapter and netting to keep algae build-up at bay.
    a. Lift mesh pieces out of the water and swish gently. Live animals will swim away and carcasses are dislodged. After all mesh is out, animals will be at the bottom of the cooler
4. Repack the collection cup (see Diagrams)
    a. Currently we are using 750 micron mesh strips, two per arm and two underneath a handmade baffle of netting, pvc, and stiff tank screens. A large piece of mesh (square in shape) sits overtop. The purpose of stuffing the cup is to create refuges from turbulence in the cup, have material that organisms can hang on to, provide shelter for organisms, and in an effort to keep organisms from directly being pushed into the hard screen backing of the collection cup out-flow arms. Other stuffing can be tried, but this has had the most success thus far. Invertebrates seem to have better survival on black soft mesh, but are likely not the correct species of Lirceolus at this site.
5. IF you begin to notice organisms are mostly dead at time of collection and/or are in poor condition (broken backs, bad injuries) most likely the net needs to be cleaned. Algae grows on the fine mesh of the net reducing the amount of water that escapes out of the sides of the net and thus increasing the force and pressure of the water coming out the terminal end of the net (the collection cup). This can be done using snorkelers and/or divers with the boat tied on to the elbow.
6. Transport salamanders into nalgenes
    a. Do NOT place small animals in the same container as large adults
    b. Do NOT place small Texas blinds and small San Marcos in same nalgenes unless also taking San Marcos
    c. Pre-fill nalgenes to the brim and place mesh inside. Use soft netting rather [see below] than stiff mesh for lining nalgene. Netting helps cushion salamanders during transport

and also provides more surface space for them to occupy. Weak and tired ones can find their feet, hang on, and begin recovering.

Soft netting for transport (cushioning, grips for babies) versus stiff mesh (750 micron)

d. Using a piece of stiff mesh gently swish water near the salamander. Usually they spook and swim up to the surface, allowing you to get underneath and lift them up on the mesh. [see below] Quickly move them to one of the pre-filled nalgenes. Place the mesh with salamander just at water's edge and wait for them to swim or slide off into the container. Occasionally some swim back up, just be patient. If they go in on their backs and float down, do not despair. AS LONG AS THEY HAVE A HEARTBEAT THEY HAVE A CHANCE!
e. This can take some time to move animals individually into nalgenes. Give the cooler a swirl with your hand and look for any carcasses or additional animals before you pack up.
f. Get a total count of how many "live/returned to station" (anything with heartbeat) and how many dead or released.



“Mesh lift method” being used to carefully move a baby salamander from its dish to its tank. Use this method to move salamanders from the cooler to nalgenes, dish to tank, tank to tank until large enough to safely handle with net.

Baby Texas blind salamander on 750-micron mesh

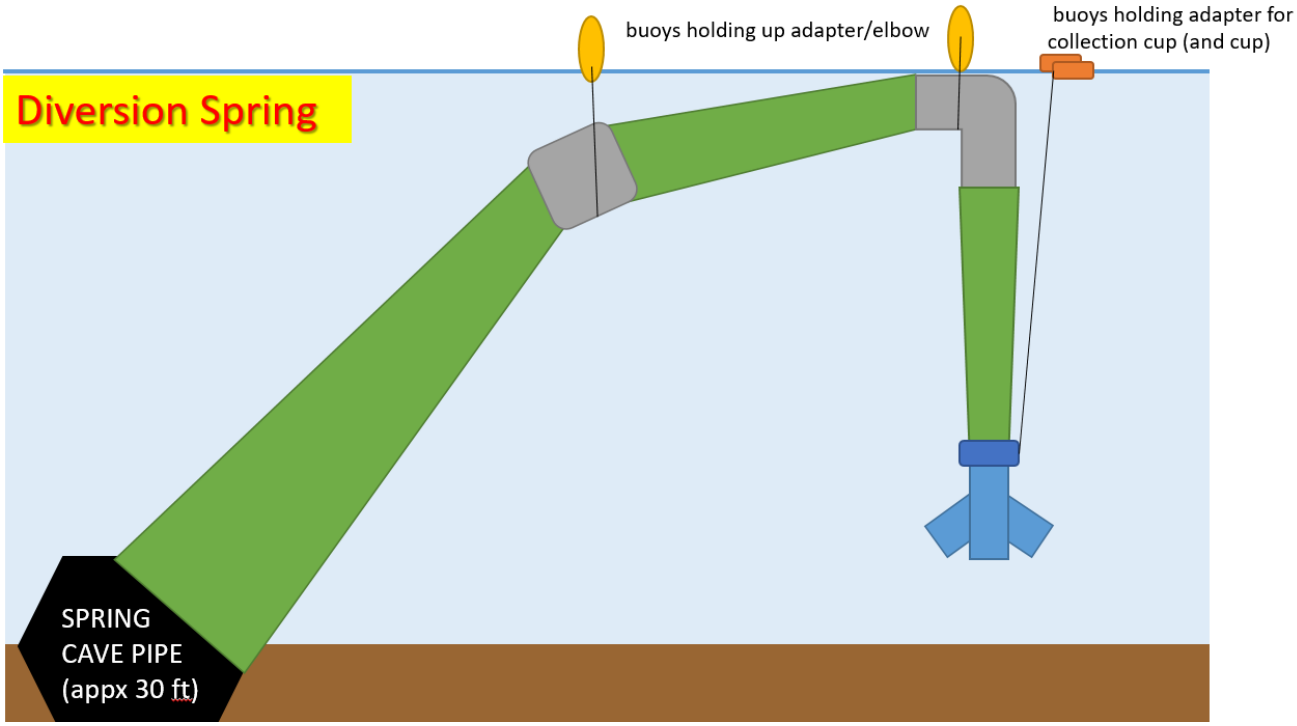
7. Released animals
    a. Texas blinds are not released at Diversion. All live animals collected are returned to station. **ANY salamander with a heartbeat is brought back. Even if it is weak, tired, or non-responsive, it may recover and should be given a chance.**
    b. If you are not taking San Marcos salamanders they are released in the shallow areas next to the boat launch by the rock wall with appropriate cover habitat. After resetting the net, paddle over and gently empty Nalgene of San Marcos into the water.
8. Dead animals
    a. Dead Texas blinds are no longer preserved, as we have more than ample genetic samples from this site. Discard in the lake and keep record of the number of dead at time of collection.
        i. In the EAA spreadsheet, Texas blinds that are dead at the time of collection are called "Released" but in USFWS documentation these are called "DEAD ON COLLECTION" and are included in total count of what was collected (live and dead)
    b. Dead San Marcos are not preserved. Discard in the lake and keep record of the number of dead at time of collection.
        i. In the EAA Spreadsheet, San Marcos that are dead at the time of collection are EXCLUDED from the total count collected. They are not called "Released". In USFWS documentation these are called "DEAD ON COLLECTION" and are included in final total count of what was collected (live and dead).
9. Arrival at station
    a. **Immediately** when arriving on station, gently pour the Nalgene with salamanders into a container (glass baking dish, plastic tub, etc.). GO SLOW. Remove the mesh gently as you pour, as many salamanders like to hang on to this. Once the water is out, rinse the cup out again and inspect the lid, as animals may still be hiding inside. Flush them out with turkey baster and tilt the nalgene.
    b. Remove the mesh and RECOUNT. Make sure you've got them all. Once you're sure, give them the mesh back.
    c. If any are on their backs use your fingers or a pipette to coax or flip them onto their feet, if possible. Onto mesh preferably. Animals on their feet show better recovery.
    d. Add fresh chilled well water from the station to start acclimation. Watch for water getting too cold from the A/C. Continue to add small amounts of chilled well water throughout the acclimation period.
    e. These small salamanders may be non-responsive, exhausted, lethargic, or shocked when they first are shot out of the aquifer and into a fast-flowing net/cup. **The most important thing is that they need to acclimate and be given time to recover in a non-turbulent holding container.** Give them time to calm down once you get back to station before you put them away. Some will not recover and die, and we make notation of those that die by the end of day or by the time they get back to station. Weak and even near-death animals often recover during this time and go on to be otherwise healthy, normal individuals. If at the end of the day one is still very weak, it might not be appropriate to put them in the small nursery tank with turbulence from the water flow. Refresh water in the dish and leave to recover for a longer period. Salamanders in

general excrete very little nitrogenous waste and that these small sizes the water quality will be at fine levels overnight. We have had salamanders who have taken up to two days to recover this way. Less weak individuals can recover in their individual small nursery tank with a low flow of water turbulence.

f. Set up housing while they are acclimating.
g. When it is time to move, use the same “Mesh Lift” method to transport them individually into their new aquaria.
h. See Husbandry and care

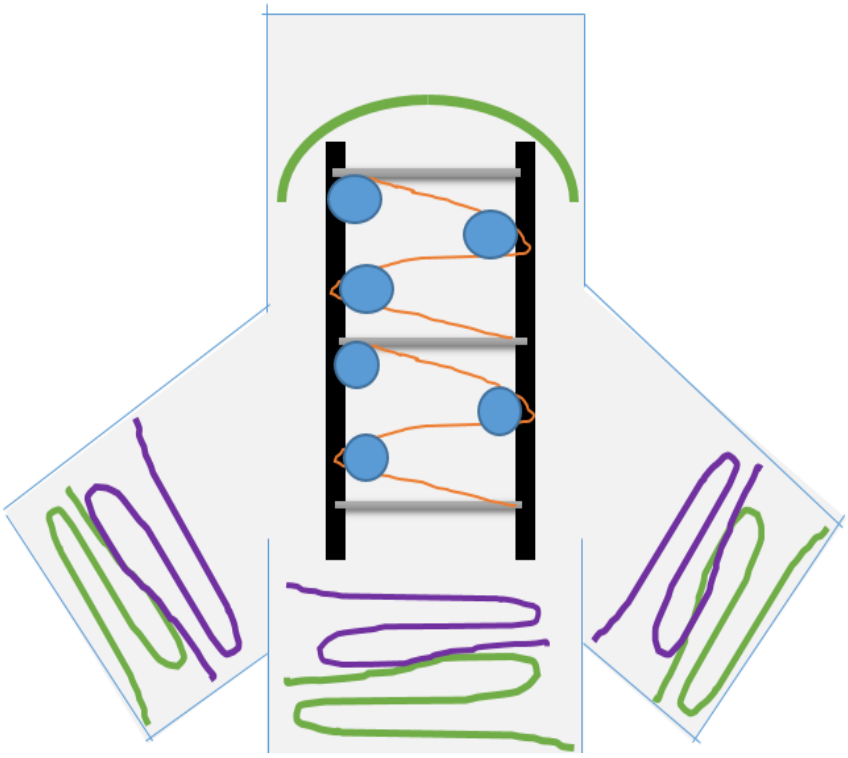
5 Texas blind salamanders (circled) the day of collection acclimate in a glass baking dish.

Diagrams



Diversion Spring (diagrammatic and underwater)

Repairing and scrubbing netting



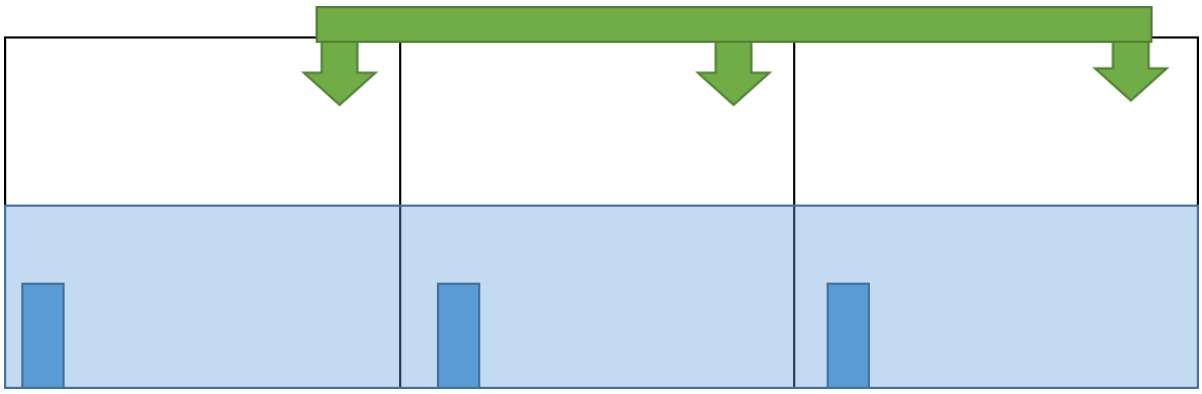
Diversion cup 4.00

- 6” double wye with all exits OPEN (not capped). Openings are covered using 750 micron mesh and chicken wire for strength.
- Loading: a “baffle” made of SCH 80 PVC bars (black, filled with fine sand for weight and sealed shut with hot glue) and soft netting (weaved back and forth, held in place with zip ties and stiff mesh rolls) sits in the main channel atop one to two pieces of 750-micron folded over itself. Two pieces of 750-micron mesh in each arm, folded over itself to take up the space and “cushion” animals. One additional piece can be added on top if you find animals are getting caught/trapped in baffle by intense flow.
    - It is best to have two cups made (so that you can clean / repair damages)
    - It is also advised to have at least two baffles made for the same reason
        - Additionally, never make these with hot glue. This falls apart fairly quickly. Silicone only (which also has a lifespan, but is significantly better than hot glue)
    - Soft netting can grow algae inside. Hard mesh should be replaced every time you check/reset with clean mesh of the same size. Mesh should be rinsed and dried before redeployed.
    - Be sure to rinse the baffle out when checking Diversion as babies can cling

HUSBANDRY AND CARE of LARVAL/JUVENILES

- **Housing (general)**
    - Salamanders do not need oxygen at any time during their care, as all oxygen is obtained through the skin and gills from the water. Texas blind salamanders live naturally in oxygen-poor environments (as evident by elaborate gill filament structure).
    - Salamanders require constant flow-through water or partial recirculation with fresh well water input. Again turnover rates of every other day are desired.
    - It is discouraged to use typical fish aquaria-type filters, as small individuals can easily be sucked in. If filters are near water surfaces, salamanders can readily climb or even jump in.
    - While they do not excrete much nitrogenous wastes themselves (as compared to fishes), excess food does. Recirculation without cleaning or lack of input of fresh water leads to dangerously toxic water quality, and can do so very quickly in the case of adult brine shrimp and artemia. The spikes in water quality caused by death of the feed can cause can kill fish and salamanders alike.
    - Watch for hydra – especially likely when feeding artemia (even more so if artemia is excessive) and tanks are dirty. HYDRA KILLS BABIES. Move salamander to a different tank if hydra is seen : babies may not withstand salt treatments.
    - Excessive amounts of gasses (through unnecessary oxygenation added by caretakers or through spikes from well water switches as in the SMARC) can cause salamanders to "gas", in which bubbles of gasses can be seen in the body. It is difficult for salamanders to maintain position on their feet and stay down, and causes distress and exhaustion as they try to fix or right themselves. Salamanders that are gassed should be given objects to cling to. Some will recover from being "gassed" but others succumb and die to it.

- **Housing babies (at least one month after capture)**
    - At the SMARC animals are held in INDIVIDUAL tanks with name and date of capture. So formerly called and used "hospital" tanks are divided into three sections, each receiving its own flow-through source and one salamander. Habitat is minimal: one to two rocks and a piece of soft mesh or netting. This makes it easier to find babies, see if they are feeding, and to find dead individuals. 750 micron mesh is sufficient for screen covers, but ensure all covers and fittings are tight as animals are small and able to escape through narrow spaces. Have covers on sump / drains as a backup!
    - If hospitals or individual tanks are not available, small groups should be used.
        - **CAUTION: Housing animals in small groups when they first arrived resulted in 50% survival at the SMARC. Housing individually improved survival of incoming babies to 85+% within the first month.**
            - This is due in part to 1) competition for food/space and 2) death of weak /injured incoming animals spreading disease to others in the tank

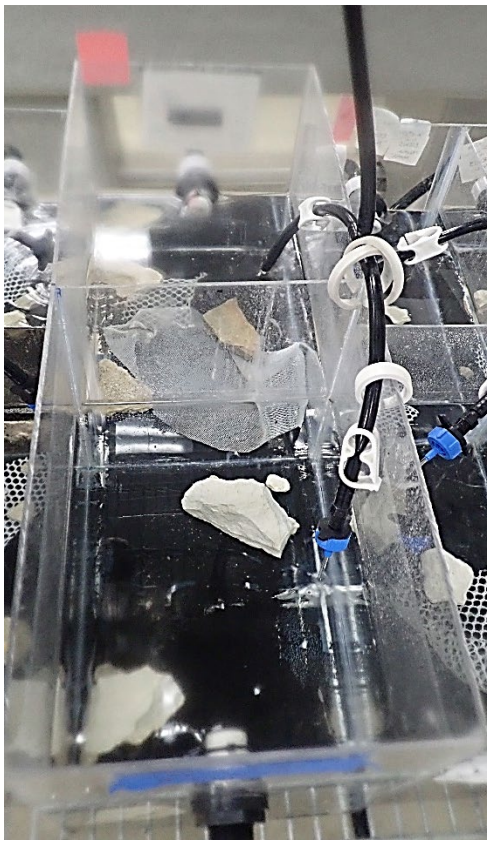


Traditional "hospital" (trifold with bottom drains) for individual salamander housing. Green = water input.

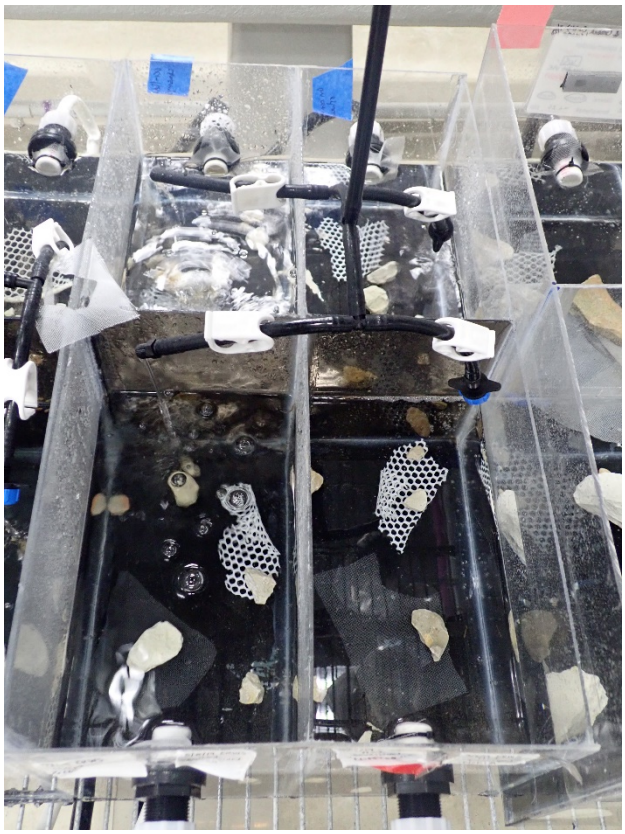
(left): Traditional trifold "hospital" (one sal per section). (right): One of the three sections in a traditional "hospital" system. Input is from the top and drain is covered with mesh tied snug with zip tie. Traditional socks can be used but be sure to check for holes as these babies CAN and DO escape if there is opportunity. 750-micron mesh coverings, clean if become clogged. Habitat is at a minimum (one soft thing, one hard thing, one rock). Gassed salamanders should have additional soft meshes added to occupy space and to give them handholds. Flow should be minimal to not push babies around but provide the recommended turnover.

- **Housing (after at least one month of isolation)**
  - Depending on your space needs, after animals are (a) eating artemia and (b) alive after 1 month in captivity they are included in your count as "refugia". **If you have the space, leave them alone in single individual tanks for as long as possible and let them grow without competition.**
  - When moving from hospital to small group nurseries, use the mesh lift method again for transport. They are still FAR too delicate (and the space too tight) for nets.

"NURSERY TANKS" (grow out) (new designs) : made with plexiglass and acrylic cement, bottoms painted with rustoleum



Duo (two larger sections), side drains

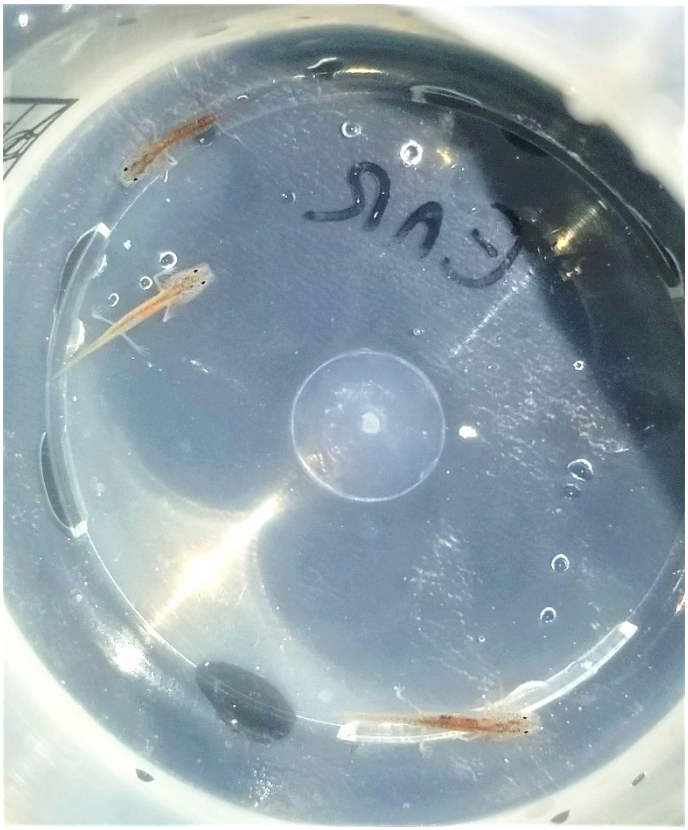


Quad design (four small sections, side drains)

- If you've a desperate need for space and/or do not need to know individual identifications, you can combine animals into small groups as soon as 1 month post arrival. Depending on tank size,

2-5 individuals is recommended. Be sure to watch groups – if you see intense aggression outside of feeding time or death occurring you need to separate them! Up your feeding for how many animals are in the tank. Still feed 2-3x/ week.

- If able (see tagging), keep individual identification for later.
- Keep salamanders in small groups for 6-12+ months before moving them into slightly larger tanks. Try to keep tank mates together if they get along. If you add more individuals watch their initial interactions very closely for aggression (as you should also do when adding adult salamanders to group tanks). Separate if needed.



Salamanders one month post collection

- Feeding
  - Do **<u>NOT</u>** expect freshly collected animals to eat (after all, even the adults don't eat right away!) For some it takes up to two weeks for yolk to fully absorb and animals to begin hunting at all. Some will <u>NEVER</u> take to food and will die within their 30-day quarantine period. The majority will take to food within 2 weeks of arrival.
  - In small individual tanks, feed each salamander 2 drops of just hatched artemia. Avoid feeding cysts or dead artemia, as they foul the water quickly and can cling to skin and gills, causing death. Artemia should be bright orange in coloration.
  - HUNGRY animals quickly learn what this is and will come running for food once they know it is there. You'll see them smelling the tank bottom, then picking their heads up and "jumping" as they inhale the artemia. They often pause between each feed and go back for more. You'll see the next day if they ate artemia at all, as their bellies fill with orange artemia and often drag the ground.

- Because they gorge themselves, they do not need food daily or often. In fact, 3x per week may be too much if artemia are densely packed.
- Once salamanders are approximately 35+ mm (at least 6-8 months in captivity) you can try to transition them to "adult" foods
    - This should ALWAYS be done WITH artemia (something they know how to eat). Use only VERY small amount of a) ostracods or b) chopped worm. **Do NOT feed amphipods until you observe them hunting successfully as they can overwhelm, injure, kill, and eat salamanders!**
    - While ostracod live quite happy in tanks, chopped worms result in foul water and a plethora of fungus. When using immersion blender, over ½ of the worms die immediately. Try very few worm bits (live ones at that!) and remove excess/dead shortly after feeding.
    - It may take several rounds of food introduction before they begin feeding on more adult foods, but continue to supplement their feeds with artemia.
        - If you have group tanks at this time, supplementing will help smaller individuals who may be behind or may be unable to hunt.
    - Texas blinds will come out and gorge on worm piles (bolus) if they want it. They'll get into ideal ambush spots (or, if really smart, under the water flow) if hunting for artemia or ostracod.
    - Something else I have had success with in transitioning to adult feed is hand feeding at the start. This AGAIN should be done at 35+ mm! Get one worm or an ostracod in the very tip of a pipette and put it in the face of a blind salamander. See if they begin to smell and strike at it before letting them have it. This also helps in group tanks if one is not getting fed or is behind growth-wise. Once they understand it is food they will begin to hunt on their own.

Juvenile Texas blind salamander the next day after gorging on artemia (approx. 15 mm TL)

- **Daily Activities**
  - Each day check for presence of flow (not much is needed, ideal turnover rates of once every other day is a good base amount)
  - Check for mortalities (usually out, curled up and fungused)

- **Weekly Activities**
  - Animals are fed artemia 2-3x per week as supplies are available
    - If artemia are very dense feed only 2x week, if sparse feed 3x
  - Every MONDAY (after weekend walkthroughs) and THURSDAY (before the weekend)
    - Use a turkey baster or a pipette and physically find EVERY single salamander.
      - This is time consuming, but worth it and has not shown negative consequences. It helps locate dead and missing (escaped). You can also clean the tank using baster/pipette while you do this (see Cleaning).
      - GENTLY blow around rocks and habitat. Blow around drain and sock/cover (they like to hide here). Watch for them to swim out, confirm they are alive. Check for injuries. Salt treatments are not recommended due to their small size.

- Using the method described above check on babies the day after you first bring them in as those that are very weak usually die the first night. **Those that survive the first 30-days will almost all make it for you if you do things correctly.** ☺

- **Cleaning**
  - Use turkey baster or pipette to "siphon" water out of individual tanks. When in larger tanks with larger individuals (not fresh captures) actual siphons can be used.
  - Animals that die in tanks should be discarded as soon as possible to avoid build-up of bacteria and fungus. At their small size, any fungus is dangerous. Items should be removed and disinfected. Remove nearly all water with a baster and let the high turnover rates replenish.
  - As in other salamander tanks, clean once weekly (more if excess food or debris present in the tank)
    - **Excessive disturbance (stressful, possible injury) is a negative for these fragile young animals. They do best when left alone.**

- **Tagging and individual identification**
  - At the SMARC Texas blind salamanders are individually identified and tagged. When they go into small groups at approx. 40 mm they are given a "ONE DOT" identification VIE tag. The space behind the tail is not large enough for the three-dot full code, so everyone gets one dot so you can tell who is who. I keep record on the tank and in notes about who is what color that way when they ARE big enough for their full code we are sure of who it is.
    - EXAMPLE
    - Animal A = purple → eventually gets orange-purple-orange (or some other combo)
    - B – red
    - C – yellow
    - D – orange
    - E – green
    - F – no tag
    - G – black
    - H – blue

One dot VIE tag in a group tank (sal size approx. 35-40 mm TL)

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/928 December 10, 2018
Memorandum

To: Kenneth Ostrand and Lindsay Campbell, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the Comal River, Texas (Case Number 19-07).

On November 6, 2018, Southwestern Fish Health Unit (SFHU) staff received 61 fountain darters (*Etheostoma fonticola*) from the Comal River (GNIS ID: 1372140), Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for fish health testing. San Marcos ARC staff recorded collection of 30 fish from latitude 29.7206° and longitude -98.1284 and 30 fish from latitude 29.7109° and longitude –98.1282. One location was recorded for reporting purposes for the NWFHS: latitude 29.7206° and longitude -98.1284° in Comal County, Texas. We received two more fish than reported in the collection information from San Marcos ARC.

Assays and examinations for the sampled fish included virology and parasitology. Viruses screened for included those listed as NWFHS targeted pathogens as well as any other viruses that may be detected in cell culture. Sixty fish were screened for viruses. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC. External examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of the left side set of gills for 10 fish. Testing was performed per the American Fisheries Society- Fish Health Section Blue Book (2016 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 9 of 10 fish examined. No viruses were detected in cell culture. The parasite data sheets that contain the specific number and type of parasites isolated from each fish are attached to the end of this memo report.

If you have any questions about test methodology or results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 19-07 for all follow up correspondence.

cc: Dave Hampton, Southwestern Fish Health Unit/ Southwestern Native Aquatic Resources and Recovery Center

FOD Parasite Data Sheet - Form P-03

Case History No. 19-07

Date examined: 11/06/2018 Date Collected: 10/29-30/2018

Collection site: Comal River

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	106	229	072	161	197	100	079	235	245	114
Total Length (mm)	25	30	22	28	30	23	22	32	30	25

Centrocestus formosanus **cysts** **Number of cysts per arch (ie 3,2,1,1)**

Mature (left gills only)	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,2,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Immature (left gills only)	L	0,4,1,1	0,0,1,0	0,1,5,0	0,1,1,0	1,3,5,4	0,2,1,0	0,1,2,1	1,13,7,7	0,1,0,2	0,0,0,0
Monogenea	L	0	0	0	0,1,0,0	0	0	0	0	0	0
Myxobolus sp.	L	0	0	0	0	0	0	0	0	0	0
Other	L	0	0	0	0	0,0,0,2 (Ich)	0	0	0,0,0,1 (Ich)	0	0

Examiner signature Jim Bundy

Revised on 9/20/2017

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/929 December 10, 2018
Memorandum

To: Kenneth Ostrand and Lindsay Campbell, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 19-08).

On November 6, 2018, Southwestern Fish Health Unit (SFHU) staff received 60 fountain darters (*Etheostoma fonticola*) from the San Marcos River (GNIS ID: 1375972), Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for fish health testing. San Marcos ARC staff recorded collection of 25 fish from latitude 29.7129° and longitude -98.1375, 20 fish from latitude 29.8900° and longitude –97.9340°, and 15 fish from latitude 29.8726° and longitude -97.9318°. One location was recorded for reporting purposes for the NWFHS: latitude 29.7129° and longitude -98.1375° in Hays County, Texas. We received one less fish than reported in the collection information from San Marcos ARC.

Assays and examinations for the sampled fish included virology and parasitology. Viruses screened for included those listed as NWFHS targeted pathogens as well as any other viruses that may be detected in cell culture. Sixty fish were screened for viruses. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC. External examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of the left side set of gills for 10 fish. Testing was performed per the American Fisheries Society- Fish Health Section Blue Book (2016 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 2 of 10 fish examined. No viruses were detected in cell culture. The parasite data sheets that contain the specific number and type of parasites isolated from each fish are attached to the end of this memo report.

If you have any questions about test methodology or results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 19-08 for all follow up correspondence.

cc: Dave Hampton, Southwestern Fish Health Unit/ Southwestern Native Aquatic Resources and Recovery Center

FOD Parasite Data Sheet - Form P-03

Case History No. 19-08

Date examined: 11/06/2018 Date Collected: 10/29-31/2018

Collection site: San Marcos River

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	151	164	222	227	266	202	422	200	419	187
Total Length (mm)	28	29	30	30	33	30	35	29	37	30

Centrocestus formosanus **cysts** **Number of cysts per arch (ie 3,2,1,1)**

Mature (left gills only)	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Immature (left gills only)	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,3,2,0	0,0,0,0	0,0,0,0	2,1,1,0	0,0,0,0	0,0,0,0

Monogenea	L	0	1,1,2,0	0	0,0,0,1	0,0,1,0	0,1,0,0	0	0	0	0,0,2,0
Myxobolus sp.	L	0	0	0	0	0	0	0	0	0	0
Other	L	0	0	0	0	0	0	0	0	0	0

Examiner signature Jim Brunty

Revised on 9/20/2017

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

January 30, 2019

In Reply Refer To:
FWS/R2/FR-SFHU/931

Memorandum

To: Lindsay Campbell, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the Fountain Darters (SNARRC Case Number 19-12)

On November 28, SNARRC staff received eight fountain darters (*Etheostoma fonticola*) from San Marcos Aquatic Resource Center. Fish were received live. These fountain darters were submitted due to increased mortalities following collection from the Comal River (upper and lower river locations) on October 30, 2018. These fish have received previous treatments with formalin following placement into isolation on October 30, 2018 and later for and ichthyobodo (costia) outbreak with follow up treatment on November 16, 2018. Clinical signs of disease reported include lesions and hemorrhaging.

Water parameters recorded at time of collection (11/27/18) were 19.4°C, 6.98 pH, 7 mg/L dissolved oxygen, salinity 0.32, specific conductivity 0.627 millisiemens/cm. Other water parameter for different nitrogen forms and alkalinity were not provided with the diagnostic submission. Fish averaged 23.5 mm in total length and 227.25 mg in weight.

Upon arrival at the Southwestern Fish Health Unit, the submitted fish were euthanized in buffered MS-222 and examined. Five fish were sampled for virology, bacteriology and parasitology and three fish were fixed in Z-fix. All clinical testing was conducted per the American Fisheries Society-Fish Health Section Bluebook and standard SFHU protocols. The fixed fish were submitted to the Washington Animal Disease Diagnostic Lab (WADDL) for histopathology.

Results:

Moderate to heavy loads of *Centrocestus formosanus* and Myxozoan spp. were observed on the gills. No parasites were observed on the skin scrapes and no bacterial pathogen were isolated from kidney. Largemouth bass virus was isolated in cell culture and confirmed by PCR.

Histopathology results from WADDL reported hypertrophy of gill epithelium. Inflammation was also described around the mouth A couple organs involved in blood formation such as the spleen and liver in this report appear to show pathology Histopathology described spleen inflammation and degradation of the structural portions of the liver.

Final Diagnosis: Fish lesions (splenitis, hepatic cord dissociation, stomatitis); branchial epithelial hypertrophy.

Hypertrophy of the branchial epithelial could be due to formalin treatment or presence of a trematode, likely *Centrocestus formosanus*. Fountain darters are routinely observed with *Centrocestus formosanus*, therefore it is unlikely this is a new cause for mortality. Causes for inflammation described around the mouth could not be discerned. Pathology reported in the spleen and liver may be attributed to the largemouth bass virus or some other virus. At this point, we cannot definitely associate fish pathology with the isolated largemouth bass virus. Histopathology and bacterial culture results agree upon absence of a bacteria pathogen as a possible cause for the mortality. Recommendation is to evaluate other water quality parameters for possible causes of mortality if this problem continues. We can also continue to investigate viral causes through histopathology for future submissions associated with case.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 19-12 for any follow-up correspondence.

cc: Dave Britton, San Marcos ARC
Ken Ostrand, San Marcos ARC

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

April 15, 2019

In Reply Refer To:
FWS/R2/FR-SFHU/942

Memorandum
To: Lindsey Campbell, San Marcos Aquatic Resource Center

From: Dave Hampton, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the Fountain Darters (SNARRC Case Number 19-23)

On January 30, SNARRC staff received 20 fountain darters (*Etheostoma fonticola*) from San Marcos Aquatic Resource Center. These darters are reported as LMBV positive, held in tanks Q2 and Q4. Observed clinical signs included hemorrhages on heads and ventral side of the bodies, and inflammation on the gills. They were submitted due to increased mortalities. These fish have received Chloramine-T treatment (15mg/L static for 1 hour) for three days as described in the fish health hand book, and mortalities continued after treatment. A total of 20 fish were received, 10 of them live, and the remaining 10 were preserved.

Water parameters recorded at time of collection (01/29/19) were 20.2°C, 6.98 pH, 64.7% dissolved oxygen, and no detectable ammonia. Other water parameter for different nitrogen forms, salinity and alkalinity were not provided with the diagnostic submission. Fish averaged 31.2 mm in total length and 377 mg in weight.

Upon arrival at the Southwestern Fish Health Unit, the submitted fish were euthanized in buffered MS-222 and examined. Five fish were sampled for virology, bacteriology and parasitology and three fish were fixed in Z-fix. All clinical testing was conducted per the American Fisheries Society-Fish Health Section Bluebook and standard SFHU protocols. The fixed fish were submitted to the Washington Animal Disease Diagnostic Lab (WADDL) for histopathology.

Results:

Low loads of *Centrocestus formosanus* were observed on the gills. No parasites were observed on the skin scrapes and no bacterial pathogen were isolated from kidney. Largemouth bass virus was isolated in cell culture and confirmed by PCR.

Histopathology results from WADDL indicated the most consistent and significant findings as the evidence of declining body condition, and skin infection (especially at the tail fin). The report emphasized that some of the histological findings might be within the spectrum of LMBV infection, however, the complete picture does not support a systemic viral infection because lack of lesions in multiple organs (e.g. spleen, kidneys, and liver).

Final Diagnosis: Decrease in body cavity fat tissue (atrophy of adipose tissue), moderate skin infection, and egg degeneration with mild ovarian infection.

Atrophy of body cavity adipose tissue is a non-specific finding suggesting decreased caloric intake or increased metabolic demand. This could be secondary to a disease, however, other factors, such as stress due to environmental factors (water quality, husbandry etc.), food competition and other factors should be considered. Changes in ovaries of female fish could reflect aging with secondary non-specific inflammation.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 19-23 for any follow-up correspondence.

cc: Dave Britton, San Marcos ARC
Ken Ostrand, San Marcos ARC

Washington Animal Disease Diagnostic Lab

P.O. Box 647034 • Pullman, WA 99164-7034
Tel: (509) 335-9696 • Fax: (509) 335-7424

Dave Hampton
Dexter Fish Health Unit
PO BOX 219
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Dexter, NM 88230
(575) 420-5711

Case#: **2019-1581**
Report Date: 15 Feb 2019
Received: 06 Feb 2019
Owner: San Marcos National Fish
Animal: 19-23
Species: Fountain darter
Breed:
Sex/Age: ,

Histopathology Report

The bodies of 3 Fountain Darters are received in fixative for histologic examination. Fish are longitudinally and cross-sectioned in their entirety and processed into 3 blocks. The fish in block # 3 was briefly decalcified prior to processing.

Fish # 1: In the kidney occasional tubules have intralumenal mineral. The coelomic adipose tissue has decreased size and number of lipid droplets (atrophy). Ovarian architecture is distorted by multiple degenerate vitellogenic follicles with infiltrates of macrophages and occasional central acicular clefts. Focally within the soft tissues of the rostral cranium there are infiltrates of small numbers of macrophages. There is minimal to mild hepatocellular anisokaryosis. Small numbers of macrophages similar to those observed in the ovary are free within the coelomic cavity. Within the epidermis and underlying soft tissues of the tail fin there are small to moderate infiltrates of neutrophils, macrophages and lymphocytes admixed with moderate numbers of extravasated erythrocytes (better observed in special stains). Luna, Fite's acid-fast and BH Gram stains are negative for microorganisms. Tissues considered to be histologically unremarkable include gill, brain, spinal cord, skeletal muscle, stomach, intestine, pancreas, eye and heart.

Fish # 2: There are multiple degenerate ovarian follicles and atrophy of coelomic adipose tissue as for Fish # 1. Myocardial endothelial cells and epicardial mesothelial cells are diffusely hypertrophic. In a focal area at the heart base there is mild deposition of fibrin with few extravasated erythrocytes and infiltrates of very small numbers of macrophages. Focally, a larger blood vessel in the pancreas has a small fibrin thrombus surrounded by macrophages. There is mild hepatocellular anisokaryosis. Fite's acid-fast and BH Gram stains are negative for microorganisms. Tissues considered to be histologically unremarkable include skin, brain, spinal cord, eye, skeletal muscle, stomach, intestine, gill and kidney.

Fish # 3: There is inflammation and hemorrhage within the epidermis and soft tissues of the tail fin as for Fish # 1. A BH Gram stain demonstrates small number of short gram-negative bacilli. There are small number of free

intracoelomic macrophages as for Fish # 1. The capsular surface of the spleen is lined by hypertrophic mesothelial cells. There are multiple degenerate ovarian follicles and atrophy of coelomic adipose tissue as for Fish # 1 and Fish # 2. There is a single small random focus of hepatocellular necrosis and mild hepatocellular anisokaryosis. Focally underlying the dorsal fin and extending into underlying musculature there are infiltrates of small numbers of macrophages. A Fite's acid-fast stain is negative. Tissues considered to be histologically unremarkable include gill, brain, spinal cord, eye, stomach, intestine, pancreas

HISTOLOGIC DIAGNOSES:

1. Atrophy of coelomic adipose tissue (Fish # 1-3)
2. Mild histiocytic coelomitis (Fish # 1 and Fish # 3)
3. Dermatitis, acute to subacute, multifocal, moderate (Fish # 1 and Fish # 3)
4. Epicarditis, fibrinous and histiocytic, focal, mild (Fish # 2)
5. Thrombosis, pancreatic blood vessel (Fish # 2)
6. Hepatocellular degeneration and necrosis, acute, focal (Fish # 3)
7. Ovarian follicular degeneration with mild histiocytic oophoritis (Fish # 1-3)
8. Mild hepatocellular anisokaryosis (Fish # 1-3)
9. Minimal nephrocalcinosis (Fish # 1)

COMMENTS: The most consistent and significant findings in this group of fish are evidence of declining body condition (atrophy of adipose tissue), dermatitis (especially of the tail fin) and evidence of possible systemic bacterial infection (epicarditis, thrombosis and hepatocellular necrosis). The atrophy of fat is a non-specific finding suggesting decreased caloric intake or increased metabolic demands. This could be secondary to disease, but other factors such as stress or food competition are also considerations. Review of husbandry and possible environmental stressors may be helpful. The cause of the dermatitis wasn't clearly evident, but trauma with secondary bacterial infection (as observed in Fish # 3) would be a consideration. Although some of the histologic findings could be within the spectrum of a viral infection such as LMBV, the complete picture is not really typical of systemic viral infections that usually have lesions concurrently in multiple organs (e.g. spleen, liver and kidneys). If results of virus isolation become available from this group I would interested in hearing the results (apessier@wsu.edu). Changes in the ovaries of these female fish could reflect aging/senescence with secondary inflammation.

WORK PENDING: None

Pathologist: Dr. Allan Pessier
Report authorized by: Dr. Allan Pessier, Senior Pathologist

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/933

Memorandum: March 13,2019

To: Kenneth Ostrand, Project Leader, San Marcos Aquatic Resource Center
From: Matthew Bagley, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final report for the fountain darters from the San Marcos River, TX (SNARRC Case Number 19-33).

On February 26,2019, the Southwestern Fish Health Unit (SFHU) received 10 fountain darters (*Etheostoma fonticola*) from the San Marcos River, TX. The receipt for donation stated that 10 fish were submitted 'San Marcos' fish. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU. Location of fish collection was recorded at latitude 29.87544497 N longitude -97.93192497 W in Hays County, Texas. The fish were examined for *Centrocestus formosanus* parasite enumeration. The final numbers are reported on the following page.

Screening for *Centrocestus formosanus* was conducted by examining the left gill arches of 10 fish. *Centrocestus formosanus* was observed infecting these fish.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 19-33 for any follow-up correspondence.

cc: Linda Moon, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 19-34

Date examined: 2/26/2019 Date Collected: 2/25/2019

Collection site: Comal River

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	241	196	135	177	156	134	215	278	167	133
Total Length (mm)	31	30	26	28	28	27	30	32	29	27

Centrocestus formosanus **cysts** **Number of cysts per arch (ie 3,2,1,1)**

Mature (left gills only)	L	0,2,0,1	0,0,0,0	0,0,0,1	1,2,1,0	0,0,0,0	1,0,0,0	1,0,1,0	1,5,3,1	1,1,1,0	1,1,2,0
Immature (left gills only)	L	0,2,0,3	2,1,0,0	0,2,0,0	0,2,2,1	1,1,0,0	0,0,0,0	0,0,0,0	0,0,0,0	1,1,0,0	0,0,0,0
Monogenea	L	0,0,0,0	1,1,0,0	0,0,0,0	0,0,0,0	1,0,0,0	0,2,0,0	0,0,0,0	0,1,0,0	1,0,0,0	0,0,0,0
Myxobolus sp.	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	1,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Other	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0

Examiner signature HK,MB

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/934

Memorandum: March 15,2019

To: Kenneth Ostrand, Project Leader, San Marcos Aquatic Resource Center
From: Matthew Bagley, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final report for the fountain darters from the Comal River, TX (SNARRC Case Number 19-34).

On February 26,2019, the Southwestern Fish Health Unit (SFHU) received 10 fountain darters (*Etheostoma fonticola*) from the Comal River, TX. The receipt for donation stated that 10 fish were submitted 'Comal' fish. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU. Location of fish collection was recorded at latitude 29.71068601 N longitude -98.12762003 W in Hays County, Texas. The fish were examined for *Centrocestus formosanus* parasite enumeration. The final numbers are reported on the following page.

Screening for *Centrocestus formosanus* was conducted by examining the left gill arches of 10 fish. *Centrocestus formosanus* was observed infecting these fish.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 19-34 for any follow-up correspondence.

cc: Linda Moon, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 19-34

Date examined: 2/26/2019 Date Collected: 2/25/2019

Collection site: Comal River

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	241	196	135	177	156	134	215	278	167	133
Total Length (mm)	31	30	26	28	28	27	30	32	29	27

Centrocestus formosanus **cysts** **Number of cysts per arch (ie 3,2,1,1)**

Mature (left gills only)	L	0,2,0,1	0,0,0,0	0,0,0,1	1,2,1,0	0,0,0,0	1,0,0,0	1,0,1,0	1,5,3,1	1,1,1,0	1,1,2,0
Immature (left gills only)	L	0,2,0,3	2,1,0,0	0,2,0,0	0,2,2,1	1,1,0,0	0,0,0,0	0,0,0,0	0,0,0,0	1,1,0,0	0,0,0,0
Monogenea	L	0,0,0,0	1,1,0,0	0,0,0,0	0,0,0,0	1,0,0,0	0,2,0,0	0,0,0,0	0,1,0,0	1,0,0,0	0,0,0,0
Myxobolus sp.	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	1,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Other	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0

Examiner signature HK,MB

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/940 June 27, 2019
Memorandum

To: Kenneth Ostrand and Lindsay Campbell, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the Comal River, Texas (Case Number 19-61).

On June 27, 2019, Southwestern Fish Health Unit (SFHU) staff received 62 fountain darters (*Etheostoma fonticola*) from the Comal River (GNIS ID: 1372140), Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for fish health testing. San Marcos ARC staff recorded collections of fountain darters from two locations on the Comal River. The location were recorded at the Lower Comal (Sclitterbahn employee parking lot) at latitude 29.7106° and longitude -98.1276° and at the Upper Comal (Upper Spring run) latitude 29.7206° and longitude -98.1285° in Comal County, Texas. Sixty of the 62 fish received were sampled for fish health testing.

Assays and examinations for the sampled fish included virology and parasitology. Viruses screened for included those listed as NWFHS targeted pathogens as well as any other viruses that may be detected in cell culture. Sixty fish were screened for viruses. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC. External examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of the left side set of gills for 10 fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2016 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 7 of 10 fish examined. The parasite data sheet containing the specific number and type of parasites isolated from each fish is attached to the end of this memo report. Largemouth bass virus was isolated in cell culture and confirmed by PCR. Additional PCR tests were performed for other NWFHS targeted viruses and aquareovirus to determining if possible coinfections exist: results were negative for other viruses. This PCR work is in addition to what is normally performed for NWFHS samples; however, this testing was completed to help diagnose additional concerns with this population due to continued fish mortality in hatchery conditions.

If you have any questions about test methodology or results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 19-61 for all follow up correspondence.

cc: Huseyin Kucuktas, Southwestern Fish Health Unit/ Southwestern Native Aquatic Resources and Recovery Center

Lindsay Campbell, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 19-61

Date examined: 4/30/19 Date Collected: 4/26/19

Collection site: Comal River, TX

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	42	114	23	60	23	120	62	44	86	47
Total Length (mm)	19	25	14	20	15	24	19	19	22	18

Centrocestus formosanus **cysts** **Number of cysts per arch (ie 3,2,1,1)**

Mature (left gills only)	L	0	0	0	0	0	0	0	0	0	0
Immature (left gills only)	L	0	0, 1, 0, 0	0	0	0	0	0	0	0	0
Monogenea	L	0	0, 0, 1, 0	0	0	0	0	0	1, 1, 0, 0	0, 1, 0, 0	0
Myxobolus sp.	L	0	0	0	0	0	0	0	0	0	0
Other	L	0	0	0	0	0	0	0	0	0	0

Examiner signature Huseyin Kucuktas

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/938 June 4, 2019
Memorandum

To: Kenneth Ostrand and Lindsay Campbell, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 19-62).

On April 30, 2019, Southwestern Fish Health Unit (SFHU) staff received 61 fountain darters (*Etheostoma fonticola*) from the San Marcos River (GNIS ID: 1375972), Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for fish health testing. San Marcos ARC staff recorded collection of fountain darters from four locations on the San Marcos River as follows: Lower San Marcos (Ramon Lucio Park) at latitude 29.8754° and longitude -97.9319; Middle San Marcos River (below falls/Salt grass steak house) latitude 29.8900° and longitude -97.9340°; Middle San Marcos (Children's Park) at latitude 29.8837° and longitude -97.9355°; and Upper San Marcos (Spring Lake Hotel) at latitude 29.7129° and longitude -98.1375° in Hays County, Texas. Sixty of the 61 fish received were sampled for fish health testing.

Assays and examinations for the sampled fish included virology and parasitology. Viruses screened for included those listed as NWFHS targeted pathogens as well as any other viruses that may be detected in cell culture. Sixty fish were screened for viruses. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC. External examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of the left side set of gills for 10 fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2016 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 5 of 10 fish examined. No viruses were isolated in cell culture The parasite data sheet that contain the specific number and type of parasites isolated from each fish is attached to the end of this memo report.

If you have any questions about test methodology or results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 19-62 for all follow up correspondence.

cc: Huseyin Kucuktas, Southwestern Fish Health Unit/ Southwestern Native Aquatic Resources and Recovery Center

Lindsay Campbell, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 19-62

Date examined: 4/30/19 Date Collected: 4/22/19

Collection site: San Marcos River, TX

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	138	31	124	101	71	473	328	207	162	99
Total Length (mm)	23	16	21	18	14	33	28	25	25	21

Centrocestus formosanus **cysts** **Number of cysts per arch (ie 3,2,1,1)**

Mature (left gills only)	L	0	0	0	0	0	0	0	0	0	0
Immature (left gills only)	L	0	0, 1, 0, 1	0	0, 0, 0, 1	0, 1, 0, 0	0	2, 8, 4, 0	2, 1, 2, 1	0, 0, 4, 0	0, 0, 2, 2
Monogenea	L	0	0, 0, 1, 0	0	0, 0, 0, 1	0	0	1, 1, 2, 0	0, 0, 0, 1	0, 1, 0, 0	0, 0, 1, 0
Myxobolus sp.	L	0	0	0	0	0	0	0	0	0	0
Other	L	0	0	0	0	0	0	0	0	0	0

Examiner signature

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

July 15, 2019

In Reply Refer To:
FWS/R2/FR-SFHU/947

Memorandum

To: Mark Yost, Uvalde NFH

From: Huseyin Kucuktas, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the fountain darters from Comal River (SNARRC Case Number 19-68)

On May 22, SNARRC staff received ten fountain darters (*Etheostoma fonticola*) from Uvalde NFH. Fish were received live. These fish were submitted due to increased mortalities following collection from the Comal River in early April, 2019. Hatchery staff reported 6-10 fish mortality per day and observed signs of bacterial infection. Fish were kept in flow-through systems with 2-3 water exchanges/hour. They were fed in the mornings, and aquarium were siphoned in the afternoons, Monday through Friday. These darters have been treated with salt upon arrival (3.0 % for 11 min), and treated sporadically with static salt bath at a concentration of 0.5 to 1.0 % for 15 minutes.

Water parameters recorded at time of collection (05/21/2019) were as following: Temperature 22.0 – 22.5 °C, pH 8.3 – 8.5, dissolved oxygen 6.9 – 7.3 mg/L, nitrite 0.05 – 0.125 [no unit]. Other water parameters were not provided with the diagnostic submission. Fish averaged 25.0 mm in total length and 206.50 mg in weight.

Upon arrival at the Southwestern Fish Health Unit, samples were euthanized in buffered MS-222 and examined. Seven fish were sampled for virology, bacteriology and parasitology and three fish were fixed in Z-fix. All clinical testing was conducted per the American Fisheries Society-Fish Health Section Bluebook and standard SFHU protocols. The fixed fish were submitted to the Washington Animal Disease Diagnostic Lab (WADDL) for histopathology.

Results:

Microscopic evaluation indicated moderate load of both mature and immature parasite *Centrocestus formosanus* on the gills. Additionally, Myxozoa spp. were observed on the gills. No parasites were observed on the skin scrapes and no bacterial pathogen were isolated from kidney. Largemouth bass

virus was isolated in cell culture and confirmed by PCR. Cell culture supernatants were collected to test for four additional viruses (IPNV, IHNV, VHSV, AquaReovirus) using PCR, however, all tests were recorded as negative.

Histopathology results indicated inflammation on skin, body cavity, nares, and the presence of an enteric nematode ova. The significance of the enteric nematode ova is not known since there is no evidence of associated abnormalities in the digestive mucosal tissue. The histological changes suggested of bacterial septicemia, however, no bacteria are observed clinically and histologically. The inflammation of nasal openings may suggest that the bacteria may have entered via the nasal pits.

Final Diagnosis: Low to moderate parasite infestation of gills and inflammation of the skin tissue.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 19-68 for any follow-up correspondence.

cc: Dr. Patricia Duncan, Dave Hampton, Jason Woodland

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

August 22, 2019

In Reply Refer To:
FWS/R2/FR-SFHU/954

Memorandum

To: Kenneth Ostrand, Project Leader, San Marcos Aquatic Resource Center

From: Matthew Bagley, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center.

Subject: Final report for the fountain darters from the San Marcos River, TX (SNARRC Case Number 19-82).

On August 20, 2019, the Southwestern Fish Health Unit (SFHU) received a total of ten fountain darters (*Etheostoma fonticola*) from the Comal River, TX. The receipt for donation stated that a total of ten fish were submitted as 'Comal' fish. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU. Location of fish collection was recorded at latitude 29.71068601N longitude -98.12762003 W in Hays County, Texas.

The fish were examined for *Centrocestus formosanus* parasite enumeration. Screening for *C. formosanus* was conducted by examining the left gill arches of all samples under light microscopy. The final numbers are reported on the following page.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to the case number 19-82 for any follow-up correspondence.

cc: Linda Moon, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 19-82

Date examined: 8/21/2019 Date Collected: 8/19/2019

Collection site: Comal River, TX

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	25	25	24	24	27	27	26	24	24.5	25
Total Length (mm)	123	110	103	99	139	170	157	110	122	155

Centrocestus formosanus **cysts** **Number of cysts per arch (ie 3,2,1,1)**

Mature (left gills only)	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Immature (left gills only)	L	0,0,0,0	1,0,0,0	0,0,0,0	0,0,1,0	0,1,1,0	0,0,0,0	0,0,0,0	0,2,0,0	0,0,0,0	0,0,1,0

Monogenea	L	0,0,0,0	0,0,0,0	1,0,0,0	0,0,0,0	2,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Myxobolus sp.	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Other	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0

Examiner signature HK

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

August 22, 2019

In Reply Refer To:
FWS/R2/FR-SFHU/955

Memorandum

To: Kenneth Ostrand, Project Leader, San Marcos Aquatic Resource Center

From: Matthew Bagley, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center.

Subject: Final report for the fountain darters from the San Marcos River, TX (SNARRC Case Number 19-83).

On August 20, 2019, the Southwestern Fish Health Unit (SFHU) received a total of ten fountain darters (*Etheostoma fonticola*) from the San Marcos River, TX. The receipt for donation stated that a total of ten fish were submitted as 'San Marcos' fish. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU. Location of fish collection was recorded at latitude 29.87544497N longitude -97.93192497W in Hays County, Texas.

The fish were examined for *Centrocestus formosanus* parasite enumeration. Screening for *C. formosanus* was conducted by examining the left gill arches of all samples under light microscopy. The final numbers are reported on the following page.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to the case number 19-83 for any follow-up correspondence.

cc: Linda Moon, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 19-83

Date examined: 8/21/2019 Date Collected: 8/19/2019

Collection site: San Marcos River, TX

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	292	353	343	387	299	166	287	146	266	120
Total Length (mm)	24	26	25	29	25	27	32	27	33	24

Centrocestus formosanus **cysts** **Number of cysts per arch (ie 3,2,1,1)**

Mature (left gills only)	L	0,0,0,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Immature (left gills only)	L	0,0,0,0	1,1,1,2	2,2,1,0	1,1,3,0	1,2,0,0	0,1,0,0	0,0,0,0	0,0,0,1	0,0,0,0	0,1,0,0
Monogenea	L	0,0,0,0	0,0,0,0	0,0,0,0	1,1,1,0	1,0,0,0	0,0,0,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Myxobolus sp.	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Other	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0

Examiner signature MB

Revised on 9/20/2017

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/959

Memorandum: October 12, 2019

To: Rachel Wirick, Uvalde NFH
From: Huseyin Kucuktas, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the San Marcos salamanders (SNARRC Case Number 20-03)

San Marcos salamanders (*Eurycea nana*) housed at the Uvalde NFH started having higher than normal mortality rates starting early October of 2019. Animals had loss of mobility and exhibited curled posture in addition to showing signs of mottled skin. Personnel collected a total of five salamanders between the dates of 1st and 5th of October 2019, and preserved them in 95% ethanol to submit directly to an animal disease diagnostic laboratory for histopathological examination (sent via FedEx on 10/08/2019.) These salamanders are housed in RE3 tank/system with a water source from Austin Chalk Aquifer. The system is a partial re-use system, and source water is filtered using a biomedia filter. Tanks are siphoned weekly to remove debris and to check water quality. Water turnover rate is reported to be as approximately three times per day. Based on the case history provided, these animals were moved into a new building/system on 08/28/2019, and wells/aquifers switched on 08/09/2019. Water quality measurements performed on 10/03/2019 was reported as; temperature reading of 21.0 ºC, dissolved oxygen as 6.79 mg/L, and a pH measurement of 8.3. Both the TAN and Nitrite measurements were reported as 0.00.

Results:

Histopathology indicated subacute to chronic, multifocal, and moderate to severe microsporidial myositis and muscle cell necrosis with mineralization in four out of five salamanders. Two of the salamanders had signs of microsporidial oophoritis, and two samples (limbs of two salamanders) had hardening/thickening of skin, focal, mild to moderate with intralesional chytrid fungal thalli (likely *Batrachochytrium dendrobatidis,* Bd).

Final Diagnosis: Microsporidial myositis (inflammation of muscle tissue due to microsporidia); ovarian microsporidiosis (presence of microsporidia in ovaries); mild to moderate hardening/ thickening of skin due to chytrid fungus (Bd).

Clinical signs are once again attributable to the microsporidial myositis previously seen from other salamanders at this facility. Additionally, the mild to moderate hardening/thickening of the skin in some of the salamanders are possibly due to presence of Bd on the limbs. Presence of Bd on salamanders are also reported from this facility previously. As discussed with the hatchery personnel during a previous phone conversation, wide variety of options exist to determine exact cause of health problems existing at this location as well as other facilities. As a matter of fact, since multiple factors are involved in aquatic animal health related issues, identification and/or determination of any underlying pathogenic/ environmental/husbandry related issues could easily be evaluated by carrying out small-scale applied research. In addition to continuous monitoring, proceeding with any type of experimental approach and/or intervention should collaboratively be performed among all involved parties for the maximum benefit.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 20-03 for any follow-up correspondence.

cc: Patricia Duncan, Uvalde NFH
Mark Yost, Uvalde NFH
Dave Britton, San Marcos ARC
Ken Ostrand, San Marcos ARC
Lindsay Campbell, San Marcos ARC

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/961 November 20, 2019
Memorandum

To: Kenneth Ostrand and Lindsay Campbell, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 20-05).

On October 22, 2019, Southwestern Fish Health Unit (SFHU) staff received 60 fountain darters (*Etheostoma fonticola*) from the San Marcos River (GNIS ID: 1375972), Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for fish health testing. San Marcos ARC staff recorded collection of fountain darters from four locations on the San Marcos River as follows: Upper San Marcos (Spring Lake Hotel) at latitude 29.71293° and longitude -98.1375; Middle San Marcos River (Below Spring Lake Dam) latitude 29.89008° and longitude -97.9340°; Middle San Marcos (Lions Club/City Park) at latitude 29.88602° and longitude -97.9358°; and Lower San Marcos (Ramon Lucio) at latitude 29.87544° and longitude -97.9319° in Hays County, Texas.

Assays and examinations for the sampled fish included virology and parasitology. Viruses screened for included those listed as NWFHS targeted pathogens as well as any other viruses that may be detected in cell culture. Sixty fish were screened for viruses. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC. External examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of the left side set of gills for 10 fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2016 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 2 of 10 fish examined. No viruses were isolated in cell culture The parasite data sheet that contain the specific number and type of parasites isolated from each fish is attached to the end of this memo report.

If you have any questions about test methodology or results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 20-05 for all follow up correspondence.

cc: Huseyin Kucuktas, Southwestern Fish Health Unit/ Southwestern Native Aquatic Resources and Recovery Center

Lindsay Campbell, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 20-05

Date examined: 10/22/19 Date Collected: _____

Collection site: San Marcos River, TX

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	107	108	360	271	206	280	142	213	309	284
Total Length (mm)	27	24	35	32	30	35	27	30	35	32

***Centrocestus formosanus* cysts** **Number of cysts per arch (ie 3,2,1,1)**

Mature (left gills only)	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,3	0,0,0,0	0,0,0,0
Immature (left gills only)	L	0,0,0,0	0,0,0,1	0,0,0,0	0,1,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Monogenea	L	0,0,1,0	0,0,1,0	0,0,0,0	0,0,0,0	0,1,0,1	0,0,0,0	1,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Myxobolus sp.	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Other	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0

Examiner signature HK, MB, JW

Revised on 9/20/2017