APPENDICES

A. 2022 EA Refugia Work Plan
B. San Marcos salamander (*Eurycea nana*) reproduction: refugia habitat and captive propagation
C. Captive husbandry and propagation of the Comal Springs riffle beetle
D. Assessing the effect of Staphylococcus exposure on Comal Springs riffle beetle (*Heterelmis comalensis*) captive survival and propagation
E. Increasing Comal Springs riffle beetle (*Heterelmis comalensis*) F1 adult production
F. Historical fountain darter (*Etheostoma fonticola*) tissue archive
G. Evaluating the effects of p-Chip tags on small-bodied salamanders (*Eurycea* spp.)
H. Treatment trials for *Batrachochytrium dendrobatidis* infections in aquatic salamanders
I. Genetic assessment of the Comal Springs riffle beetle in Landa Lake
J. Monthly reports
K. Fish health unit reports

5.1.1 Refugia Program

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 Edwards Aquifer Habitat Conservation Plan (EAHCP). The tasks and subtasks that follow provide the details for the services to be performed in 2022, which provide for the maintenance of a refugia population of the Covered Species (Table 1), including 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
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Comal Springs salamander	*Eurycea pterophila*	Petition Rescinded
Texas troglobitic water slater	*Lirceolus smithii*	Petitioned

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, prey, competitors, pathogens, parasites; or on their habitat, including food, water, and shelter.

2022 Assumptions

As work plans are developed almost a year prior to implementation, it is possible that methods described herein will 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.

The following potential situations could necessitate methodology adjustments.

- Target numbers for standing and refugia stocks to be housed at both the UNFH and SMARC deviate from those established by the USFWS-EAA Refugia Contract (Contract # 16-822-HCP).
- Species capture rates fall short of historic values.
- Mortality rates of specimens held in captivity exceed historic values.
- Staff member vacancies occur at either of the two Service facilities during the performance period.
- A pandemic or other emergency prevents scheduled collections.

Target for 2022 (Deliverables and Methods by Task):

Task 1. Refugia Operations

Standing Stocks: 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 contains the target species numbers.

Table 2. Target refugia numbers and census by species.

Species	Standing Stock	Refugia Stock	Salvage Stock	Anticipated SMARC census (Jan 2022)	Anticipated SMARC census (Dec 2022)	Anticipated UNFH census (Jan 2022)	Anticipated UNFH census (Dec 2022)
Fountain darter (Comal)	1000	1000 including specimens within the standing stock	2000	*	*	*	*
Fountain darter (San Marcos)	1000	1000 including specimens within the standing stock	2500	500	500	500	500
Texas wild-rice	430	430 including specimens within the standing stock	1500	215	215	215	215
Texas blind Salamander	500	500 including specimens within the standing stock	500	250	250	60	60
San Marcos salamander	500	500 including specimens within the standing stock	500	250	250	250	250
Comal Springs salamander	500	500 including specimens within the standing stock	500	135	150	105	135
Peck's cave amphipod	500	500 including specimens within the standing stock	500	250	250	250	250
Comal Springs riffle beetle	500	500 including specimens within the standing stock	500	75	75	75	75

Comal Springs dryopid beetle	500	500 including specimens within the standing stock	500	*	*	*	*
Edwards Aquifer diving beetle	500	500 including specimens within the standing stock	500	*	*	*	*
Texas troglobitic water slater	500	500 including specimens within the standing stock	500	*	*	*	*

We will not collect Comal fountain darters until we have a better understanding of their mortality rates
*catch rates and hatchery survival are uncertain given the rarity of the species

Collection: In 2022, the USFWS will collect Covered Species as required to reach and maintain target standing and refugia stock numbers as shown in Table 2. The USFWS will coordinate species collections with other ongoing HCP activities (e.g. Biological Monitoring Program) so that collections for refugia do not adversely impact other efforts. The USFWS will carry out species collections through a variety of passive and active collection methods and will minimize aquatic invasive species transfer by conducting collections in accordance a Hazard Analysis Critical Control Point plan. The USFWS will document and report collection efforts to the EAA. The USFWS will distribute captured organisms 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. The USFWS will hold all species in respective quarantine areas until their health has been assessed. Staff will incorporate quarantined organisms into the general refugia population once they have determined that such specimens are healthy and free from invasive species. The USFWS will share reports, including test results, produced as part of the quarantine process.

The following sections briefly describe planned 2022 collection, maintenance, and propagation efforts for each species.

Fountain Darters:

Collection: In 2022, the USFWS will collect Fountain Darters from the San Marcos River in coordination with the Spring and Fall Biomonitoring events. This will be more efficient than separate collection events and will reduce habitat disturbance. For refugia purposes, USFWS staff will retain Fountain Darters collected by biomonitoring staff via drop nets. Staff will collect fish proportionally from the three sections of the San Marcos River: 1) Upper = Spring Lake, 2)

Middle = Spring Lake dam to Rio Vista dam, and 3) Lower = below Rio Vista dam to Cape's dam. The USFWS will thoroughly investigate unusual mortality events. The USFWS will include summary reports to the EAA as part of the monthly reports. Collections will target sufficient fish so to account for regular, expected mortality, such that the captive population should remain at or above the target.

Due to the detection of largemouth bass virus (LMBV) in Comal fountain darters throughout the Comal River, the USFWS will maintain all fountain darters from Comal River in quarantine facilities, in consideration of other species on the two stations. We have continued concern over higher mortality rates of incoming Comal fountain darters, as no root cause has been identified despite extensive testing and evaluation with the USFWS Fish Health Unit. Until we have a better understanding of the high mortality rates of incoming Comal fountain darters we will conduct limited collections from the wild, unless salvage is needed.

As part of quarantine procedures, the USFWS will send a subset of fish (maximum of 60 per river) to the Southwestern Fish Health Unit or equivalent facility for pathogen (bacteria, virus, and parasite) testing prior to incorporating collected animals into the general refugia population. The USFWS will follow standardized methods outlined within USFWS and AFS-FHS (2016) and AFS-FHS (2005) protocols and provide Fish Health reports to the EAA.

Maintenance: The USFWS will monitor water quality (i.e., temperature, pH, dissolved oxygen, total dissolved gasses) and record these data weekly. Staff will feed fountain darters a mix of live and frozen foods reared or purchased. The USFWS will rear zooplankton and amphipods in ponds and tanks for food. We do not generally examine food items for pathogens. However, if they are suspect and tested for pathogens, the USFWS will include all diagnostic results to the EAA within monthly reports.

Propagation: The USFWS will maintain standing and refugia stocks for each river to produce captive-bred fish for research purposes, as necessary and approved. Staff will maintain fish by their geographical collection location. If reintroduction is warranted, the USFWS will communally spawn subsets from each geographical location. The USFWS will cull subset groups to an equal number of progeny prior to release.

Texas wild-rice:

Collection: USFWS staff will collect Texas wild-rice tillers from San Marcos River reaches (Figure 1), with a break during summer months when collected wild rice does not fare well due to heat stress. In 2022, staff will target stands that are not already part of the refugia population or require supplementation in collections for SMARC and UNFH. The refugia populations will reflect the wild populations in both their respective proportion, based on the most recent Texas wild-rice survey data, and historical genetic diversity (2021 genetic assessment and Wilson et al. 2016). During tiller collection, the USFWS will record the geographic coordinates, area coverage, and depth of the stand or individual plant. USFWS staff will collected tillers by

wading and SCUBA diving. The USFWS will consider georeferenced aerial imagery to help identify distinct TWR stands used for tiller collection.

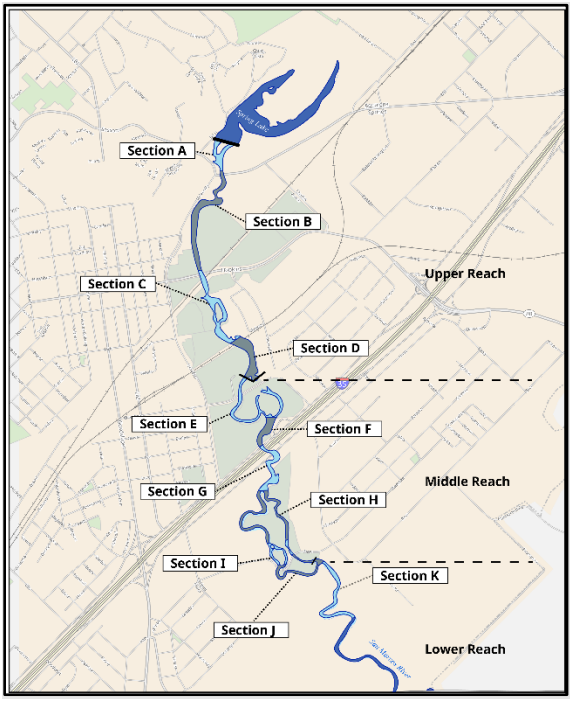


Figure 1. Letters define designated San Marcos River reaches where Texas wild rice is collected for refugia populations.

Maintenance: Once tillers have successfully rooted, USFWS staff will tag and maintain with their collection date and location information.

Propagation: USFWS staff will maintain plants to prevent sexual reproduction within the refugia population, unless EAHCP triggers occur. If reintroduction is warranted, USFWS staff will produce seeds and tillers from each geographical location. During reintroduction, staff will transplant refugia plants produced from seeds and tillers to their original source location, delineated by river section (Figure 1).

Texas blind salamanders:

Collection: USFWS will collect Texas blind salamanders using nets and traps. Staff will deploy traps quarterly for approximately 14 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, staff will only collect 1/3 of salamanders observed from each of these locations during quarterly sampling events. Staff will also collect salamanders 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. We will retain all specimens from this site, under the assumption that any Texas blind

salamander leaving a spring orifice that enters a stream or lake environment will ultimately succumb to predation. We will check these sites up to three times per week when applicable. Staff will transport all specimens alive and maintain them in the SMARC or UNFH refugia. Texas State University staff generally check drift nets on Sessom Creek and Texas State University Artesian Well; Texas State University transfers live Texas blind salamanders to SMARC according to their permits, when appropriate. USFWS staff may periodically check nets on these sites when they are not being checked by Texas State University staff.

As part of quarantine procedures, USFWS staff will swab all large Texas blind salamanders. If they are too small to be swabbed, then we will do a representative batch swab of group-housed salamanders once they are large enough to be safely swabbed. USFWS staff will process these samples at SMARC or other facility 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. Staff will retain duplicate swabs in case further testing is warranted. Staff will hold all salamanders 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 salamanders) have regularly 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 North America. Staff would retain such salamanders in quarantine until further study and recommendations from FWS Fish Health.

Maintenance: USFWS staff will individually tag salamanders to retain information on collection location, date, and other life history events. Staff will monitor water quality and record data weekly. Staff will feed salamanders live and frozen foods, either reared or purchased. Staff will utilize ponds and tanks to produce amphipods on site.

Propagation: Staff will maintain standing and refugia stocks to encourage reproduction. Staff will maintain all progeny separately by generations. If reintroduction is warranted, an attempt will be made to produce offspring from each geographical location.

San Marcos salamanders:

Collection: USFWS staff will collect San Marcos salamanders quarterly from below Spring Lake dam and with SCUBA teams in Spring Lake (Table 5). Staff will check the drift net on Diversion Springs routinely and keep specimens from this location as space in quarantine and need allows. We will avoid collections close to the HCP Biological Monitoring Program assessment events. Staff will transport all specimens alive and maintain these in the SMARC and UNFH refugia.

As part of quarantine procedures, USFWS staff will swab all large San Marcos Salamanders. If they are too small to be swabbed, then we will do a representative batch swab of group housed salamanders once they are large enough to be safely swabbed. USFWS staff will process these samples at SMARC or other facility 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. Staff will retain duplicate swabs in case further testing is warranted. Chytrid testing will occur in batches where groups of five swabs will be pooled for analysis. Staff will hold all salamanders 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 salamanders) have regularly tested positive for Bd. Positive testing for Bsal will be treated more cautiously as it has not yet been documented in North America. Staff would retain such salamanders in quarantine until further study and recommendations from FWS Fish Health.

Maintenance: Staff will monitor water quality and record data weekly. Staff will feed salamanders live foods, either reared or purchased, mixed with purchased frozen food sources if necessary. Staff will utilize ponds and tanks to produce amphipods on site.

Propagation: USFWS staff will maintain salamander standing and refugia stocks to encourage reproduction. We will separate all progeny by generation. If reintroduction is warranted, staff will employ pairwise and group mating to produce offspring. Staff will initiate stocking once juveniles have reached 30 mm total length.

Comal Springs salamanders:

Collection: USFWS staff will collect Comal Springs salamanders quarterly from Comal Spring Runs 1-3 and Spring Island and surrounding areas (Table 5) by hand, with dipnets, using snorkelers. We will coordinate with the HCP biological monitoring program in order to ensure that, to the degree practicable, refugia collections do not overlap with specific EAHCP long-term monitoring locales. In the event overlap of sampling areas is unavoidable, we will collect Comal salamanders at a rate of no more than 10% of salamanders observed in those specific locales per daily sampling trip. We will employ a SCUBA team for a portion of these collection efforts if necessary.

As part of quarantine procedures, USFWS staff will swab all large Comal Springs salamanders. If they are too small to be swabbed, then we will do a representative batch swab of group housed salamanders once they are large enough to be safely swabbed. USFWS staff will process these samples at SMARC or other facility 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. Staff will retain duplicate swabs in case further testing is warranted. Chytrid testing will occur in batches where groups of five swabs will be pooled for analysis. Staff will hold all salamanders 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 salamanders) have regularly 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 North America. Staff would retain such salamanders in quarantine until further study and recommendations from FWS Fish Health.

Maintenance: Staff will monitor water quality and record data weekly. Staff will feed salamanders live and frozen foods, either reared or purchased. Staff will utilize ponds and tanks to produce amphipods on site.

Propagation: USFWS staff will maintain salamander standing and refugia stocks to encourage reproduction. We will separate all progeny by generation. If reintroduction is warranted, staff will employ pairwise and group mating to produce offspring. Staff will initiate stocking once juveniles have reached 30 mm in total length.

Comal Springs riffle beetle:

Collection: USFWS staff will collect Comal Spring riffle beetle for standing and refugia stocks four times a year from a variety of locations, including Spring Run 1, Spring Run 3, the Western Shore, and areas surrounding Spring Island (Table 5). Staff will collect riffle beetles with cotton lures following EAHCP standard operating procedures (Hall 2016) and from wood, as needed. Staff will follow protocols established by the CSRB Work Group in 2019:

1. Staff will not sample the same spring orifice two times in a row.
2. Staff will collect all riffle beetle adults and larvae from lures.
3. Standing stock numbers will be reduced to 75 per station until USFWS has established sufficient propagation methods, and we have better understanding of population numbers to derive meaningful standing stock targets.

The Comal Springs Riffle Beetle Work Group Standing will evaluate standing stock numbers yearly. Additional collections for research purposes may be required outside of standing stock collections.

Maintenance: USFWS staff will maintain specimens by collection date. Staff will hold Comal Springs riffle beetles within custom built aquatic holding units and feed them detrital matter and matured biofilms colonized on cotton lures, wood dowels, and leaf matter.

Propagation: Propagation methods for this species are being developed.

Peck's cave amphipod:

Collection: USFWS will conduct Peck's cave amphipod collection for standing stock four times annually (Table 5). Staff will collect adult Peck's cave amphipods with drift nets and by hand at a variety of locations (drift nets: Spring Run 3, N = 2; Spring Island and associated Spring Lake habitats: hand collection).

Maintenance: Staff will maintain specimens by collection date within custom-built aquatic holding units and feed amphipods with commercial flake fish food.

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

Comal Springs dryopid beetle:

Collection: USFWS will collect Comal Springs dryopid beetles primarily through the use of wooden lures and hand picking from submerged wood found in the Comal Spring system. If staff find dryopid beetles on cotton lures used for Comal Spring riffle beetles, these will also be retained (Table 5). We will potentially conduct two trapping events with bottle traps in Panther Canyon Well during the year as access to the well and staff time allows. Staff will check these traps weekly for a month.

Maintenance: USFWS will combine collected Comal Springs dryopid beetles, regardless of collection location. Staff will hold Comal Spring dryopid beetles within custom built aquatic holding units and feed them detrital matter and matured biofilms colonized on cotton lures, wood dowels, and leaf matter.

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

Edwards Aquifer diving beetle:

Collection: Staff will collect Edwards Aquifer diving beetles with drift nets (Table 5). Staff will set drift nets 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). USFWS staff will deploy and check drift nets at the Artesian Well when as Texas State University allows.

Maintenance: USFWS will combine collected Edwards Aquifer diving beetles, regardless of collection location. Staff will transfer captured specimens to the SMARC or UNFH and house them in custom-made aquatic holding systems. Edwards Aquifer diving beetles are predators; staff will feed them 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 slaters are primarily found in Artesian Well on Texas State Campus. Recent research by Will Coleman (Texas State University) suggests that this is a deep aquifer species, rarely found at the surface. Mr. Coleman was unable to keep any alive, as all specimens he collected were injured. USFWS will continue to work with invertebrate experts to determine what might be the optimum way to collect this species. USFWS staff will deploy and check drift nets in the Artesian Well as Texas State University allows.

Maintenance: Staff will transfer captured specimens to the SMARC and house them in custom aquatic holding systems. Staff will feed Texas troglobitic water slaters detrital matter, matured biofilms colonized on cotton lures, and flake fish food to supplement their diet.

Propagation: Staff need to determine propagation methods for this species, to be conducted as part of normal refugia operations.

Table 5. A tentative schedule for all species sampling during 2022. Collections listed here are subject to change with extenuating circumstances such as weather and coordination with external partners. USFWS will notify EAA and partners of sampling dates as they become known or changed.

Edward's Aquifer Species Collection Plan 2022			
Date (month)	**Interval**	**Location**	**Target Species**
January	14 Consecutive days with traps checked 2-3 times a week	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
January	1 day sampling event, hand pick from downed wood	Landa Lake	Comal Springs dryopid beetle
February	14 Consecutive days with traps checked 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
February	Set lures	Spring Run, Landa Lake	Comal Springs riffle beetle, Comal Springs drypid beetle, Peck's cave amphipod, Texas troglobitic water slater
February	1 day sampling event	San Marcos River	Texas wild rice
February	1 day sampling event	San Marcos River	Texas wild rice
March	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
March	Collect Lures	Spring Run, Landa Lake	Comal Springs riffle beetle, Comal Springs drypid beetle, Peck's cave amphipod, Texas troglobitic water slater
March	1 day sampling event, hand pick	Landa Lake	Peck's Cave amphipod
March	1 day sampling event	Comal Springs	Comal Springs salamander

Edward's Aquifer Species Collection Plan 2022			
Date (month)	**Interval**	**Location**	**Target Species**
March	1 day sampling event, hand pick from downed wood	Landa Lake	CSDB
April	Check 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
April	1-2 day sampling event	Spring Lake and below dam	San Marcos salamander
April	1 day sampling event	San Marcos River	Texas wild rice
April	Throughout, coincide with bio-monitoring	San Marcos River	Fountain darters
April	Drift net, donated from bio-monitoring	Comal Springs	PCA
May	Set lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs drypid beetle, Peck's cave amphipod, Texas troglobitic water slater
May	14 Consecutive day with traps check 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
May	1-day sampling event	San Marcos River	Texas wild-rice
June	Collect lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs drypid beetle, Peck's cave amphipod, Texas troglobitic water slater
June	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
June	1 day sampling event, hand pick	Landa Lake	Peck's Cave amphipod

Edward's Aquifer Species Collection Plan 2022			
Date (month)	**Interval**	**Location**	**Target Species**
June	1 day sampling event	Comal Springs	Comal Springs salamander
June	Set lures	Western Shore	Comal Springs riffle beetle, Comal Springs drypid beetle, Peck's cave amphipod, Texas troglobitic water slater
July	14 Consecutive days with traps check 2-3 times a week	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
August	Set lures	Western Shore	Comal Springs riffle beetle, Comal Springs drypid beetle, Peck's cave amphipod, Texas troglobitic water slater
August	14 Consecutive days with traps check 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
August	1-2 day sampling event	Spring Lake and below dam	San Marcos salamander
September	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
September	1 day sampling event, hand pick	Landa Lake	Peck's Cave amphipod
September	1 day sampling event	Comal Springs	Comal Springs salamander
September	Collect lures	Western Shore	Comal Springs riffle beetle, Comal Springs drypid beetle, Peck's cave amphipod, Texas troglobitic water slater
October	14 Consecutive days with traps checked 2-3 times a week	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander

Edward's Aquifer Species Collection Plan 2022			
Date (month)	**Interval**	**Location**	**Target Species**
October	Throughout, coincide with bio-monitoring	San Marcos River	Fountain darters
October	Drift net, donated from bio-monitoring	Comal Springs	Peck's Cave amphipod
October	1 day sampling event	San Marcos River	Texas wild-rice
October	1 day sampling event, hand pick from downed wood	Spring Runs, Landa Lake	Comal Springs dryopid beetle
November	14 Consecutive days with traps checked 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
November	1 day sampling event, hand pick	Landa Lake	Peck's cave amphipod
November	1 day sampling event	Comal Springs	Comal Springs salamander
November	Set lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs drypid beetle, Peck's cave amphipod, Texas troglobitic water slater
December	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
December	1 day sampling event	San Marcos River	Texas wild rice
December	Collect lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs drypid beetle, Peck's cave amphipod, Texas troglobitic water slater

Refugia Stocks:

Collection: Standing Stock numbers contribute to Refugia Stock numbers. Collections will continue until Standing stock targets are attained. In the event that Refugia Stock triggers, outlined in the contract, are reached and Standing Stock are not at full capacity, USFWS will conduct special targeted collections to increase Standing Stock.

Maintenance: USFWS will conduct maintenance in a similar manner described for standing stocks.

Propagation: Propagation for stocking is not anticipated during 2022.

Salvage Stocks:

Collection: If specific salvage triggers defined in the EAHCP are reached, the Refugia Program, in consultation with the EAA, will accommodate salvaged organisms no more than twice during the 12-year contract period. If triggers for multiple species are simultaneously reached, species collections during salvage operations will be prioritized based upon the perceived impacts of reduced river and spring flow and habitat degradation on Covered Species (i.e. EAHCP triggers). Those species that are river obligate species (i.e., fountain darters and Texas wild rice) or that occupy spring orifice and interstitial ground water habitats (i.e., San Marcos and Comal Springs salamanders, Peck's cave amphipods, Comal Springs dryopid beetles) are presumed to be affected first as flows decrease. Those that reside solely within the aquifer (i.e., Edwards Aquifer diving beetles, Texas troglobitic water slaters and Texas blind salamanders) are presumed to be affected subsequently.

Maintenance: The Refugia Program will maintain organisms collected during salvage operations at the SMARC or UNFH for up to one-year or until their disposition is determined. The Refugia Program may suspend or terminate research if space is required for salvaged organisms. Research may also be suspended if personnel are directed to collect and maintain salvage stocks.

Propagation: Likewise, production of species would be limited to no more than twice during the 12-year contract period if species extirpation occurs. USFWS propagated species at the SMARC or UNFH would be held for up to one year or less if stocking is required. We may suspend or terminate research activities if space is required to house cultured species. Research may also be suspended if personnel are needed to reproduce, maintain, or stock progeny.

Construction/Renovation/Infrastructure/Facility:

The USFWS will report any non-routine maintenance for the program buildings to the EAA as they occur.

The USFWS will institute all reasonable and practical security measures to safeguard EAA refugia facilities, equipment, and species.

For 2022, the refugia is asking for the use of $160,000 Task 1 rollover funds to make improvements to the refugia systems at both the Uvalde National Fish Hatchery ($80,000) and

the San Marcos Aquatic Resource Center ($80,000). Six monitor and control units and the associated equipment will be purchased for each facility. These units will record water parameters and controlling equipment (chillers, CO_2 injectors) on up to 12 systems at each facility. These systems will assist in maintaining water parameters and alert staff if values deviate from specified levels. In addition to the controllers, CO_2 injection systems will be installed to assist in maintaining a consist pH and reduce calcium buildup on equipment. Mechanical filters and UV sterilizers will be added to the systems for increased flexibility, where each system can function as flow-through or 100% recirculating. Were needed, old water pumps will be replaced with more energy efficient pumps. These improvements will minimize the potential for catastrophic system failure, alert staff to problems with individual systems, and add redundancy into the functioning of the refugia systems.

In addition to the amount above we are asking for $5,282.03 from Task 1 roll over funds for the purchase of a portable water velocity meter to be used for field measurements.

Staffing/Labor/Personnel:

The two program Leads (Research and Husbandry/Collections) will mentor and train lower-graded employees, oversee facility maintenance and repair, develop, and implement budgets, and organize activities that relate to all contract activities. The program leads will manage and coordinate research, propagation, culture, and field activities related to the refugia. The leads are expected to provide proper and efficient use of facilities and staff resources. These leads will work with the Center Director and the Deputy Director to ensure that contractual obligations are met in a timely manner. In coordination with the Deputy Center Director, they will prepare all the required written materials required for the reimbursable agreement reporting. Likewise, the leads 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 leads will continue to work and communicate regularly with partners, USFWS personnel and other researchers to meet USFWS and contract goals.

Under the direction of the Lead Biologist at UNFH, five biological science technicians, two at SMARC and three at UNFH, 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 culture and experimental 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.

Under the direction of the Lead Biologist at SMARC, an SCA Student Intern will be hired to conduct the San Marcos fountain darter historical tissue archive research project. This SCA intern will catalog and organize all historical darter collections at SMARC and UNFH and will start the process of assessing the collections suitability in future genetic analysis.

Under the direction of the Lead Biologist at UNFH, two SCA interns will be hired to assist with day-to-day husbandry tasks; one located at SMARC and the other at UNFH.

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 will practice biosecurity procedures in Refugia and Quarantine areas and conduct appropriate biosecurity procedures on field equipment.

Bd/Bsal Testing:

Water from sampling locations, water bodies in the SMARC and UNFH area, and the wells at the SMARC and UNFH will be test for Bd/Bsal. Wild stock and F1 salamanders in refugia will be tested for Bd/Bsal to determine the extent of Bd occurrence in the Standing/Refugia stock. Extended Bd/Bsal testing will ensure that any salamander brought on station would not further contribute to or modify the occurrence of Bd/Bsal in the locations salamanders are sampled from.

Husbandry Pilot Studies:

PCA Exclusion – Peck's cave amphipod does not readily produce offspring that survive to adulthood mostly due to cannibalism by the brooding female. EARP staff currently separate brooding females from main housing and put them into a separate container to reduce cannibalism by the larger population, but cannibalism still occurs by the brooding female. Exclusion chambers will be constructed to separate the offspring from the brooding female. The success of each exclusion chamber design will be assessed by comparing the number of offspring recovered. Each design will be compared to each other and to the currently used brooding chamber.

CSRB Dowel Condition – It takes about a month for a dowel to develop sufficient biofilm to support Comal Springs riffle beetles. This extended time period can cause delays in research and potential lack of food sources for refuge populations if materials are not replenished on a strict schedule or if a system were to fail. We suspect the time required for biofilm to develop on the dowels will decrease if more surface area is available for biofilms to develop. Dowels will be etched and set to condition alongside dowels that are not etched, under the same conditions. The

dowels will be checked daily, and the number of days floating will be recorded. Pictures of biofilm development will be taken weekly. Preliminary testing shows that dowels that are etched sink faster than dowels that are not etched. This study will quantify the number of days required for etched dowels to develop biofilm relative to unetched dowels. If etched dowels develop biofilm faster than unetched dowels (days floating) and produce equal or better biofilm (subjective assessment using photos), then etching dowels will be incorporated into the Comal Spring Husbandry SOP.

Fountain Darter Diet - Fountain darters (*Etheostoma fonticola*) from the Comal and San Marcos Rivers have been successfully bred and reared in captivity at both the Uvalde National Fish Hatchery and the San Marcos Aquatic Resources Center. However, the survival rate during the first few weeks after hatch is often variable and low. Low survival of recently hatched fish can often be attributed to several factors, such as improper diet nutrition of the broodstock, improper diet nutrition of the hatchlings, improper prey size for the hatchlings, tank design, and pathogens. For this project we will focus on comparing diet size. We will rear San Marcos River fountain darters and monitor survival, body length, body depth, and mouth gape of the fish from hatching to 1-month-old, relative to three different diets: 1) current SOP diet of recently hatched live *Artemia* (~400-500um length), 2) live rotifers (~150 – 350 um length), and 3) a mix of live rotifers and *Artemia*. Results from this study will allow us to improve the fountain darter rearing SOP for the EARP and can lead to future work on nutritional needs.

Research Pilot Study:

Tagging Comal Springs riffle beetles – Tagging refugia species provides valuable information on the long-term status and longevity of individuals. Tagging also informs reintroduction strategies and success. Larger species, such as salamanders and Texas wild rice, are relatively easy to individually tag and have many options for effective tagging. Comal Springs riffle beetles are particularly difficult to tag due to their small size, and most available tags are too large or would negatively impact survival. Previous studies at the SMARC used paint to tag Comal Springs riffle beetles and field mark-recapture efforts have shown promising results. The EARP is partnering with Dr. Shannon Brewer with U.S. Geological Survey, Alabama Cooperative Fish and Wildlife Research Unit to conduct a pilot study. The pilot study will use 0.5mm x 0.5mm p-Chips to tag F1 refugia produced adult Comal Springs riffle beetles. The study will determine if p-Chips are a viable tag option or the Comal Springs riffle beetles. Successful tagging will inform a larger tagging study.

Task 2. Research

The Research Plan for 2022 will involve a series of projects designed to improve culture protocols and the health, survival, and propagation of captive populations. We have nearly all we

need for a fully functioning Fountain Darter *ex situ* refuge, but an evaluation of the genetic diversity in the standing stock is needed. We will assess the quality of historical samples for future DNA analysis to assess the genetics of wild and refugia populations over time. To inform refugia collections, we will conduct a population genetic analysis of Comal Spring riffle beetles. Progress will continue to be made in Comal Spring riffle beetle propagation through a continuation of 2021 pupation trials. A handbook will be generated describing the advancements made toward successful collection and pupation. Salamander reproductive disfunction will be further investigated through habitat modification and Bd treatment trials. If successful, Bd treatment trials for aquatic salamanders will reduce refugia mortality and allow for transfers between SMARC and UNFH.

The total cost for proposed 2022 research, given the following projects, is approximately $515,969. Call for proposals from external partners to continue San Marcos salamander reproduction and Comal Springs riffle beetle pupation work will advertised and, if appropriate for Refugia needs, will be funded in 2022.

The following section describes the basic components of each of these proposed 2022 activities.

Project 1:

Title: Propagation of Comal Springs riffle beetles
Species: *Heterelmis comalensis*
Principal: BIO-WEST with FWS staff
Overview: A fully functional refugia requires predictable propagation. Based on evidence gleaned from previous research, we will calculate a target number of beetles then scale-up earlier attempts, propagating CSRB larvae at suitable densities with wild cultivated biofilm to test if we are able to meet our predicted targets.
Budget: $93,747.71
Benefit to the Refugia: This research will provide confirmation of progress toward a fully function refugium for this species.
Expected Results: We will produce a report for the EAA.

Project 2:

Title: Genetic assessment of Comal Springs riffle beetle
Species: *Heterelmis comalensis*
Principle/Co PI: FWS Staff
Overview: Little is known about the population structure and genetic diversity of the Comal Springs Riffle Beetle. A population-wide assessment can provide population metrics to inform future conservation and refugia needs. FWS will work with a partnering biologist, who is conducting an n-mixture model study on the abundance of Comal Springs riffle beetles, to collect adult Comal Springs riffle beetles across spring openings in Landa Lake and the Comal River. FWS staff will use high-throughput genome wide sequencing to make population measurements at the genetic level.

Budget: $ 141,344.64
Benefit to the Refugia: In combination with the occurrence study, the genetic assessment of the entire Comal Springs Riffle Beetle population will provide valuable information to the level of genetic variation and population structure in the wild. We do not yet know the extent of movement across spring openings at Landa Lake or how much genetic diversity is shared. The existence of distinct sub-populations would require different levels of representation in the refugia in order to reflect wild populations. Additionally, a range-wide genetic assessment can provide an estimate of the effective number of breeders, which would provide information to the minimum number of individuals that would need to be kept in refugia to accurately represent the wild population. This effort will greatly contribute to achieving a more complete refugia.
Expected Results: A report will be presented to the EAA and a peer-reviewed publication will be submitted.

Project 3:

Title: Handbook for the captive propagation of Comal Springs riffle beetles
Species: *Heterelmis comalensis*
Principal/Co PI: FWS Staff; BIO-WEST Support
Overview: The SMARC, BIO-WEST, and collaborating researchers have completed many investigations into the life history, collection, and husbandry of the Comal Springs Riffle Beetle. At this point in time, a document is needed that summarized the body of work that has been completed to date and provides a handbook for Riffle Beetle collection and captive holding. FWS and BIO-WEST will gather the data collected from field observations and collections and combine that with the data gathered through captive holding observations and research to develop a guide outlining what we know about Riffle Beetle life history and captive husbandry.
Budget: $59,735.15
Benefit to the Refugia: This document will provide SMARC biologists and partners with background knowledge of life history, as well as a standard set of SOPs for collection and captive husbandry. This document will be used as a training and reference tool for future SMARC staff and FWS partners.
Expected Results: A Report and an SOP for propagating Comal Springs riffle beetles will be presented to the EAA and a peer-reviewed publication will be submitted, if appropriate.

Project 4:

Title: Improve efficacy of tagging of small-bodied salamanders using p-Chip tags
Species: *Eurycea nana*
Principal/Co-PI: FWS staff
Overview: Previous tagging studies at the SMARC have shown improved efficacy of visible implant elastomer (VIE) tags over passive integrated transponder (PIT) or visible

implant alpha (VIA) tags for use in salamanders, being most effective in Texas blind salamanders. Although VIE tags can be used in smaller-bodied salamanders, there is a higher tag reading error rate and tag rejection rate. P-Chip tagging, a new tagging technology, is successfully used in small-bodied fish with very little morbidity or mortality. Additionally, the tags can be scanned and read without having to extensively handle the individual, reducing stress and potential physical harm. SMARC staff will test tag retention and readability of p-Chip tags in the small-bodied salamander, *Eurycea nana*.

Budget: $21,858.40

Benefit to the Refugia: Increased success in tagging small-bodied salamanders , and the ability to track each organism as an individual can improve refugia efforts and reduce stress to captive held animals. p-Chips are much smaller and less invasive than currently used tagging methods, which could reduce stress and potential morbidity to tagged individuals. Tracking organisms as individuals will inform basic life history aspects such as longevity and number of reproductive events per year. In future efforts, the genetic information of each individual can be collected non-lethally and associated with the individual's p-Chip ID. This will assist in developing higher level restocking strategies through ensuring the genetic diversity of refugia produced F1 offspring is representative of wild populations. Additionally, the refugia would no longer need to separate individuals by year or collection site, increasing refugia space for more individuals.

Expected Results: The results of the study will be presented as a report to the EAA, an updated tagging SOP, and a peer-reviewed publication (if applicable).

Project 5:

Title: Continuation of San Marcos salamander habitat modification and propagation manual (carry over from 2021)

Species: *Eurycea nana*

Principal: FWS staff

Overview: This study will continue 2021 efforts assess the effects of habitat manipulation on reproductive success of San Marcos salamanders. A San Marcos salamander propagation handbook will be developed. The handbook will provide a protocol for San Marcos salamander propagation with the best available information gathered through research and husbandry efforts.

Budget: $21,126.59

Benefit to the Refugia: Continued refinement of salamander reproduction and propagation. Information gained will guide additional research and inform reintroduction strategy.

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

Project 6:

Title: Fountain darters tissue catalog and DNA viability

Species: *Etheostoma fonticola*

Principal/Co-PI: FWS staff, SCA Student

Overview: An SCA Student, under the direction of SMARC Staff will inventory and catalog the many fountain darter tissue samples that have been preserved and kept on station from the 1990s to now. Taking inventory of these tissues and extracting their DNA would provide a valuable resource to compare genetic diversity in the San Marcos and Comal Springs fountain darter populations over time as well as compare contemporary diversity to historical diversity.
Budget: $29,818.60
Benefit to the Refugia: Provide the resources necessary to make comparisons between historic and contemporary population level genetic diversity of fountain darters.
Expected Results: The results of the study will be presented as a report to the EAA and a peer-reviewed journal article.

Project 7:

Title: Testing Bd treatments for aquatic salamanders
Species: *Eurycea nana*
Principal/Co-PI: FWS
Overview: Chytrid fungus, such as *Batrachochytrium dendrobatidis* (Bd), is a health concern for amphibians, including the aquatic salamanders associated with the Edwards Aquifer. Bd infections in amphibians are usually associated with reddened skin and tissue degradation of the toes and tail. In aquatic salamanders, issues with osmoregulation are also observed. Although San Marcos salamanders routinely test positive for Bd, we have yet to investigate Bd infections' potential impact on long-term aquatic salamander health. A common mortality observed in San Marcos salamanders held in refugia is rupturing of the abdominal cavity, potentially related to Bd infections. We will investigate the efficacy of Bd treatment options that have been pilot tested in other aquatic salamanders. We will record Bd infection status pre- and post-treatment as well as any long-term effects of treatment.
Budget: $35,736.78
Benefit to the Refugia: We will identify a treatment method for Bd in aquatic salamanders and develop an SOP for treating salamanders when they are collected and brought into the refugia.
Expected Results: Bd positive individuals will be Bd negative post treatment.

Project 8:

Title: Continuation of Comal Springs riffle beetle *Staphylococcus* exposure
Species: *Heterelmis comalensis*
Principal/Co-PI: Dr. Camila Carlos-Shanley (Texas State University)/FWS
Overview: Previous research has shown distinct differences in the microbial community of wild and captive held riffle beetles and biofilm food materials. Additionally, potentially harmful bacteria spp. (such as *Staphylococcus aureus*) were identified in higher abundance in captive held beetles. It is unclear if the increased relative abundance of bacteria, like *S. aureus*, is detrimental to beetle larvae survival and subsequent pupation. In 2021, we tested beetle survival after *Staphylococcus* exposure. This 2022

effort is continuation of the 2021 efforts. Samples were sent off for sequencing in 2021 but were lost in shipping. There are larvae from each treatment group on hand to continue the sequencing effort. The aim is to sequence the microbiome of the larvae exposed to *staphylococcus*, *Bacillus*, and a no bacteria added control to determine how high untypical bacteria exposure impacts the microbiome, which then can be correlated to overall survival and pupation rates of larvae in each treatment.
Budget: $19,557.43
Benefit to the Refugia: Determine if more strict biosecurity measures need to be in place to reduce bacterial exposure to beetle larvae. This study would also add to the overall understanding of how changes in the microbiome impact beetle survival and pupation in captivity.
Expected Results: There will be significant differences in the microbial communities of each treatment group.

Task 3. Species Propagation and Husbandry

Development and refinement of SOPs for animal rearing and captive propagation: SMARC and UNFH will 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, SMARC and UNFH will further develop draft Captive Propagation Plans for all species.

Task 4. Species Reintroduction

Reintroduction Plan for term of contract:
SMARC and UNFH continue to refine the Reintroduction Strategy as new information becomes available.

Reintroduction Plan for 2022: None

Any anticipated triggers being prepared for: Given current weather predictions, spring flows, and the Edwards Aquafer water level, no anticipated triggers are anticipated during the 2022 performance period.

Task 5. Reporting

5.1 Species specific Propagation plans (SOPs): Refine throughout year as needed
5.2 Species specific Genetic Management plans: Texas wild-rice, contingent on when genetic study results are finished.
5.3 Species specific Reintroduction plans: Refine as needed
5.4 2022 EAHCP Annual Program reporting– A year-end report of 2022 activities will be provided to the EAA no later than 1/31/2022.
5.5 Program reporting as required by ITP and TPWD. TPWD Scientific Research Permit Report will be filed July 31, 2022.
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
  - Professional Scientific Meetings

Monitoring:

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

Cost estimate:

U.S. Fish and Wildlife Service 2022		**Task Budget Amount**	**Total Task Budget Amount**
TASK 1	**Refugia Operations**		$836,473.48
	SMARC Refugia & Quarantine Bldgs.		
	Equipment & Building Maintenance	$ 15,000	
	Utilities	$ 14,000	
	UNFH Refugia & Quarantine Bldgs.		
	Equipment & Building Maintenance	$ 15,000	
	Utilities	$ 35,000	

	SMARC Species Husbandry and Collection Salaries	$ 150,851	
	UNFH Species Husbandry and Collection Salaries	$ 185,000	
	Water Quality System	$ 12,000	
	Divers Salaries	$ 3,500	
	Fish Health	$ 10,000	
	SMARC Reimbursable	$ 100,000	
	UNFH Reimbursable	$ 145,283	
	Subtotal	$ 685,634	
	Admin Cost Subtotal	$150,836.48	
TASK 2	**Research**		$515,968.84
	BIO-WEST: CSRB Propagation (2021 Rollover)	$ 49,451.71	
	BIO-WEST: CSRB Propagation	$ 30,000	
	BIO-WEST: CSRB Handbook contribution	$ 22,000	
	Texas State Research	$ 19,557.43	
	USFWS Research		
	Materials	$ 142,790.90	
	SMARC Staff	$ 142,839.54	
	UNFH Staff	$ 16,285.69	
	Subtotal	$ 422,925.28	
	Admin costs for Task 2	$ 93,043.56	
TASK 3	**Species Propagation and Husbandry**	-	-
	Subtotal	-	
TASK 4	**Species Reintroduction**	-	-
	Subtotal	-	
TASK 5	**Reporting**		$ 78,506.68
	SMARC Staff	$ 35,770.08	
	UNFH Staff	$ 28,579.66	
	Subtotal	$ 64,349.74	
	Admin costs for Task 5	$ 14,156.94	
TASK 6	**Meetings and Presentations**		$ 16,987.08
	SMARC Staff	$ 10,811.78	
	UNFH Staff	$ 3,112.06	
	Subtotal	$ 13,923.84	

	Admin costs for Task 6	$ 3,063.24	
	TOTAL	**$1,447,936.08**	

Projected (2022) Budget Summarized by Task:

Task 1: $836,473.48
Task 2: $515,968.85
Task 3: $0
Task 4: $0
Task 5: $78,506.68
Task 6: $16,987.08

Projected (2022) Subcontractor Expenses Summarized by Task

Task 1:
Task 2: BIO-WEST $101,451.71
Task 2: Texas State University $19,557.42
Task 3: $0
Task 4: $0
Task 5: $0
Task 6: $0

Timeline of 2022 Milestones

January	Continue with species collection
	2022 Specific Research Study Plans finalized
May/June	Subcontract research awards executed
July	Submit and renew TPWD permit
September to	Draft Research Reports
December	Draft Annual report

Literature Cited

AFS-FHS (American Fisheries Society-Fish Health Section). 2005. Model Quality Assurance/Quality Control Program For Fish Health Laboratories, 2016 edition. Accessible at: http://afs-fhs.org/bluebook/bluebook-index.php.

Hall, R (Edwards Aquifer Authority). 2016. 2016 Comal Springs Riffle Beetle SOP Work Group: Attachment 2: Existing CSRB Cotton Lure SOP. Available at: http://www.eahcp.org/index.php/administration/work_groups/2016_comal_springs_riffle_beetle_sop_work_group

Wilson, W. D., J. T. Hutchinson, K. G. Ostrand. 2016. Genetic diversity assessment of in situ and ex situ Texas wild-rice (*Zizania texana*) populations, an endangered plant. Aquatic Botany 136:212-219.

USFWS and AFS-FHS (U.S. Fish and Wildlife Service and American Fisheries Society-Fish Health Section). 2016. Standard procedures for aquatic animal health inspections. *In* AFS-FHS. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2016 edition. Accessible at: http://afs-fhs.org/bluebook/bluebook-index.php.

San Marcos Salamander Reproduction Handbook

2023 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Desiree Moore, Shawn Moore, Dr. David Britton, Eleanor E. Krellenstein, Richelle Jackson, and Dr. Katie Bockrath

San Marcos Aquatic Resources Center

Table of Contents

Introduction ..... **Error! Bookmark not defined.**

Housing 5

Food 6

Environment and water quality 9

Reproduction 10

Reproductive dysfunction 11

Eggs and hatchlings 14

Summary of procedures 15

References 16

Introduction

There are several endangered and vulnerable species in the Edwards Aquifer in Central Texas, including the federally threatened San Marcos salamander (*Eurycea nana*; U.S. Department of the Interior 1980). The Edwards Aquifer supplies Spring Lake, San Marcos, TX with water through multiple spring openings. San Marcos salamanders exclusively inhabit Spring Lake, the headwaters of the San Marcos River, and the first 150 m of the San Marcos River below Spring Lake Dam (Nelson 1993). The water in Spring Lake is thermally stable year-round (21-22 ºC), with dissolved oxygen approximately 4 mg O2/L and pH of 7.1-7.6 (Ogden et al. 1985). The San Marcos salamander population size has been estimated to be from 21,000 (Tupa and Davis 1976) to 54,000 (Nelson 1993). Since the population numbers are low and the geographic range of the species is limited, the San Marcos salamander is considered threatened.

San Marcos salamanders are small, neotenic salamanders in the family Plethodontidae (Mackay 1952). Their life span is unknown, but they have been documented to live to at least three years in captivity after collection from the wild (Campbell and Anderson 2020). There is some disagreement concerning the existence of a true breeding season (i.e., when viable eggs are laid) for the San Marcos salamander. Mackay (1952) postulates that the San Marcos salamander's breeding season is late spring (May or June), but there may be a second breeding season in late fall. However, some researchers hypothesize San Marcos salamanders lay viable eggs year-round (Tupa and Davis 1976; Nelson 2001; Najvar 2001). Wild San Marcos salamanders can grow up to 56 mm in total length (TL; Nelson 1993). There is some uncertainty regarding the body length of the San Marcos salamander at sexual maturity. Tupa and Davis (1976) described wild females and males as sexually mature at a snout-to-vent length (SVL) of approximately 21 mm and 19 mm, respectively. However, Mackay (1952) found that wild males generally reach sexual maturity at a TL of 35 mm, and Bishop (1941) states that wild San Marcos salamanders are sexually mature when they reach 41 mm TL.

Knowledge of reproductive morphology, physiology and behavior is critical to the survival of threatened and endangered species. Reproductive success could determine if an endangered or threatened species is able to persist and recover from a perturbation or becomes extinct. Knowledge of San Marcos salamander propagation in captivity is important if the goal is to have a fully functional refugia population. A fully functional refugia population represents the identity and range of genetic diversity found in wild populations and can reproduce effectively in captivity. Wild stock salamanders are held in captivity to propagate F1 salamanders (i.e., first generation individuals produced in captivity). The F1 generation could be reintroduced into the wild in the event of extirpation or extinction in the native habitat. Generally, salamanders of the F2 (i.e., produced by F1 generation individuals) and later generations are considered "hatchery-adapted" and thought to not be able to survive and reproduce successfully in the wild. It is important that the refugia salamanders are as

physiologically healthy and reproductively sound as possible in the event mass reproduction is needed to supply reintroduction efforts.

San Marcos salamander eggs have never been observed in the wild (Tupa and Davis 1976; Nelson 1993), although juvenile San Marcos salamanders are commonly found. Therefore, all available information about San Marcos salamander eggs is based on reproduction in captive populations. A captive female San Marcos salamander can deposit up to 73 eggs per clutch (Najvar 2001), 176 eggs per year, and over 500 eggs in a lifetime (Najvar et al. 2007). The eggs take 16-24 days to hatch (Najvar et al. 2007). It is believed that San Marcos salamanders lay their eggs on the undersides of rocks or in interstitial spaces among gravel near springs in the wild (Nelson 1993). In captivity, they commonly lay their eggs on rocks and aquatic vegetation, but eggs have been observed on all available surfaces in tanks. More eggs are laid in captive systems with upwelling water rather than no upwelling water (Najvar 2001), which supports the theory that wild San Marcos salamanders lay their eggs around spring vents. In captivity, substrate such as small limestone rocks or glass marbles, through which upwelling water flows at a rate of 1 cm/s, is optimal for San Marcos salamanders (Fries 2002). It is beneficial to mimic natural conditions in captivity as closely as possible to enhance propagation efforts.

The San Marcos Aquatic Resources Center (SMARC) has held San Marcos Salamanders in an *ex situ* refugium for decades. Between 1995 and 2015 the SMARC averaged 112 San Marcos salamanders per year on station. The average survival rate for San Marcos salamanders from 1996 through 2015 was 73%. An official partnership with the Edwards Aquifer Authority began in 2017, placing the U.S. Fish and Wildlife Service under contract to maintain a refuge population of San Marcos salamanders at each of two stations. Uvalde National Fish Hatchery (UNFH) was selected as the second station. Under the new Edwards Aquifer Habitat Conservation Plan, our target was to hold 500 individual San Marcos salamanders, split evenly, between the two stations. In 2017, new staff were hired at both stations to cover the duties of the new Edwards Aquifer Refugia Program (EARP). The new staff achieved a 74% survival rate for San Marcos salamanders at the SMARC, and a 90% survival rate at the UNFH their first year.

Reproductive dysfunction, or increased difficulty with propagation, has been observed in captive San Marcos salamanders, with a higher mortality rate in gravid females than in males and non-gravid females (Anderson and Campbell 2019). These observations were recorded unofficially in USFWS standard operating procedure documents prior to EAHCP reproduction efforts but were not published. In 2017, 50% of adult San Marcos salamander mortalities were observed in gravid females (Campbell and Anderson 2018). It is currently unknown why reproductive dysfunction occurs or if this phenomenon also occurs in the wild (Anderson and Campbell 2019).

Male San Marcos salamanders may have “multiple testes,” which is a phenomenon that occurs in only large individuals (Mackay 1952). This is a condition that involves a single testis with multiple lobes or a single testis with multiple functioning regions divided by non-

functioning regions (Mackay 1952). More sperm can be produced and deposited by these individuals, which may positively affect reproduction. San Marcos salamanders are sexually dimorphic, with only males having acinar glands in their lower jaws and at the bases of their tails (Sever 1985). Males initiate courtship of females by rubbing pheromones from the acinar glands in their jaws on the females (Campbell and Anderson 2020). Then, the male performs the "tail-straddle walk" (TSW) courtship ritual, in which he approaches the female from behind, stands with his upper body straddling her hindquarters, and walks along with her (Campbell and Anderson 2018). TSW has been observed to occur in captivity for up to 45 minutes (Campbell and Anderson 2018). The gravid female deposits her eggs and the male deposits spermatophores, or sperm packets (Campbell and Anderson 2018). Both males and females exhibit mate choice (Thaker et al. 2006).

Housing

In their natural habitat, San Marcos salamanders are found under rocks and among aquatic vegetation near spring upwellings in Spring Lake and in the first 100 yards below the headwaters of the upper San Marcos River (Nelson 1993). In both wild and captive settings, salamanders aggregate under rocks or other types of cover in groups of two or more.

San Marcos salamanders are not conditioned to bright overhead light or high disturbance conditions (Anderson and Campbell 2019). In an aquarium under fluorescent lights, salamanders react to movement around the tank by moving further under shelters or areas of cover. These conditions are typical within the refugia and difficult to mitigate due to the nature of research and animal care. Some environmental stress may be reduced by covering tanks and avoiding movement around aquaria unless necessary. These measures are especially important for salamanders that have been recently collected from the wild and brought into the refugia. Minimizing the stress of newly collected wild salamanders is integral to their health and survival.

Figure 1. A PVC pipe cut in half is placed on tank dividers to prevent salamanders from moving between sections.

It is recommended captive salamander populations not exceed a density of one salamander per gallon and tanks should not be filled more than three-quarters full to prevent escape (Anderson and Campbell 2019). Tanks

of any size that meet these two parameters can be used to house San Marcos salamanders safely. However, tanks with a higher surface area to volume ratio are preferred because San Marcos salamanders tend to stay near the tank floor. San Marcos salamanders are not held in tanks separated based on collection site based on a genetic analysis that suggested no population structure within their native range (Lucas et al. 2009). Additionally, PVC half pipe "corrals" can be placed on tanks with dividers to prevent salamanders from escaping or travelling between tank sections (Figure 1).

All housing components are cleaned regularly to preserve good water conditions and avoid potential illnesses. All excess food is removed the day following feeding. Algae is scrubbed from tank surfaces every two to four weeks depending on growth. Algal growth increases in tanks that get more direct light and have higher densities of salamanders. Housing components are descaled manually or chemically one to two times annually. Chemical descaling is conducted by using a acidic solution with pH 4 for 24 hours (i.e., 20% acetic acid or 30% muriatic acid diluted with water). Descaling is needed more often in tanks where calcification builds more quickly.

This species does not demonstrate strong social interactions beyond cohabitation and mating rituals. Aggressive behavior among conspecifics has not been observed in adults of this salamander species (Thaker et al. 2010). A lack of territory overlaps and mobility between areas of cover creates a scarcity of mates in natural conditions which drives the need for reproduction (Anderson and Campbell 2019). When male and female salamanders are housed together in captivity the demand to reproduce is thought to be decreased by the constant availability of mates. Researchers have had some success in triggering reproductive events by separating salamanders by sex for several months, then recombining salamanders in heterosexual pairs (Campbell and Anderson 2020). It is unknown if San Marcos salamanders rely on visual or hormonal cues to trigger mating behavior.

Food

The San Marcos salamander diet is largely invertebrate based. Analyses of stomach and intestine contents indicate wild San Marcos salamanders in Spring Lake most commonly consume tendipid larvae and pupae and amphipods (Tupa and Davis 1976). When examining the gut contents of San Marcos salamanders in Spring Lake and the headwaters of the San Marcos River, it was found that these salamanders are generalist aquatic invertebrate predators with amphipods, caddisflies, and ostracods making up most of their diet (Diaz 2010). Furthermore, Diaz (2010) found no difference in diet based on salamander size, sex, or diel variation.

In captivity, San Marcos salamanders receive a variety of frozen and live feed that mimic their wild diet as much as practically possible. Frozen feeds are sometimes more practical because they can be reliably obtained and safely stored for long periods of time. San Marcos

salamanders receive varying diets of artemia, amphipods, *Daphnia*, blackworms, *Mysis*, copepods, and bloodworms based on the life stage of the salamander. Juvenile salamanders receive live artemia, or brine shrimp. This food source is best suited for their size as it is small enough to fit into their mouths and is easy for them to capture. Once juveniles grow to approximately 25.4 mm SVL, they are transitioned to live *Daphnia* and amphipods as a larger source of prey. Adult salamanders in the refugia receive a variety of food sources that are rotated throughout the week. *Mysis* and bloodworms arrive frozen and are thawed before feeding. Copepods are refrigerated. *Daphnia*, amphipods, and blackworms are fed live to allow the salamanders to hunt for their prey.

Some feeds are used as supplements when others are not readily available. Amphipods are fed to captive salamanders in place of *Daphnia* if *Daphnia* availability is low. The amphipods are harvested from one of the culture ponds at the SMARC. However, it can be difficult to maintain cultured stock due to seasonal variation and overharvesting, and *Daphnia* culture was developed as a more reliable feed at times of low amphipod production. Additionally, refrigerated copepods were tested as a substitution for *Mysi*s. This alternative was successful, and copepods are fed when *Mysis* stores are low and as a supplement to frozen *Mysis* solution.

San Marcos salamanders are fed twice weekly, and feeds are portioned according to the number and stage (juvenile or adult) of individuals within each tank. A disposable pipette is used to measure and distribute feed. Salamanders are fed 0.25 mL of each food item per individual. *Mysi*s and *Daphnia* are void of air bubbles and blackworm portions are drained of water. Salamanders are fed twice per week on Tuesdays and Fridays. Juveniles are fed artemia both days. Adults are fed *Mysis* and/or copepods and amphipods or *Daphnia* on Tuesdays and blackworms and amphipods or *Daphnia* on Fridays.

Recently, the SMARC began introducing frozen bloodworms into the San Marcos salamander diet by mixing them with *Mysis*. It was decided that *Mysis* would be the best medium to mix with as both are frozen. The introduction of bloodworms into the San Marcos salamander diet was suggested because blackworms are not always readily available and it provides some variety without having to rely on live cultured feeds. Bloodworm introduction began with a 1:4 ratio of bloodworms to *Mysis*, and the quantity of bloodworms will gradually increase until a 1:1 ratio is reached. San Marcos salamanders are observed to predominately feed on the bloodworms and eat around the *Mysis*. Therefore, *Mysis* is hypothesized to be less palatable to salamanders than bloodworms.

The feeds selected are limited by the gape size of the San Marcos salamander. Red worms were considered as an alternative live feed, but the integration into feedings was not successful because their diameter is too large for successful hunting by San Marcos salamanders.

There are no existing data showing a direct relationship between diet and reproduction in San Marcos salamanders, but it is known having proper nutrition and general health is

necessary for successful reproduction (Keulen and Janssens 2017). High levels of barium and inflammation of the adipose tissue (steatitis) are potential health concerns that arise from the diet in salamanders at the SMARC.

A diet incorporating blackworms is associated with higher levels of barium found within captive stock salamanders (Anderson and Campbell 2019). High concentrations of barium have been shown to negatively affect the ability to reproduce in amphibians. Wild salamanders were shown to have a significantly lower level of barium in their systems in comparison to their captive counterparts (Anderson and Campbell 2019). Additionally, the barium level within an individual tends to increase the longer the salamander is in captivity. It is hypothesized that the large quantity of blackworms in the captive salamander diet causes bioaccumulation of barium over time.

Steatitis is an inflammation within fatty tissues and can be caused by contaminated feed or imbalances in the diet or pathogens (Wright and Whitaker 2001). The Texas blind salamanders in the captive population at the SMARC have been diagnosed with the non-pathogenic form of steatitis. Although it is inconclusive what causes this illness in the Texas blind salamanders, it is possible the steatitis was due to contaminated feed, too much saturated fat in the diet, or insufficient vitamin E consumption (Wright and Whittaker 2001). San Marcos salamanders have not shown visible symptoms of steatitis, but if the condition is related to diet in the Texas blind salamanders, it is possible that San Marcos salamanders would also be affected. It is possible that the San Marcos salamander's heavier pigmentation and propensity for hiding makes it difficult to observe signs of infection (e.g., yellow appearance, lethargy, loss of appetite). Due to the diagnosis in the Texas blind salamanders, steps were taken to improve salamander diets. All *Mysis* supplies were thrown out and replaced in case contamination occurred or the *Mysis* had gone rancid. Feed containers with smooth sides were purchased to ensure that there were no ridges and all surfaces could be properly disinfected. SMARC staff also supplemented Vitamin E to a subset of Texas blind salamanders via soaked *Mysis*. The *Mysis* was soaked in a diluted vitamin E solution for several minutes before feeding

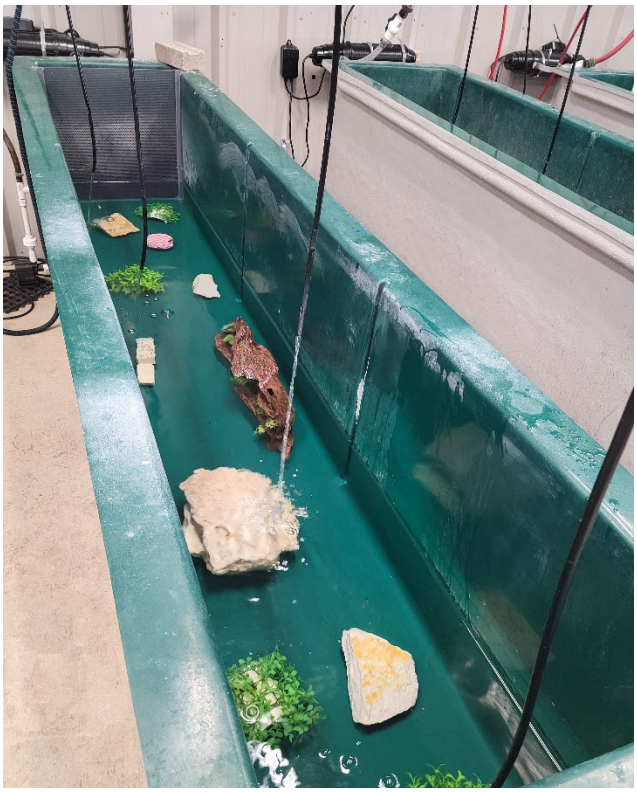


Figure 2. San Marcos salamander tank with rock piles, artificial plants, and aquarium habitat

to salamanders, and this procedure was implemented once weekly over the course of a month. The supplementation yielded no clear positive or negative results. Overall, the other preventive measures (smooth containers and replaced *Mysis*) yielded less mortality and morbidity.

Environment and water quality

San Marcos salamanders are commonly found in areas with bedrock or gravel substrate and aquatic moss or filamentous algae (Diaz 2010). The refugia uses rock piles, mesh tubes, and artificial plants or aquarium habitat to provide salamanders with adequate shelter and structures to deposit eggs (Figure 2; Anderson and Campbell 2019). Artificial vegetation and nonporous structures are preferred for biosecurity and ease of cleaning. San Marcos salamanders seek cover under large rocks, in gaps within rocky substrate, and in mats of filamentous algae (Diaz 2010). They use substrate and vegetation to lay their eggs in areas with consistent flow. In captivity, salamanders have been observed using rocks, marbles, and artificial vegetation for oviposition (Najvar 2001). In a study comparing different flow sources and types of cover, upwelling-flow tubes surrounded by moss and marbles were preferred by San Marcos salamanders for oviposition (Najvar et al. 2007).

Staff at the SMARC try to imitate the water quality conditions of the wild as much as practically possible. In the wild, San Marcos salamanders are found near spring openings, presumably due to the higher availability of amphipods. A laboratory study found that San Marcos salamanders prefer slow flowing conditions, with water flow of 1 cm per second (Fries 2002). The refugia uses an overhead flow system on most tank systems. Several studies have been conducted on the use of upwelling-flow tubes to increase egg deposition and clutch viability, but upwelling-flow is not necessary for reproductive success. Additionally, upwelling-flow tubes are more difficult to maintain and impeded organism inventories. Adequate water flow is necessary to oxygenate eggs as they develop (Fries 2002).

Water from the San Marcos Springs is typically 21.0°C -21.5°C but can reach temperatures as high as 23°C during warmer weather. The San Marcos River has a dissolved oxygen content of approximately 4 mg/L at spring openings and ranges from 3 mg/L to 5 mg/L downstream (Najvar, 2001). The pH of the San Marcos River water ranges from 6.9 to 7.8 (US Fish and Wildlife Service 1996). The water conditions of the San Marcos and Uvalde refugia closely resemble those of the San Marcos River because well water is pumped directly from the Edwards Aquifer for organism care. Water circulation within organism tanks is crucial to keep ammonia levels low, pH stable, and oxygen saturation adequate. The water is monitored for temperature changes daily, and pH changes monthly. Heater-chiller units assist in keeping the water a constant temperature. This consistency is especially necessary during extreme weather that often occurs in late summer and winter.

Reproduction

Plethodontid salamanders perform complex courtship behaviors. Male San Marcos salamanders contain glands that produce courtship pheromones on their chins and premaxillary teeth that are used to rub the pheromones on female salamanders (Najvar 2001). Campbell and Anderson (2018) details the courtship dances of San Marcos salamanders. Briefly, the courtship pattern is approach-rub, TSW, and tail waggle. After courtship, it is assumed males deposit the spermatophore, and females pick it up. Several methods to trigger reproduction in San Marcos salamanders were examined, but none were found to increase reproduction.

In 2018, the EARP conducted a study to compare the number of egg clutches laid by pairs and groups of San Marcos salamanders using the separation/reunion technique. The study found that more frequent reproduction occurs in groups of salamanders rather than mating pairs, but groups must be selected carefully to reduce the chance of inbreeding. This study also found that courtship behavior was most intense immediately following the reunion of separated salamanders and slowed as time passed thereafter.

Based on observations made in the 2018 reproduction study, SMARC staff conducted a pilot study in 2019 using only grouped San Marcos salamanders with removal of males after 72 hours. Staff recorded one pair and one group from three angles to capture spermatophore deposition on video. 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. Despite efforts, no oviposition occurred in that study.

In 2020, San Marcos salamanders at the SMARC were treated with a hormone to examine its effects on reproduction. Following the advice of Dr. Ruth Marcec-Greaves (DVM, Ph.D., and Director of the National Amphibian Conservation Center, Detroit Zoological Society), SMARC staff tested the use of Luteinizing Hormone Releasing Hormone (LHRH, also known as gonadotropin releasing hormone or GnRH) in a pilot study, to be scaled-up to full experiment if successful. After no deleterious effects from LHRH were observed in a pilot experiment, staff conducted a large-scale experiment using all mature captive wild stock San Marcos salamanders. LHRH was applied at 50 µg/g only to males. No increase in clutches laid after the application of LHRH was observed.

In 2021, a study examining the effects of darkened tanks and textured tank bottoms on San Marcos salamander reproduction was held at the UNFH. Pairs of salamanders were held in aquaria instead of groups due to salamander availability. A quarter of the tanks were covered with a film to reduce light penetration, a quarter had textured tank bottoms attached, a quarter were covered with the film and had textured tank bottoms, and a quarter had neither. No oviposition occurred over the duration of this study, indicating that darkened tanks and textured tank bottoms do not trigger reproduction in San Marcos salamanders.

Egg production by San Marcos salamanders varies greatly between the SMARC and the UNFH (Table 1). Although the UNFH had a higher number of eggs per clutch in 2022, the resulting number of eggs overall was still much lower than at the SMARC. Additionally, no clutches were laid by San Marcos salamanders at the UNFH in 2021. It is unknown what drives this dramatic difference in reproduction between the two facilities. SMARC and UNFH staff are working to examine any differences in husbandry between the facilities and develop standardized husbandry practices. The differences in the aquifer water pumped to each facility might contribute to some variation, but that is not a factor currently within staff's control. The decrease in average percent of eggs hatched at the SMARC from 2021 to 2022 is partly due to several clutches laid in all-female tanks. These clutches were not fertilized and 0% of them hatched. It is interesting that pheromones were not the trigger for these females to produce these clutches, but the 2018 reproduction study did note several instances of courtship behavior between females. These numbers indicate that 200-300 viable eggs can be produced at the SMARC within a year if good conditions are provided. It is possible these were particularly good years with some unknown factor contributing to the fecundity of the captive San Marcos salamanders. However, the data is not available to accurately compare to previous years' production. Staff at the SMARC and UNFH will continue to monitor these metrics carefully to provide a more complete picture of oviposition trends to be expected.

Table 1. The oviposition information for San Marcos salamanders at the SMARC and UNFH in 2021 and 2022. Average number of eggs per clutch and average percent of eggs hatched does not include information on salamanders donated to research partners.

	2021	2022
SMARC		
Number of clutches	21	25
Average eggs/clutch	18.6	16.8
Average % hatched	78%	40%
UNFH		
Number of clutches	0	2
Average eggs/clutch	-	27
Average % hatched	-	72%

Reproductive dysfunction

SMARC staff first reported abdominal ruptures in female San Marcos salamanders in 2017. Unable to release or reabsorb eggs naturally, the abdomens of some female San Marcos salamanders bloat with fluid and eventually rupture, releasing eggs and fluid from the body cavity. A veterinarian was asked to investigate the issue to determine potential cause and possible treatments. Deaths due to complications of female abdominal rupture accounted for 34% of all SMARC San Marcos salamander mortalities and 50% of adult mortalities.

In 2018, salamanders were held in groups differentiated by size. Staff witnessed no major losses, gains, or disease outbreaks for the San Marcos salamander refugia. However,

mortality due to abdominal ruptures continued at the SMARC, accounting for 35% of all adult mortality, which was an improvement from 2017. Staff noted that abdominal rupture occurred predominantly with San Marcos salamanders of unknown or older ages rather than newly collected and presumably younger individuals. A USFWS veterinarian examined salamanders with swollen abdomens (coelomic effusion) from the UNFH and noted the condition may have been associated with a difficulty in maintaining osmotic balance or declining nutritional condition leading to hypoproteinemia (low levels of protein in the blood). Inflammation of the ovaries (oophoritis) was also noted. The USFWS veterinarian expressed concerns with ambient dissolved oxygen (DO) availability at the UNFH and recommended adjustments to husbandry to increase DO levels for improved health of the salamanders.

At the SMARC, staff reported a marked difference in total survivor rates (i.e., including males and females) between San Marcos salamanders that were collected in the fall of 2017 through 2018 compared to those collected before the fall of 2017. These older salamanders were already at the facility before the EARP started. These were designated "heritage" salamanders. The survival rate of the heritage group of San Marcos salamanders was 54% in 2018. The survival rate of the more recently collected (non-heritage) salamanders at the SMARC was 88%. At UNFH, we held no heritage salamanders, and the average survival rate of San Marcos salamanders was 83%.

Staff sent mortalities at each facility to the USFWS Fish Health Unit for analysis. Necropsies revealed complications from microsporidiosis, an infection of unicellular microsporidian parasites causing necrosis and atrophy in the pelvic girdle area and the gonads. Microsporidia and chytrid fungus have previously been reported in this and related species. There is no known treatment.

In 2018, a USFWS veterinarian reported that these mortalities appear to be a facility problem rather than a ubiquitous issue such as microsporidia due to the severity of the lesions and the lack of comparable lesions in animals from Uvalde.

In 2019, the cases of egg-related mortality continued to decline but still occurred in refugia populations at both facilities. The survival rate of the heritage group of salamanders was 42.2% in 2019 at the SMARC. In contrast, the survival rate of the more recently collected (non-heritage) salamanders was 85.5%.

SMARC water was tested for endocrine disrupting compounds and other deleterious compounds. 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. We found unusually high levels of barium in captive individuals, that increased corresponding with time in captivity. We also found that micropsoridial infection rates were much higher in captive salamanders than in wild populations. Micropsoridial infections tended to be concentrated in the ovaries and reproductive organs of the salamanders (personal communication, USFWS Fish Health Unit).

In 2020, staff at the SMARC discontinued blackworms as a food source for one year after finding that blackworms were high in barium. Due to the low availability of other live feeds and no definitive indications that the barium harms San Marcos salamanders, blackworms were reintroduced into their diet in 2021. The EARP also hired a person to lead husbandry and collections at both the SMARC and UNFH. The husbandry lead evaluated both stations and began to implement improvements.

Our veterinarian reported that mycobacteriosis appears to be the primary cause of illness in these animals. Mycobacteriosis is a persistent and chronic pathogen and there is currently no treatment. Its pattern centered in the reproductive tract could indicate an ascending infection from the environment. Any additional animals added to systems with mycobacteria are likely to become infected.

Survival rate in 2020 was 60% at the SMARC and 81% at the UNFH. The cases of egg-related mortality continue to decline but were still found in refugia populations at both facilities. Individuals from other younger populations (collected in 2017 and 2018) began to show similar issues as the older, heritage group. Staff hypothesized that reproductive-related death may increase with animal age or time in captivity.

Survival rate in 2021 was 56% at the SMARC and 82% at the UNFH. Reproductive-related death continued to increase with animal age and time in captivity. The salamanders held at the UNFH continue to have higher survival rates than those at the SMARC, although husbandry practices are becoming more similar between facilities. It is important to note that egg production is much higher at the SMARC than the UNFH, and it has been hypothesized that this increased production might be part of the reason egg-related mortality is also increased. It is unknown if this egg-related mortality occurs in older individuals in the wild.

Our efforts to improve and predict reproduction in San Marcos Salamanders has not revealed a solution to these problems. We tried observational studies, manipulating the presence of conspecifics, adjusted food, assessed water quality, applied reproductive hormones, and manipulated habitat characteristics. We found no evidence that any of these improve reproduction or its predictability. Meanwhile, we continued to observe bacterial (Mycobacteria), fungal (Microsporidia, Saprolegnia, and Batrachochytria), and parasitic (Tremetodal) infections in our captive populations.

Table 2. Annual survival of all San Marcos salamanders in captivity at the San Marcos Aquatic Resources Center (SMARC) and the Uvalde National Fish Hatchery (UNFH) from 2017 through 2022.

	2017	2018	2019	2020	2021	2022
SMARC	77%	71%	77%	60%	56%	60%
UNFH	90%	83%	75%	81%	82%	84%

Eggs and hatchlings

Once a clutch of San Marcos salamander eggs is observed, staff moves them from the parent tank to a separate system designed for eggs. The cannibalism of eggs (oophagy) has been observed in San Marcos salamanders at the SMARC (i.e., staff observations). Therefore, it is important to move the eggs as soon as possible to prevent loss. Housing is similar to that of the adult salamanders; however, less space and well water input is needed. The eggs only need enough surface area in the tank to keep them from touching one another and at least two-three inches of depth to give hatchlings room to begin swimming. However, the tanks currently used for egg and juvenile rearing are much deeper provide space for salamanders to begin swimming (Figure 3). Eggs should be kept from touching one another to reduce

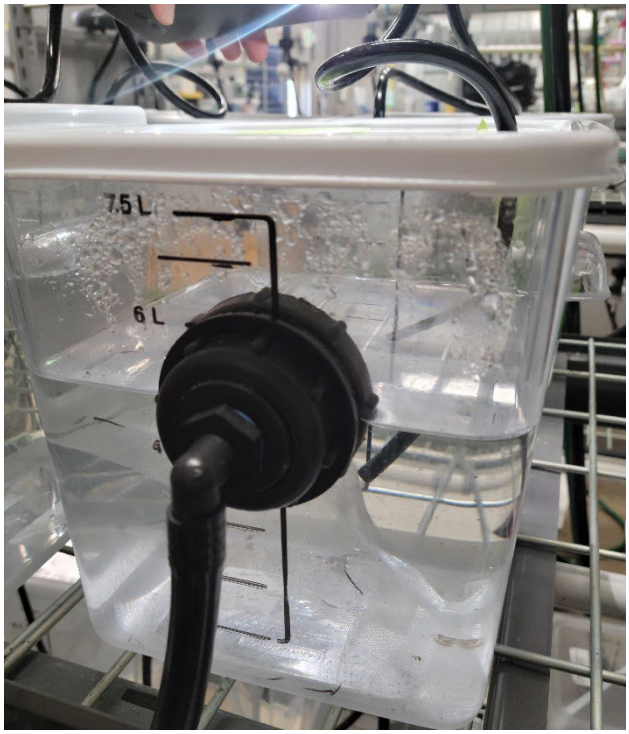


Figure 3. Newly hatched juvenile San Marcos salamanders in their hatch tank with mesh habitat.

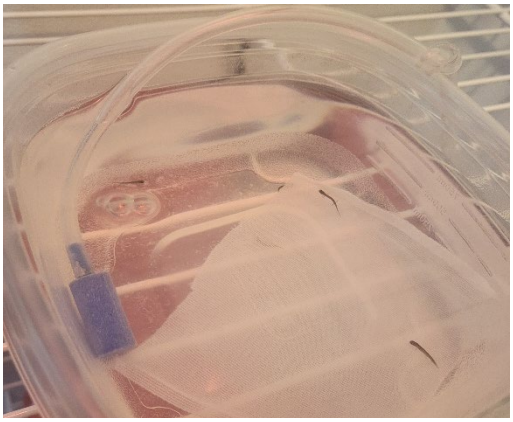


Figure 4. Static San Marcos salamander egg and juvenile enclosure with mesh for habitat.

the chance of fungus infection spreading among eggs. Fungus commonly occurs on unfertilized and undeveloped eggs, but healthy developing eggs can be infected with fungus if they are touching an infected egg. Fungused eggs are removed daily to prevent fouling the water. It is thought that placing the eggs on a small piece of mesh can also reduce the spread of fungus. The mesh also provides habitat for the salamanders once they hatch. The eggs consume less oxygen and excrete less waste than hatched salamanders, requiring only a small amount of well water input. It is also possible to house the eggs in a static system with an air stone if two to three water changes are performed each week (Figure 4).

Hatched salamanders can feed on their yolk for a short time before they must be provided supplemental nutrition. Hatchlings are fed artemia at two weeks post-hatching. It is also possible to cut up and carefully feed blackworms or bloodworms to the hatchlings using forceps. Excess feed must be removed from the tank quickly after feeding to prevent fouling the water. Alternatively, the water flow can be increased to flush the food out without removing it manually. Salamanders are incorporated into the refugia stock at ten weeks post-hatching. Prior to ten weeks, mortality is elevated due to birth defects, competition, injuries, etc.

Summary of procedures

Although reproductive success is varied at the EARP, we suggest the following guidelines for successful reproduction based on program records thus far:

- As little disturbance in and around tanks as is possible,
- Tanks with a high surface area to volume ratio where the water is filled three-quarters full or less,
- Densities of one salamander per gallon,
- A regular cleaning schedule based on feeding, algal growth, and calcification rates,
- Separating salamanders by sex for a month or more and then recombining the sexes to trigger courting,
- Feeding a variety of food items to provide a nutritionally variable diet until we know more about the nutritional needs of this species,
- Providing a variety of habitat items (e.g., rock piles, mesh, artificial plants, aquarium habitat) for cover and oviposition structure,
- Providing flowing water to tanks or completing regular water changes (two to three per week) if flowing water is not possible,
- Providing water quality conditions (e.g., temperature, pH) as close to the natural habitat as possible,
- Removing eggs as soon as possible after oviposition,

- Keeping eggs from touching one another and removing any sources of fungus, and
- Feeding juveniles starting two weeks from their hatch date.

These steps have been shown to improve overall reproductive success for San Marcos salamanders in the EARP. More research is needed to refine and add to these steps to further improve the standard operating procedures for San Marcos salamander reproduction.

References

Anderson, K., and L. Campbell. 2019 Edwards Aquifer Refugia Program research report: salamander reproductive dysfunction. https://www.edwardsaquifer.org/wp-content/uploads/2020/04/2019-Refugia-Appendices.pdf.

Bishop, S. C. 1941. Notes on salamanders with descriptions of several new forms. Occasional Papers of the Museum of Zoology 451:1–25.

Campbell, L., and K. Anderson. 2018. 2018 salamander reproduction research report: investigating San Marcos salamander reproduction in captivity. https://www.edwardsaquifer.org/wp-content/uploads/2020/04/2018-Refugia-Appendices.pdf.

Campbell, L., and K. Anderson. 2020. Continuation of captive reproduction techniques in San Marcos salamanders (*Eurycea nana*). https://www.edwardsaquifer.org/wp-content/uploads/2022/05/2022-EAA-Work-Plan-Am ended.pdf.

Diaz, P. H. 2010. Diet and mesohabitat associations of the threatened San Marcos salamander (*Eurycea nana*). Texas State University, San Marcos, Texas.

Keulen, J., G. Janssens. 2017. Nutrition of captive amphibians. Master's Dissertation, Ghent University.

Lucas, L. K., J. N. Fries, C. R. Gabor, and C. C. Nice. 2009. Genetic variation and structure in *Eurycea nana*, a federally threatened salamander endemic to the San Marcos Springs. Journal of Herpetology 43(2):220-227.

Fries, J. N. 2002. Upwelling flow velocity preferences of captive adult San Marcos salamanders. North American Journal of Aquaculture 64(2):113-116.

Najvar, P. A. 2001. The effects of diel water quality fluctuations on reproduction and growth in the San Marcos salamander. Unpublished M.S. thesis, Southwest Texas State University, San Marcos.

Najvar, P. A., J. N. Fries, and J. T. Baccus. 2007. Fecundity of San Marcos salamanders in captivity. The Southwestern Naturalist 52(1):145-147.

Nelson, J. M. 1993. Population size, distribution, and life history of *Eurycea nana* in the San Marcos River. Southwest Texas State University. https://www.edwardsaquifer.org/wp-content/uploads/2019/02/1993_Nelson_Eurycea-nan a.pdf

Ogden, A. E., A. J. Spinelli, and J. Horton. 1985. Hydrological and hydrochemical data for the Edwards Aquifer in Hays and Comal counties, October 1981 to September 1983. Edwards Aquifer Research and Data Center, Southwest Texas State University, San Marcos, Texas. EARDC Number R1-85.

Sever, D. M. 1985. Sexually dimorphic glands of *Eurycea nana*, *Eurycea neotenes*, and *Typhlomolge rathbuni* (Amphibia: Plethodontidae). Herpetologica 41:71-84.

Thaker, M., C. R. Gabor, and J. N. Fries. 2006. Sensory cues for conspecific associations in aquatic San Marcos salamanders. Herpitologica 62:151-155.

Thaker, M., J. Fries, K. Epp, and C. Gabor. 2010. Cohabitation patterns of the San Marcos salamander (*Eurycea nana*). Amphibia-Reptilia 31:503-508.

Tupa, D. D., and W. K. Davis. 1976. Population dynamics of the San Marcos salamander, *Eurycea nana* Bishop. Texas Journal of Science 27:179-195.

U.S. Department of the Interior. 1980. Endangered and threatened wildlife and plants; listing of San Marcos salamanders as threatened, the San Marcos gambusia as endangered, and the listing of critical habitat for Texas wild rice, San Marcos salamander, 51 San Marcos gambusia, and fountain darter. Federal Register 45(14 July 1980):47355- 47364.

U.S. Fish and Wildlife Service. 1996. San Marcos and Comal Springs and associated aquatic ecosystems (revised) recovery plan. U.S. Fish and Wildlife Service ecological services office, Austin, Texas.

Wright K., and R. Whittaker. 2001. Amphibian medicine and captive husbandry. Krieger Publishing, Malabar, FL.

Captive Husbandry and Propagation of the Comal Springs Riffle Beetle

2022 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Randy Gibson, Ameila Hunter, Desiree M. Moore, Jennifer Whitt, Braden R. West, Adam Daw, and Dr. Katie Bockrath.

San Marcos Aquatic Resources Center
U.S. Fish and Wildlife Service

TABLE OF CONTENTS

2022 Research Report for the Edwards Aquifer Authority 0
1 Introduction 2
2 Life History and Ecology 3
2.1 Habitat 3
2.2 Diet 4
2.3 Life History 5
2.3.1 Eggs 6
2.3.2 Larvae 6
2.3.3 Pupae 7
2.3.4 Adults 8
3 Genetics 8
4 Collection 9
5 Captive Husbandry 9
5.1 Evolution of Housing 9
5.1.1 Box Containers 10
5.1.2 Flow-through Tubes 14
5.2 Current /Future Improvements 14
5.3 Assessment of Water Quality Needs 15
5.4 Feeding materials 15
5.5 Effects of Handling and Light Exposure 17
6 Captive Propagation 17
6.1 Seasonality 18
6.2 Flow 18
6.3 Timing for Generation Separation 18
7 Conservation Efforts 19
7.1 Endangered Species Act 19
7.2 Edwards Aquifer Recovery Implementation Program Habitat Conservation Plan 19
8 References 21

1 INTRODUCTION

The Comal Springs riffle beetle, *Heterelmis comalensis* (Bosse et al. 1988), was described from Comal Springs (Comal County, Texas) and later collected in the headwaters of San Marcos Springs (Hays County, Texas) during status assessment surveys of nine central Texas springs (Figure 1; Barr 1993). The Comal Springs riffle beetle (CSRB) was listed as federally endangered due to threats of reduced groundwater quantity and quality because of human activities (e.g., groundwater withdrawal) and their restricted distribution to spring habitats (USFWS 1997; USFWS 2013). The Edwards Aquifer Authority (EAA) was formed in 1993 (Chapter 626 1993) to manage and issue permits for the withdrawal of groundwater from the Edwards Aquifer and charged with protecting terrestrial and aquatic species, domestic and municipal water supplies, the operation of existing industries, and the economic development.

This handbook will provide an overview of CSRB life history and ecology, the evolution of captive husbandry and propagation at the US Fish and Wildlife Service Edwards Aquifer Refugia Program (EARP), and description of current conservation measures for CSRB. This handbook will serve as a training and guide tool for researchers and husbandry biologists to inform future research needs and ensure smooth captive husbandry care during staff transitions.

Figure 1. General locations for each spring run reach in the Comal Springs system in Comal County, Texas where the Comal Springs riffle beetle has been collected (Bosse, Tuff & Brown 1988; Barr 1993; Coleman, Gibson, and Norris, 2022, pers. comm.).

2 LIFE HISTORY AND ECOLOGY

2.1 HABITAT

Riffle beetles (Family Elmidae; ca. 100 species, 28 genera in North America; Merritt et al. 2019; Barr et al. 2015) typically occur in swifter portions of relatively clean rivers and streams feeding on biofilm (microorganisms and debris) scraped from surfaces such as rocks, submerged wood, and vegetation (Brown 1987; Bowles et al. 2003; Elliott 2008). BIO-WEST (2002a) found that CSRB mainly occurred in areas with gravel and cobble and not in areas of high sedimentation. Areas of little to no flow, resulting in lower dissolved oxygen and sedimentation, lowered plastron respiration efficiency (Brown 1987; Flynne and Bush 2008).

CSRB congregate in spring outlets (BIO-WEST 2002a; Cooke et al. 2015), display positive rheotaxis (BIO-WEST 2002b), prefer temperatures between 22.5 to 25.5°C, avoid low concentrations of carbon dioxide, avoid higher flows, prefer supersaturated groundwater and dark spaces (Cooke et al. 2015), and have narrow tolerance to short-term temperature fluctuations (stenothermal; Nair 2019). Compared to its sister taxa, CSRB have the narrowest thermal tolerances and display even greater adaptation to permanent groundwater habitat because it is the only species of *Heterelmis* with truncated non-functional wings, significantly limiting its dispersal capabilities (Nowlin et al. 2017b, Nair et al. 2023). CSRB occur on gravel and cobble, are negatively associated with silt (Bowles et al. 2003; BIO-WEST 2002a), and require stable water temperatures (BIO-WEST 2014; BIO-WEST 2015; Nowlin et al. 2017b; Nair et al. 2021, Nair et al. 2023). This species may prefer areas free of silt near spring outflows and is not limited to high-velocity spring orifices as previously believed (BIO-WEST 2007).

In 2020, two adults and two larvae were caught at spring run 4 (Figure 1) and were confirmed by a second survey (Coleman, Gibson, and Norris 2022, pers. comm.). No CSRB were collected from these spring orifices during subsequent surveys (Gibson unpublished data). It was previously believed this species may not exist at spring run 4 because of the lentic conditions and domination of silt substrate (Bowles et al. 2003). Another possibility is the CSRB never occupied run 4 and were introduced by recent activity without establishing a population or were extirpated during drought from this highest elevation spring isolated in the headwater regions of Landa Lake.

CSRB larvae mortality rates are likely higher in drifting larvae due to the danger of dispersal to sub-optimal habitats (Elliott 2008). Norris (2002) found CSRB (mostly larvae) drift rate to be as much as 18 beetles/day in Comal Spring Runs 1 and 3. Elmid adults are often found drifting in streams using a terminally extruded air bubble (Buchwalter et al. 2019). CSRB adults are not often observed to display drifting behavior (e.g., splaying legs or terminal bubble) commonly seen on stream associated elmids including *Heterelmis vulnerata*. This CSRB relative (i.e., sister taxa) occupies areas directly downstream of both Comal and San Marcos springs impoundments and has functional wings but has not been observed to co-occur (i.e., sympatric overlap) with CSRB.

CSRB likely inhabit interstitial spaces within spring sources feeding on roots and woody debris associated with developed riparian zone. Burrowing behavior has been recorded in the wild, and CSRB are likely to seek out sources of water when spring flows decrease (BIO-WEST 2007). Furthermore, CSRB were found to prefer gas saturated groundwater in the lab in the absence of food sources whereas a related stream species, *H. vulnerata*, preferred degassed water (Cooke at al. 2015). This utilization of subterranean

groundwater habitat by CSRB likely aided in survival at Comal Springs during the 1950s drought when flow ceased and surface water temperatures increased (Bowles 2003). Additionally, because CBRB larvae have gills instead of a plastron, they are more resistant to lower dissolved oxygen levels and increased sedimentation, both of which are associated with drought conditions. Specific spring flow requirements and how much subterranean habitat this species uses is unknown; management relies on assuring historical conditions are maintained within the natural habitat for the species (LBG-Guyton Associates 2004).

2.2 Diet

Riffle beetles in the genus *Heterelmis* are detritovores often occurring on submerged wood (Brown 1976), presumably using it as a source of nutrition, and several undescribed species have been collected in large numbers from streams, seeps, and springs in central and west Texas (Gonzales 2008). CSRB feed on detritus (i.e., microbial growth on roots, woody debris, and leaf litter) within spring-influenced riparian zones (Gibson et al. 2008; USFWS 2013; BIO-WEST 2015; Nowlin et al. 2017a) and derives >80% of its essential amino acids from bacterial sources (Nair et al. 2021).

Wild CSRB have rotifers (Figure 2) basally attached around the mouthparts and head, presumably feeding on organic debris created by the beetle's mandibles during feeding (Gibson, pers. obs.). This is similar to observation by Brown (1987) as a common occurrence of attached ciliates on elmids. The rotifers do not persist in captivity.

CSRB typically occur at the surface on decaying woody debris, leaves, and roots over spring sources. Presumably, CSRB prefer biofilm growing within the saturated groundwater flow.

Figure 2. CSRB ventral view with rotifers attached near mouth area. Picture by Mike Quinn.

2.3 Life History

CSRB life cycle has been fully documented in captivity (Figure 3), with adult lifespans up to a year and an average time of two years between two consecutive generations of captive populations, but further research is needed (Bowles 2003; EAA 2017b). Wild-caught adults tend to live longer but produce fewer offspring at lower levels of supplied nutrients (Gibson, pers. obs.). Other species of riffle beetle have survived for several years in captivity (Brown 1973; Buchwalter et al. 2019).

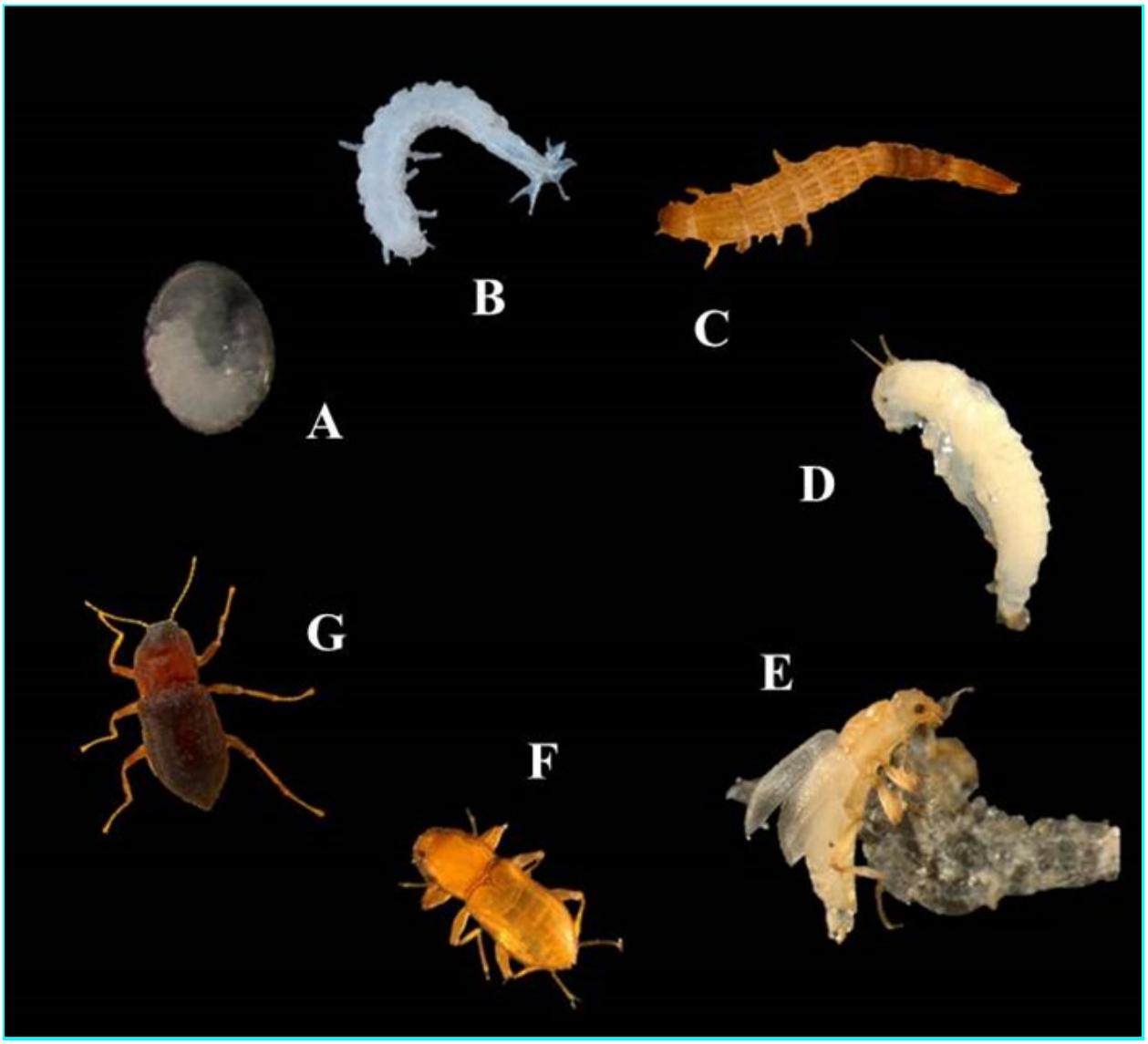


Figure 3. Life cycle of the Comal Springs riffle beetle (Heterelmis comalensis). Starting as an egg (A), a riffle beetle hatches into a soft-bodied, early instar larva (B) and develops into a later instar larva (C). Afterwards larvae develop into pupae (D) and if successful, eclose out of the pupal case (E). Immediately after the last molt, the adult stage begins with immature coloring and soft body in a teneral state (F) and matures to a fully adult riffle beetle (G). Courtesy of A. Hunter 2018.

2.3.1 Eggs

Captive CSRB deposit eggs (diameter ≈150 µm) singly or in clutches preferably on leaves compared to wood or cotton substrate; egg production declines over time in captivity possibly due to available nutrition or natural senescence. CSRB egg incubation time is around three weeks (BIO-WEST 2017). Brown (1987) reported short incubation times of 5-15 days for elmids. Subsequent work of two species from Texas and Arkansas (Phillips 1997a; 1997b) incubate 18-46 days. Four British species held ≤ 16°C had an incubation mean time of 29-30 with a range of 23-38 days (Elliott 2006; 2008). Incubation time likely depends on temperature and size of beetle similar to larval development rate (Brown 1987; Phillips 2017). Offspring production by female CSRB in captivity was found to be a function of longevity measured as 0.37 larvae/day, mean = 29.3 (range 0 – 121) /female (Kosnicki 2022).

Egg development starts with globular bodies like early cells of a zygote (3 days), to more cell division and smaller cells developing (7 days), to tissue differentiation with an embryo visible and budding appendage (14 to 18 days), to a fully developed larva observable inside the egg with a faint red eye (21 days) and hatching from the egg after 25 days (BIO-WEST 2017). Hatching success depended on the nutritional quality females received in captivity; the inclusion of cotton cloth contributed the most to producing healthy eggs viable to become larvae (BIO-WEST 2017).

2.3.2 Larvae

Characteristic of the elmid family, larvae have gills and are aquatic, often inhabiting similar habitat as adults subsisting on microorganisms and debris scraped from substrate (Brown 1987). CSRB larvae feed on allochthonous material and gain nutrients from the associated microbial communities, especially bacteria (Nair et al. 2021; Mays et al. 2021). CSRB larvae are mostly cylindrical in shape (Figure 3) often found together with adults. Elmid larvae with this body shape (rounder in cross-section) often burrow in gravel or wood (Elliott 2008).

The gills of elmid larvae can be expanded and contracted to increase ventilation when oxygen levels are lower (Short and White 2019). This allows CSRB larvae to inhabit areas of lower dissolved oxygen and higher sedimentation where adults are not typically found (Gibson *pers. obs.;* Elliott 2008; Walters and Post 2011). Later instar larvae develop tracheal air sacs allowing control of specific gravity and apply this to drift downstream, likely for escape or method for finding better living conditions (Brown 1987).

Other elmid genera have larval stage durations of 6 to 36 months and 5 to 8 instars, both varying with temperature, body size, and food availability (Brown 1987; Elliott 2008; Short and White 2019). Larval instar determination is set by measuring the head capsule width of a larva (Cooke 2012; BIO-WEST 2017). CSRB have seven developmental instars (Figure 4) based on measurements of preserved specimens and later through captive rearing (Cooke 2012; BIO-WEST 2017). In captivity, larvae developed into the final instar in four months, then persisted for four additional months before pupation. Observations of both increased and greatly inhibited larval development at ca. 23°C in captivity have occurred, presumably due to the unavailability to integrate nutrients to survive the pupation process, inadequate habitat conditions, or food quality (BIO-WEST 2017). Temperature was not found to affect larval survival, tested 19 to 25˚° C (BIO-WEST 2017).

A developing CSRB will go through 7 different instars before pupation (BIO-WEST 2017).

- 1st Stage – at approximately three weeks (21-25 days), the larva will emerge from the egg

- 2nd Stage – at around four weeks, the larva is translucent with long tail filaments. The larvae will morph through to the 6th instar in this stage.
- 3rd stage – at about four months, the larva will spend approximately four months (possibly longer) in its third stage as it processes through the 7th instar. The beetle larva now resembles most riffle beetles in this stage with a hard segmented exoskeleton. This stage has a poor survival rate that is believed to be due to the rapid development requiring a higher amount of nutrient-dense food.
- 4th stage – at approximately 9-11 months, the larva will molt into its pupa stage
- 5th stage – pupa emerges
- 6th stage – at around a year, the pupae molts into a teneral adult (freshly pupated).
- 7th stage – Adult stage. CSRB eclose to the adult stage after an approximate one-year larval stage. Adults live an additional year in captivity

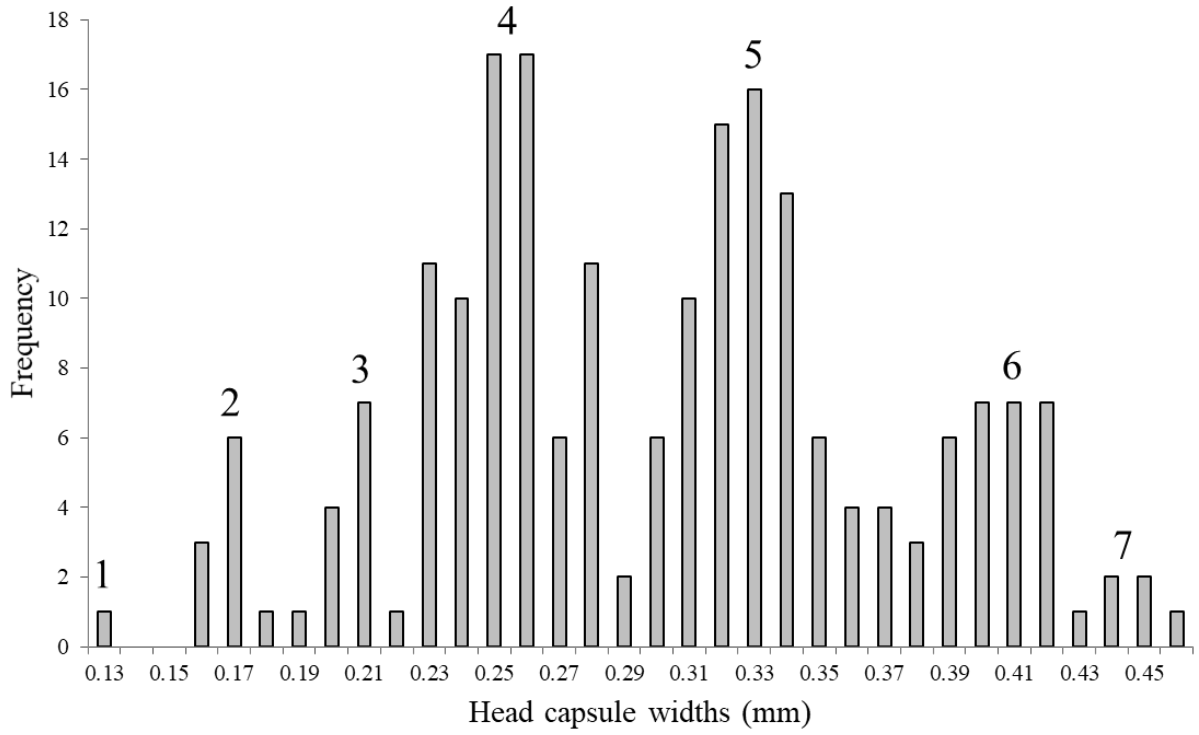


Figure 4. CSRB instar determination using width measurements of larval head capsules preserved from field collections adapted from Cooke (2012).

2.3.3 Pupae

Pupae are pale in color (Figure 2), with legs and wingpads that project loosely from the pupal body, and with fine setae on the pupal surface (Huston and Gibson 2015). These delicate setae might give the pupa its highly hydrophobic qualities and potentially generate a bubble around the body, acting like a plastron when underwater (Huston and Gibson 2015).

Riffle beetles typically pupate above the water line in moist soil, under rocks, or in rotting wood (Brown 1987; Elliott 2008; Buchwalter et al. 2019) Because Comal Springs groundwater is supersaturated, gas

bubbles constantly release from the spring sources when pressure is reduced near the surface. As a result, trapped bubbles within the interstices of the spring sources could provide air spaces under the water surface and act as potential pupation sites. Pupation time is unknown for CSRB and most species of riffle beetle. White (1978) reported pupation time of 11-12 days for *Stenelmis sexlineata,* and Elliott (2008) found mean pupation times for four British elmid species to be 11-13 days.

Before pupae become adults, they undergo a process called eclosion (molt), which takes a month (BIO-WEST 2017). Pupae for this species are capable of eclosing both underwater and at the surface of the water (Huston and Gibson 2015). Soon after eclosion to adult, individuals are light yellow in color (teneral) and slowly darken to an orange-brown (Figure 2). During this early stage of adulthood, it is very difficult to see the internal abdominal structure for determining sex.

2.3.4 Adults

Adult CSRB are relatively slow moving (cannot swim) and respire through a plastron (gas film produced by area of dense water repelling hairs), which limits them to habitats with higher dissolved oxygen (Hinton 1976; Brown 1987; Elliott 2008; Buchwalter et al. 2019). The plastron acts as a physical gill, with oxygen diffusing directly in from the surrounding water, and allows riffle beetles to stay submerged indefinitely (Harpster 1944; Thorpe and Crisp 1949; Elliott 2008; Buchwalter et al. 2019).

Female adult CSRB are iteroparous (can reproduce multiple times in a year) with up to 121 larvae (29 larvae on average) produced in their lifetime in captivity (Kosnicki 2022). In a captive study, more than half of the eggs that produced larvae were reared in treatments including biofilm poly-cotton cloth, suggesting an important nutritional requirement for egg development (BIO-WEST 2017).

Seasonal field surveys by Bowles et al. (2003) determined CSRB to have nonseasonal overlapping, asynchronous generations due to the stable environment. Other elmids with stable environmental conditions can affect emergence timings and oviposition based on changes in water velocity or temperature and food availability (Passos et al. 2003). There are no indicators or mechanisms for emergence of the CSRB. Males and females are similar in size and differ in length of 5th abdominal sternite and have a skewed sex ratio with males being 2/3^{rds} of the wild population (BIO-WEST 2017; Nair et al. 2019).

3 GENETICS

Although CSRB is a genetically distinct species, it is the most closely related to but significantly divergent from *Heterelmis glabra*, a species capable of flight associated with rivers and streams (Gonzales 2008). Bosse et al. (1988) speculated that the CSRB likely evolved from an isolated population of *Heterelmis glabra*, which was substantiated by Gonzales (2008). Genetic studies have shown some degree of isolation of CSRB populations within Comal Springs (Spring Runs and Landa Lake) and San Marcos Springs populations and relatively good migration within the Comal Springs complex (Gonzales 2008; TPWD 2015; Lucas et al. 2016). Dye tracing studies showed a different water source for each of the three high-variance populations and supports the occurrence of bottlenecks during extensive drought periods (LBG-Guyton Associates 2004; Johnson and Schindel 2008; Musgrove and Crow 2012). Ongoing genetic studies at Texas State University show an even greater degree of isolation among CSRB populations (W. Coleman, unpublished data). This isolation is due to the lack of recent gene flow, but

historically they had a common ancestral population (Gonzales 2008). The Comal Springs runs and the backwater spring populations have dried up during drought periods and genetic bottlenecks were apparent (Gonzales 2008).

4 COLLECTION

CSRB are best captured within or around spring orifices, even at shallow water depths (Bowles et al. 2003; Gibson et al. 2008; Cooke et al. 2015). These beetles have low dispersal abilities because they are flightless, limiting them to crawl or drift downstream to habitats that have adequate food resources and conditions within their preferred physicochemical range.

Larger and more reliable numbers of beetles are collected using techniques developed by Huston et al. (2015) using poly-cotton cloth (i.e., 60% cotton; 40% polyester) inserted into spring sources for at least four weeks. Biofilms associated with roots and surrounding allochthonous material grow on the poly-cotton cloth buried within the spring sources and result in beetles congregating on this trap over time. Cooke et al. (2015) using meter long cotton traps buried at 6 sites (3 shoreline seeps, 3 upwellings) found CSRB concentrated near (most within 20 cm and none more than 80 cm from) the spring source where the total gas saturation is higher and potentially different biofilm growth occurs. Gases (including elevated levels of CO_2) bubble out of solution at the surface, changing water quality as water flows downstream. These cotton traps had observable differences in the microbial growth near the spring source as compared to farther from the spring source. Beetle numbers using hand collection are unpredictable and sparse, especially during lower flows (BIO-WEST 2002b).

Larvae are caught in lower numbers during biomonitoring using the poly-cotton cloth method, suggesting they either occupy different habitats or a sampling bias where the biofilms produced on the cloth are not desired by this life stage (BIO-WEST 2005). Assembling leaves in a similar fashion to the cloth method did not result in a statistically significant preference for leaves over cloth lures (Kosnicki 2021).

5 CAPTIVE HUSBANDRY

Captive husbandry is defined as maintaining the health and welfare of an organism in an enclosed environment. Best husbandry practices recognize a species' biological, environmental, nutritional, and social needs to foster a species' growth and development in captivity. It is important to note that the objective of captive husbandry may not include the active propagation of a species. If a species is maintained in adequate conditions, it will potentially propagate *ad libitum*. Thus, there may be overlap between the captive husbandry methods of a species and those use to propagate it. Different setups or methods may be required if the goal of a program is to optimize propagation versus maintaining a species.

5.1 EVOLUTION OF HOUSING

The evolution of the culture units used in housing wild-caught CSRB has diverged into two different designs. Both systems are designed to have adequate substrate and biofilm material in the container while maintaining proper water flow. One design utilizes a square container, and the other design

utilizes an enclosed tube to house the CSRB. Both designs try to mimic the upwelling and spring orifice opening of their natural habitat.

Source water used for maintaining the CSRB in captivity at both the Uvalde National Fish Hatchery (UNFH) and San Marcos Aquatic Resources Center (SMARC) has been well water. Each location has multiple wells on site and will switch between them. At the SMARC the wells pump water from the Edwards Aquifer and the wells at the UNFH pump water from either the Edwards Aquifer or a mix of the Edwards Aquifer and Trinity Aquifer. Well water at both locations has been used without modification but currently at the SMARC the water is chilled before reaching the husbandry systems.

In general, the husbandry systems are comprised of a multi shelf rack to hold the CSRB containers. Straight or pre-chilled well water enters a sump tank below the rack and is then pumped through a chiller/heater and then through distribution lines above the racks, from which it enters the CSRB containers. Water exiting the containers is directed back into the sump. A standpipe in the sump maintains the water level and allows excess water to be discarded.

CSRB culture began at SMARC in 1996 with limited success. Beetles were held in 3-gal aquaria containing anacua leaves, limestone rocks placed in the middle and flow-through well water and recirculated chilled water directed to one side producing circular flow around the central substrates. Thirty-eight larvae were recovered none of which survived past 8 months and a single adult collected from the wild survived for 19 months in captivity (Fries 2003). Starting in 2003, survival and larval production increased using 10-gal covered aquaria (reducing light and algal growth) with anacua leaves, placed between rocks stacked at one end of the aquarium and both well and recirculated water directed toward the glass above the substrate. Beetles were more abundant on the side of the aquarium fed by the well water compared to the recirculated water. The first successful pupation of a captively produced larva to an adult was first observed October 2003 and later in July 2004, when two more were produced. Cotton substrate was added between the rocks as a biofilm substrate, which provided additional nutrient sources beginning in 2005.

5.1.1 Box Containers

The EARP utilized the Easy-Upwelling system (Figure 5. Huston and Gibson 2015) supplied with flow-through well water until November 2019. Black shade cloth was placed around containers to reduce light penetration and stress for light-sensitive species. (Figure 6) Container height was reduced by moving from 64-quart to 32-quart containers while maintaining both length and width measurements. This change allowed staff to effectively double usable space on a shelf.

Figure 5 Easy-Upwelling system (Huston and Gibson 2015) utilizes stacked limestone rocks, conditioned leaves, and conditioned cloth as CSRB habitat. Water enters from PVC spray bar (1/16" equidistant holes drilled lengthwise) and exits through a PVC outflow pipe wrapped in 200-220µm mesh screen.

Figure 6. Black shade cloth was placed around sides of containers holding light-sensitive species.

Anecdotal evidence and observations from refugia biologists suggested that increased vertical habitat had an insignificant relationship with CSRB habitat utilization. Height of stacked substrate was reduced from as many as five layers of limestone rocks to two to three layers of rocks interspersed with conditioned sycamore, anacua, pecan, and black walnut leaves. This change reduced the chances of mortality during inventory, due to crushing. Substrate was spread out across a greater portion of the container base to maintain amount of habitat provided.

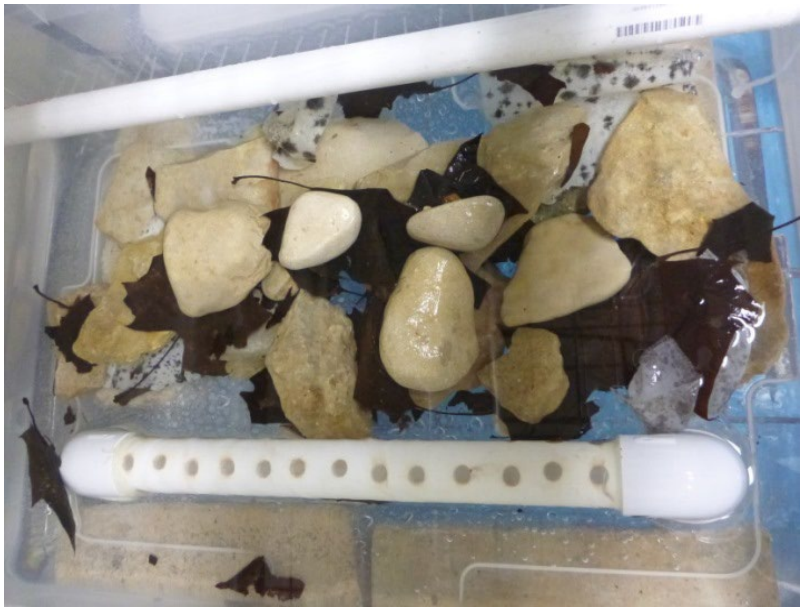


Figure 7. Height of stacked limestone, leaf, and cloth substrate was reduced and spread out further.

Small pebbles were secured to larger limestone rocks using aquarium-safe silicone or hot glue. The use of hot glue or aquarium-safe silicone was found to require leaching for up to one week to ensure no residual chemicals leached into the system. This reduced anoxic conditions in containers. Densely packed materials blocked flow within housing containers and increased the occurrence of anoxic conditions, especially cloth between rock layers.

Figure 8. Small pebbles were secured to larger limestone rocks using silicone or hot glue. Space under substrate is key to preventing anoxic conditions from forming.

Refugia biologists began incorporating 20-quart opaque black plastic boxes in 2019. Transitioning to opaque boxes eliminated the necessity of covering of systems and containers with light-blocking material.

In November 2019, refugia biologists made the decision to move from fresh well water to partially recirculating well water supplied by a variable speed drive pump. Utilizing a pump and collection sump allowed for greater modulation of temperatures and water quality in the system. A 40-gallon sump was installed to further slow changes in water quality and temperature.

Starting in 2020, the habitat boxes at the SMARC were transitioned to an upwelling-style flow system. Under this new design, partially recirculated well water entered habitat containers through 1/16” holes drilled in PVC pipes. Pipes were located near or on the bottom of the container and holes faced upward and inward, which better emulated spring upwellings. Habitat boxes at the UNFH kept the spray bars just above the water surface in the box. All habitat boxes were transitioned to the completely opaque 20-quart habitat containers.

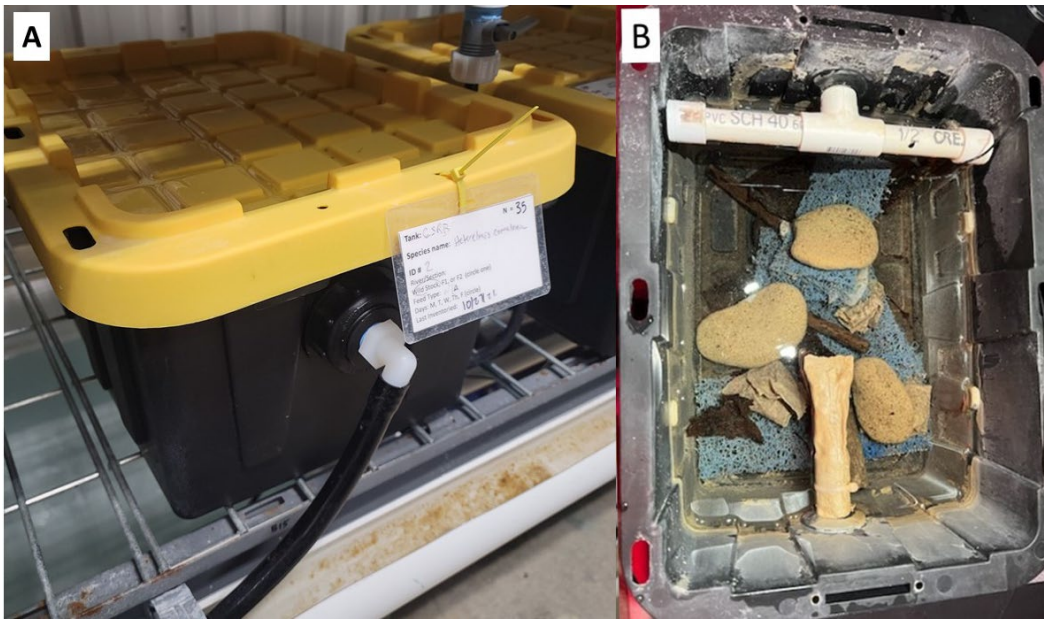


Figure 9. The current box design outer view (A) and inside view (B).

Current boxes at the UNFH have water entering and exiting the housing unit through a ½" bulkhead fitting placed centered on the short ends of the culture box placed at about two thirds the height of the container (Figure 9). The inflow bulkhead is situated slightly higher than that of the outflow. The inflow spray bar is level with the inflow bulkhead. Originally, the inflow PVC pipes had one row of 1/16" holes drilled even distances lengthwise across the spray bar. A second row has been added to the flow bar 90 degrees from first row to increase flow to the container while keeping the laminar flow through the culture box. The water from one row of outlets of the spray bar is directed at the water surface about halfway down the box, with the other pointed down and slightly back towards the back wall of the box. A horizontal PVC pipe covered in 200-220 µm mesh casing is used for outflow to keep adults and larvae from being lost. Due to the potential sensitivity of CSRB to toxic leaching in most adhesives, the mesh

screen is constructed using hot glue. High density filtration media is added to the boxes for habitat structure, and rocks are added to keep habitat and food items from floating to the surface.

The 20-quart culture boxes can house up to 50 adult beetles or 250 larvae.

5.1.2 Flow-through Tubes

Flow-through tubes are built from 2" internal diameter x 6" in length, opaque, threaded PVC pipe. Female adapters with 100 to 250-µm mesh added to the inside acts as a permeable barrier for proper water flow while containing the larvae in the tube (BIO-WEST 2017). A 6" by 8.5" piece of rigid mesh (2 to 4mm) with hot glue covering the rough ends is rolled into a tube shape to be placed in the middle of the flow-through tube. Feeding materials are layered around the rigid mesh tube where dowels are closest to the mesh, then leaves and cloth is placed in last. This configuration allows for proper flow and access to the feeding materials. All systems are flushed and preconditioned before adding CSRB to the system to reduce debris or leaching of plastic chemicals. Recent research shows at least 40 larvae can be held in each flow-through tube (Moore et al. 2022, in progress). Because wide temperature fluctuations are common in small volumes of water such as in the flow-through tubes, a system was built to provide a stable temperature. A heater-chiller unit closely controls the temperature of the water in a sump, and a hose with heating coils attached is placed in the sump and supplies water to the tubes. The water in the sump does not pump through the tubes; the sump water only promotes heat exchange to achieve a stable temperature for the water running through the hose. This system allows staff to select the optimal water temperature for the organisms while still being able to provide flow-through water. This is important because larvae have a higher pupation rate with saturated flow-through water compared to degassed recirculating water (Moore et al. 2021). To minimize potential system failure, the system is designed so that either well water, recirculated system water, or a mix of the two can be used.
Design of flow-through tubes used for research and captive propagation purposes has remained relatively unchanged. A December 2018 instructional photo shows sycamore leaves, large mesh (~500µm), and multiple poplar dowels completely filling the diameter of the tube. Historically, tubes were positioned vertically, relying on gravity to pull water through the contents of the tube. Research conducted in 2019 showed that horizontal positioning of tubes allowed formation of a small air pocket necessary for pupation (Kosnicki 2019).

In 2021, a SMARC study compared the flow-through tubes to flow-through boxes of similar size (Kosnicki et al. 2021). The box design had overflow problems and clogged frequently. Due to the enclosed nature of the flow-through tubes, overflow was not possible. However, the tubes also clogged regularly. Many larvae went missing from the boxes, and researchers determined the tubes were more efficient. However, the boxes described in the section above are larger and have a smaller chance of overflow. No study has compared the larger box design to the flow-through tube design thus far.

5.2 Current /Future Improvements

Improvements to the current system design currently being made are the addition of U.V. sterilizers to disinfect the water between the sump and the inlet to the containers. Course filtration by filter floss of the water returning from the sump from the CSRB containers. The systems are being retrofitted to allow either 100% well water or 100% recirculated water being used, or any ratio of the two. The well water at both locations has high dissolved CO_2 which off gasses once it enters the sump/containers. Due to the effect that dissolved CO_2 has on water pH, the amount of well water going into the system significantly

influences the pH of the system. To minimize fluctuations, a CO_2 dosing system is being added to the systems to regulate system pH.

5.3 Assessment of Water Quality Needs

The utilization of gills for respiration in their larval stage allows most riffle beetles to tolerate lower flows than in their adult stage (Walters and Post 2011; Cooke et al. 2015). Due to the species' inability to swim, CSRB spends most of its time crawling along benthic surfaces. However, the ability to control the specific gravity of their body develops in the later instar stages of the larva. The species develops a tracheal air sac at this point in its growth, allowing the larvae to drift with the flow (Brown 1987; Cooke et al. 2015). Flow aids late-instar CSRB larvae as they seek the proper environment for pupation. The housing developed for the EARP has considered these different factors in the system designs.

The recent water temperature of Comal Springs falls between 19-24°C year-round with an average pH of 6.5-7.7. CSRB have a narrow thermal tolerance range (Cooke et al. 2015; Nair et al. 2023). However, CSRB larvae had no significant relationship in the survival rates under continuous 22°C water temperatures compared to groups where the water was more variable but kept in tolerance range (BIO-WEST 2017). CSRB have a positive rheotaxis behavior, wanting to move into the current, and a positive response to flow (BIO-WEST 2002). With these parameters in mind, the EARP husbandry team strives to mimic the native water quality in a captive setting. Therefore, the refugia invertebrate systems are maintained daily at a flow rate of approximately 15 mL/s, water temperatures at 22°C (±1), and pH at 7.0-8.0.

In a habitat study, Cooke et al. (2015) found that CSRB showed a preference for well water over recirculating water. Additionally, pupation was noted to increase in flow-through tubes with well water compared to partially recirculating water (Moore et al. 2021). Well water emulation is closer to that of Comal Springs as the water from the two sources go through similar natural processes when brought to the surface resulting in water with higher dissolved oxygen (DO) and CO_2 resulting in lower pH than recirculating systems (Cooke et al. 2015). Recirculating systems are often used to combat thermal regulation issues brought about by the extreme ambient temperature gradients. However, it is possible to regulate water temperature using a temperature-controlled sump (see section 5.1.2 for details).

5.4 Feeding materials

CSRB are considered non-selective grazers, with 70-90% of their diet composed of bacterial biofilm associated with terrestrial coarse particulate organic matter (BIO-WEST 2017, Nair et al 2021). Leaves and poly-cotton cloth (biofilm matrix) are used as primary food and habitat sources in standard EARP husbandry practices. Culture containers that contain wood have a 56% pupation rate (BIO-WEST 2017). The husbandry staff incorporate wooden dowels to the habitat standards and UNFH staff incorporate woody debris. During quarterly inventories, feeding materials that have become anoxic, excessively worn, or have unwanted fungal growth are replaced. A 2019 study examined the possibility of providing CSRB with an extruded nutritional mash (Hunter et al. 2019). This feeding strategy was not successful, but more work on this subject might reveal more successful methods.

Leaves – The Refugia primarily uses sycamore leaves for captive propagation, but tree leaves from black walnut, pecan, sycamore, or anacua trees have been previously used. To diminish the accidental introduction of non-target species, leaves from the collection sites are brought to the station, cleaned,

and quarantined for at least two weeks, and sanitized in a laboratory oven. After the leaves are oven dried, they are stored in a sealed dry container until needed. As needed, leaves are conditioned (i.e., submerged) in a biofilm-conditioning box within a partially recirculating or flow-through water system. The leaves are conditioned after approximately two weeks and can be used in CSRB enclosures for two to three months thereafter.

Cloth – The cloth used in the collection process and husbandry practices of CSRB is a 40% cotton/60% polyester blend with a thread count of 200. Poly-cotton sheets are cut into squares no larger than 15 cm^2, rinsed to remove any manufacturing residues, and placed in a conditioning box with leaves to promote the production of biofilm growth. These poly-cotton cloths are shown to increase overall egg production when used in conjunction with leaves (BIO-WEST 2017).

Woody material – Anecdotal evidence from attending biologists showed that larvae present in refugia containers burrow into woody substrate. Although this finding was not shown to be statistically significant, survival of larvae in conditioned wood treatments was found to be over double that of treatments not containing conditioned wood. Poplar wood dowels are purchased from hardware stores and submerged woody debris is collected at the CSRB collection sites. Wood dowels are rinsed and placed in the biofilm box with leaves and cloth. The dowels are conditioned after approximately three months and can be used for several months thereafter, depending on CSRB density. Woody debris is sometimes collected from the wild and brought to the refugia. The woody debris is placed in quarantine for at least 30 days to remove any unwanted organisms. After quarantine, woody debris is conditioned in the biofilm box with the other feeding materials to promote biofilm growth. Quarantine boxes are set up without screens in the outflow to allow non-target species to flush through and into the chlorine-treated outflow sump. Although the feeding materials are handled similarly between facilities and conditioned in Edwards Aquifer water, the biofilm produced in captivity might be different than wild biofilm. The gut microbiome composition of wild adult CSRB was found to be different from adult CSRB that were collected from the wild and then housed at the SMARC for five to six months (Mays et al. 2021). The water at the SMARC might be different from the water at the springs in which CSRB live. The water at the SMARC is pumped from deep in the aquifer (320-402 ft), which might account for the differences in microbiomes. However, the microbiomes of adult CSRB housed at the UNFH did not differ from those of wild CSRB (Carlos-Shanley et al. in progress). It might be that the water pumped from the aquifer at the UNFH is more similar to the water at Comal Springs than SMARC water. These differences in the gut microbiome might be able to account for some of the differences in survival and pupation rate between facilities. Differing methods for conditioning biofilm might be needed between facilities.

Alternatively, there might be a human component involved in the gut microbiome differences in wild and captive CSRB. Adult CSRB at the SMARC contain gut microbiomes with an overgrowth of *Mammaliicoccus sciuri* (previously *Staphylococcus sciuri*) compared to wild CSRB (Mays et al. 2021). This species is often found in human environments, and it is possible staff expose CSRB to this bacterium during collections, inventories, and feeding material conditioning. SMARC staff now take extra measures to ensure all items are disinfected and there are fewer opportunities to expose captive CSRB. For example, inventories are conducted less frequently, going from monthly to every three months. Staff also change gloves more often. It is not yet clear if these measures reduce the gut microbiome differences, and it is unknown if these factors play a role in the bacterial overgrowth.

Although they are provided the same feeding materials currently, adult and larval CSRB might require different biofilm compositions. It was hypothesized that the overgrowth of *Mammaliicoccus sciuri* might inhibit pupation in captive larvae. Due to the overgrowth found in adults, SMARC staff and Dr. Camila Carlos-Shanley (Texas State University) designed an experiment to examine the effects of exposing larvae to *Mammaliicoccus sciuri* on survival and pupation (Moore et al. 2021). No difference was found in survival or pupation of exposed larvae, but larvae gut microbiomes were then analyzed. The results showed that gut microbiomes were different between CSRB larvae and adults (Carlos-Shanley et al. in progress). These results might indicate that larvae and adults need different biofilms. However, these larvae were housed at a lab at Texas State University, and it is possible these results just show a difference in aquifer water composition at that facility.

5.5 Effects of Handling and Light Exposure

The frequency of inventory procedures has decreased over the past year. Historically, beetle containers were inventoried once monthly. However, a recent study found that frequent handling of CSRB causes increased mortality over time (Nowlin 2021). Due to these results, the inventory frequency of refugia CSRB has decreased to once every three months. Overall survival of CSRB has anecdotally increased since this change was implemented.

No preference for dark over light environments has been recorded for adult beetles in their natural habitat. However, in captivity these beetles exhibit light avoidance behaviors (e.g., hiding under feeding materials, faster movement, crawling onto one another) (Cooke et al. 2015). Red light causes the least amount of stress and behavioral changes in stygobitic invertebrates (Nowlin et al. 2016). To reduce any light stress, CSRB are housed in opaque boxes or tubes and are only handled in areas where red-light sources are used. Headlamps and flashlights are used whenever possible during inventories or daily system checks.

6 Captive Propagation

The EARP aims to establish protocols for successful captive propagation of CSRB. Captive CSRB produce larvae in abundance, but the progression of the F1 generation into their adult stage has been limited in captivity (Kosnicki 2020).

The presence of a male is required for female CSRB to produce eggs (BIO-WEST 2017; Kosnicki 2020). CSRB propagate at varying densities from 20 or more per tube to as few as a single breeding pair. Estimating the sex ratio required in a population to maintain a captive breeding population in refugia is essential. As few as 11 F1 females with access to a mate can produce a self-propagating community, assuming fecundity does not decrease over generations (Kosnicki 2020).

Egg deposition in captivity happens soon after their introduction into the refugia population, with larvae found during quarterly inventories. Biofilm protein matrices are essential to female beetles for egg production. A 2017 study by BIO-WEST found that poly-cotton cloth was an important factor in the size of clutches and the number of eggs produced by mating pairs. However female CSRB showed a substrate preference of leaves for egg deposition, where 82% of 65 eggs that were laid during the study period were deposited on leaves (BIO-WEST 2017). Egg production and survival decreases with time parent CSRB are held in captivity (BIO-WEST 2017). It takes approximately three weeks for CSRB eggs to

incubate (BIO-WEST 2017). Under optimal conditions (e.g., unlimited access to mates), 10 females are estimated to produce 185 larvae over 60 days, and it is estimated that 185 larvae would produce 22 adults, 11 of which would be females (Kosnicki 2020). Survival and pupation rates are highly variable among groups of larvae (BIO-WEST 2017; Kosnicki 2020; Moore et al. in progress). Therefore, it might be prudent to have redundancies in the refugia.

Recent studies were developed with the goal of increasing pupation rates in captive CSRB larvae. Holding larvae at higher densities in flow-through tubes was examined as an option to increase pupation rates. Historically, CSRB larvae were held at a density of up to 20 larvae per tube. Increasing the density to 30 and 40 larvae per tube was found to produce similar rates of pupation (Moore et el. in progress). Although pupation rate did not increase, more adults could be produced in the same number of tubes. In a related study, larvae were provided with wild-cultivated or SMARC-cultivated biofilm to determine if supplying wild biofilm can increase pupation rates. The larvae provided with wild-cultivated biofilm pupated at a lower rate than larvae with SMARC-cultivated biofilm (Moore et al. in progress). However, it was not possible to determine the mechanism driving this relationship. SMARC staff hypothesized the relationship could be due to the larvae being adapted to SMARC conditions or some unknown factor that was not observed.

6.1 SEASONALITY

Early observations of CSRB in the wild are multivoltine with the presence of adults and larvae in multiple instar stages simultaneously (Bowles et al. 2003). Lack of apparent seasonality in emergence is likely due to their natural spring-fed environment where water quality conditions show minimum changes throughout the year (BIO-WEST 2015). These overlapping asynchronous emergence patterns are also observed in captivity.

6.2 FLOW

Adequate flow is essential for CSRB to achieve pupation and eclosion into their adult stage. A variable-flow artesian-spring emulator (VFASE) was created to test the response of CSRB adults under varying flow regimes with differing locations for food resources (Kosnicki and Julius 2019). CSRB moved toward food resources, against the flow if required. CSRB tended to stay with a food resource if placed with it at the beginning of the trial. In a 2020 study, a 15 mL/sec flow rate target was optimal for larvae to pupate (Kosnicki 2020). Flow rates lower than 10 mL/sec caused stagnant conditions with high mortality and no pupation while flow rates greater than 30 mL/sec were thought to push larvae away from air pockets and, in some instances, resulted in physical harm.

6.3 TIMING FOR GENERATION SEPARATION

Currently, the EARP separates the F(x) larvae from the adults upon inventory conducted every three to four months. This process not only gives the larvae adequate habitat for growth but allows the program to maintain genetic integrity among separate generations.

7 CONSERVATION EFFORTS

7.1 ENDANGERED SPECIES ACT

Under the Endangered Species Act (ESA), the CSRB is listed as endangered since 1997 (62 FR 66295). An endangered species is one that is in danger of extinction throughout all or a significant portion of its range. The ESA implements measures to protect the species from adverse effects of Federal activities (through Section 7 consultations), authority for the Service to develop recovery plans and purchase important habitat, restrictions to transporting, selling, or taking; and Federal aid to State and Commonwealth wildlife agencies to have cooperative agreements with the Service (16 U.S.C. 1531-1544). The CSRB is not included in the latest recovery plan because the species was listed after the publication of this plan (Service 1996).

The Service revised critical habitat for the CSRB on November 22, 2013, in areas of occupied, spring-related aquatic habitat (USFWS 2013). Twenty-two hectares (54 acres) of surface critical habitat were designated without additional subsurface designation because this species is restricted to surface waters (USFWS 2013). The original designation was surface critical habitat of 12.3 hectares (30.3 acres) of surface habitat without subsurface (USFWS 2007). Springs, associated streams, and underground spaces immediately inside of or adjacent to springs, seeps, and upwellings are the primary components of the physical or biological features essential to the conservation of this species (50 CFR 17.95; USFWS 2013).

Section 7(a)(2) of the ESA authorizes incidental take of listed species under activities that will not likely jeopardize the continued existence of listed species or destroy or adversely modify designated critical habitats (50 CFR 402). There are three consultations for the San Marcos ecosystem and multiple consultations for the Comal Springs ecosystem. The Service authorizes the ongoing operations of the Service's National Fish Hatchery in Uvalde and Fish Technology Center in San Marcos, Texas, which supports the eleven covered species refugia for the Edwards Aquifer Recovery Implementation Program Habitat Conservation Plan (EARIP HCP). This consultation determined that the considered actions would jeopardize listed species or result in destruction or adverse modification of designated critical habitat.

7.2 EDWARDS AQUIFER HABITAT CONSERVATION PLAN

The EAHCP was finalized in 2012 which authorizes incidental take of eleven Covered Species, including CSRB, for groundwater withdrawal, recreation, and other activities through 2028 (Recon Environmental et al. 2012). The EAHCP includes measures to minimize and mitigate impacts (e.g., water quality) and contribute to the recovery of the Covered Species, including CSRB, to ensure spring flows persist during a repeat drought of record (Recon Environmental et al. 2012; Payne et al. 2019).

As part of the EAHCP execution of their scientific components, a contract (# 16-822-HCP) was awarded to the Service's National Fish Hatchery in Uvalde and Fish Technology Center in San Marcos, Texas to operate the Aquifer Refugia Program for the EARIP HCP's Covered Species (Service 2017; Payne et al. 2019). This program ensures protection of extirpated populations and preserve the capacity of the species to be re-established after catastrophic events. The EAA has implemented other measures that ensure good habitat quality for the Covered Species such as minimizing recreation, modeling climate change futures to incorporate into aquifer management, land protection through easements, habitat monitoring, mitigation and protection from groundwater contamination (in association of the Texas

Commission on Environmental Quality), development of regulations, nonnative species management, and working with city partners to establish Watershed Protection Plans.

The EAHCP long-term biological goals for the CSRB are to:

(1) maintain silt-free gravel and cobble substrate in ≥ 90 percent of three areas in the Comal system (qualitative habitat component) and
(2) maintain specific median beetle population densities (as measured by numbers per lure) in the same three areas (quantitative population measurement). The areas are Spring Run 3 (≥ 20 CSRB/lure), the western shoreline of Landa Lake (≥ 15 CSRB/lure), and Spring Island (≥ 15 CSRB/lure).

The EAHCP long-term biological objectives for the CSRB are to:

(1) maintain a long-term average total Comal Springs discharge above 225 cfs with a minimum of 30 cfs that is not to exceed six months in duration, followed by at least 80 cfs for three months (flow component).
(2) maintain water quality issuing from the spring openings within 10 percent of historical conditions at the three study locations (water quality component).
(3) restore riparian habitat adjacent to spring openings to reduce siltation (habitat component).

The EAHCP biological monitoring program was developed to ensure the EAHCP biological goals are being meet for each covered species. To meet the quantitative population measurement goal, median densities for the three representative reaches were produced and serve as an indicator of population stability (Recon Environmental et al. 2012). Biomonitoring sampling for all benthic macroinvertebrates, including the CSRB, in the Comal Springs ecosystem was established in 2000 using drift nets and the introduction of targeted spring orifice sampling using poly-cotton cloth traps in 2003 (BIO-WEST 2003; BIO-WEST 2004). CSRB has been consistently collected since 2003 within the Comal Springs system (BIO-WEST 2007).

8 REFERENCES

Chapter 626. 1993. Acts of the 73rd Texas Legislature, HB3042. https://lrl.texas.gov/LASDOCS/74R/HB3042/HB3042_74R.pdf.

Barr, B. 1993. Survey for two Edwards Aquifer invertebrates: *Stygoparnus comalensis* Barr and Spangler (Coleoptera: Dryopidae) and Peck's cave amphipod *Sygobromus pecki* Holsinger (Amphipoda: Crangonyctidae). Final Report. Prepared for the U.S. Fish and Wildlife Service Ecological Services.

BIO-WEST. 2002a. Comal Springs riffle beetle habitat and population evaluation. Final Report. Prepared for the Edwards Aquifer Authority.

BIO-WEST. 2002b. Comal Springs riffle beetle laboratory study: evaluation under variable flow conditions. Final Report. Prepared for the Edwards Aquifer Authority.

BIO-WEST. 2003. Comprehensive and critical period monitoring program to evaluate the effects of variable flow on biological resources in the Comal Springs/River aquatic ecosystem. Annual Report. Prepared for the Edwards Aquifer Authority.

BIO-WEST. 2004. Comprehensive and critical period monitoring program to evaluate the effects of variable flow on biological resources in the Comal Springs/River aquatic ecosystem. Annual Report. Prepared for the Edwards Aquifer Authority.

BIO-WEST. 2005. Comprehensive and critical period monitoring program to evaluate the effects of variable flow on biological resources in the Comal Springs/River aquatic ecosystem. Annual Report. Prepared for the Edwards Aquifer Authority.

BIO-WEST. 2007. Variable flow study: seven years of monitoring and applied research. Final report. Prepared for the Edwards Aquifer Authority.

BIO-WEST. 2015. Comal Springs riffle beetle habitat connectivity study. Report. Prepared for the HCP Science Committee.

BIO-WEST. 2017. Comal Springs riffle beetle (*Heterelmis comalensis*): life history and captive propagation techniques. Final Report. Prepared for the Edwards Aquifer Authority.

Bosse, L.S., D.W. Tuff, and H.P. Brown. 1988. A new species of *Heterelmis* from Texas (Coleoptera: Elmidae). The Southwestern Naturalist 33:199-203.

Bowles, D.E., C.B. Barr, and R. Stanford. 2003. Habitat and phenology of the endangered riffle beetle *Heterelmis comalensis* and a coexisting species, *Microcylloepus pusillus*, (Coleoptera: Elmidae) at Comal Springs, Texas, USA. Archiv für Hydrobiologie 156:361-384.

Brown, H.P. 1973. Survival records for elmid beetles, with notes on laboratory rearing of various dryopids (Coleoptera). Entomological News 84:278-284.

Brown, H.P. 1976. Aquatic dryopid beetles (Coleoptera) of the United States.

Brown, H.P. 1987. Biology of riffle beetles. Annual Review of Entomology 32:253-273.

Buchwalter, D.B., V.H. Resh, G.A. Lamberti, and W.C.E.P. Verberk. 2019. Aquatic insect respiration. Pages 43-63 *in* R.W. Merritt, K.W. Cummins, and M.B. Berg, editors. An introduction to the aquatic insects of North America. Kendall Hunt Publishing Company, Dubuque, Iowa.

Carlos-Shanley C., K.D. Bockrath, D.M. Moore, and S. Tye. In progress. Microbiome of Comal Springs riffle beetle. Final Report. Prepared for the Edwards Aquifer Authority.

Cooke, M. 2012. Natural history studies on the Comal Springs riffle beetle (*Heterelmis comalensis*). Thesis, Texas State University, San Marcos, Texas.

Cooke, M., G. Longley, and R. Gibson. 2015. Spring association and microhabitat preferences of the Comal Springs riffle beetle (*Heterelmis comalensis*). The Southwestern Naturalist 60:110-121.

Ellott, J.M. 2006. Critical periods in the life cycle and the effects of a severe spate vary markedly between four species of elmid beetles in a small stream. Freshwater Biology 51:1527-1542.

Elliott. J.M. 2008. The ecology of riffle beetles (Coleoptera: Elmidae). Freshwater Reviews 1:289-203.

Flynn, M.R., and J.W. Bush. 2008. Underwater breathing: the mechanics of plastron respiration. Journal of Fluid Mechanics 608:275-296.

Fries, J.N. 2003. Possible reproduction of the Comal Springs riffle beetle, *Heterelmis comalensis* (Coleoptera: Elmidae), in captivity. Entomological News 114:7-9.

Gibson, J.R., S.J. Harden, and J.N. Fries. 2008. Survey and distribution of invertebrates from selected springs of the Edwards Aquifer in Comal and Hays Counties, Texas. The Southwestern Naturalist 53:74-84.

Gonzales, T.K. 2008. Conservation genetics of the Comal Springs riffle beetle (*Heterelmis comalensis*) populations in central Texas, with examination of molecular and morphological variation in *Heterelmis* sp. Throughout Texas. Thesis. Texas State University, San Marcos, Texas.

Harpster, H.T. 1944. The gaseous plastron as a respiratory mechanism in *Stenelmis quadrimaculata* Horn Dyropidae. Transactions of the American Microscopical Society 63:1-26.

Hinton, H.E. 1976. Plastron respiration in bugs and beetles. Journal of Insect Physiology 22:1529-1550.

Huston, D.C., and J.R. Gibson. 2015. Underwater pupation by the Comal Springs riffle beetle, *Heterelmis comalensis* Bosse, Tuff, and Brown, 1988 (Coleoptera: Elmidae), with an update on culture techniques. The Coleopterists Bulletin 69:521-524.

Huston, D.C., J.R. Gibson, K.G. Ostrand, C.W. Norris, and P.H. Diaz. 2015. Monitoring and marking techniques for the endangered Comal Springs riffle beetle, *Heterelmis comalensis* Bosse, Tuff, and Brown, 1988 (Coleoptera: Elmidae). The Coleopterists Bulletin 69:793-798.

Johnson, S.B., and G.M. Schindel. 2008. Evaluation of the option to designate a separate San Marcos pool for critical period management. Report. Prepared for the Edwards Aquifer Authority.

Kosnicki, E. 2019. Comal Springs riffle beetle (*Heterelmis comalensis*) pupation enhancement. Interim report. Prepared for the Edwards Aquifer Authority.

Kosnicki, E. 2020. Increasing pupation success in the Comal Springs riffle beetle in a captive setting. Final Report. Prepared for the Edwards Aquifer Authority.

Kosnicki, E. 2021. Comal Springs riffle beetle cotton luring analysis. Final Report. Prepared for the Edwards Aquifer Authority.

Kosnicki, E. 2022. Fecundity of first-generation captively reared *Heterelmis comalensis* (Coleoptera: Elmidae). Journal of Insect Science 22:1-3.

Kosnicki, E., and E. Julius. 2019. Life-history aspects of the Comal Springs dryopid beetle (*Stygoparnus comalensis*) and notes on life-history aspects of the Comal Springs riffle beetle (*Heterelmis comalensis*). Final Report. Prepared for the Edwards Aquifer Authority.

Kosnicki, E., D. Moore, and K. Bockrath. 2021. Increasing Comal Springs riffle beetle (*Heterelmis comalensis*) F1 adult production at the Refugia level. Interim Report. Prepared for the Edwards Aquifer Authority.

LBG-Guyton Associates. 2004. Evaluation of augmentation methodologies in support of in-situ refugia at Comal and San Marcos Springs, Texas. Report. Prepared for the Edwards Aquifer Authority.

Lucas, L.K., Z. Gompert, J.R. Gibson, K.L. Bell, C.A. Buerkle, and C.C. Nice. 2016. Pervasive gene flow across critical habitat for four narrowly endemic, sympatric taxa. Freshwater Biology 61:1-14.

Mays, Z., A. Hunter, L.G. Campbell, and C. Carlos-Shanley. 2021. The effects of captivity on the microbiome of the endangered Comal Springs riffle beetle (*Heterelmis comalensis*). FEMS Microbiology Letters 368: fnab121.

Merritt, R.W., K.W. Cummins, and M.B. Berg, editors. 2019. An introduction to the aquatic insects of North America. Kendall Hunt Publishing Company, Dubuque, Iowa.

Moore, D., K. Bockrath, and I. Prewitt. In progress. Increasing Comal Springs riffle beetle (*Heterelmis comalensis*) F1 Adult Production. Final Report. Prepared for the Edwards Aquifer Authority.

Moore, D., C. Carlos-Shanley, and K. Bockrath. 2021. Assessing the effect of *Staphylococcus* exposure on Comal Springs riffle beetle captive survival and propagation. Interim Report. Prepared for the Edwards Aquifer Authority.

Musgrove, M., and C.L. Crow. 2012. Origin and characteristics of discharge at San Marcos Springs based on hydrologic and geochemical data (2008-10), Bexar, Comal, and Hays Counties, Texas. Report. U.S. Geological Survey, Reston, Virginia.

Nair, P. 2019. Ecophysiology and food web dynamics of spring ecotone communities in the Edwards Aquifer, USA. Dissertation. Texas State University, San Marcos, Texas.

Nair, P., P.H. Diaz, and W.H. Nowlin. 2021. Interactions at surface-subterranean ecotones: structure and function of food webs within spring orifices. Oecologia 196:235-248.

Nair, P., A.H. Hunter, M.L.D. Worsham, M. Stehle, J.R. Gibson, and W.H. Nowlin. 2019. Sexual dimorphism in three species of *Heterelmis* Sharp (Coleoptera: Elmidae). The Coleopterists Bulletin 73:1075-1083.

Nair, P., J.R. Gibson, B.F. Schwartz, and W.H. Nowlin. 2023. Temperature responses vary between riffle beetles from contrasting aquatic environments. Journal of Thermal Biology112:1-8.

Nice, C., and L. Lucas. 2015. Genetic demography of endemic and endangered taxa in springs of the Edwars Plateau. Final Report. Prepared for the Texas Parks and Wildlife Department.

Norris, C.W. 2002. Effects of variable flows on invertebrate drift in Comal Springs, Texas. Thesis. Southwest Texas State University, San Marcos, Texas.

Nowlin, W. H. 2021. Factors affecting pupation in the endangered Comal Springs riffle beetle. Final Report. Prepared for the Edwards Aquifer Authority.

Nowlin, W.H., and R. Gibson. 2016. Refugia research: development of husbandry and captive propagation techniques for invertebrates covered under the Edwards Aquifer Habitat Conservation Plan. Final Report. Prepared for the Edwards Aquifer Authority.

Nowlin, W.H., D. Hahn, P. Nair, and F. Alfano. 2017a. Evaluation of the trophic status and functional feeding group status of the Comal Springs riffle beetle. Final Report. Prepared for the Edwards Aquifer Authority.

Nowlin, W.H., P. Nair, and B. Schwartz. 2017b. Evaluation of the long-term elevated temperature and low dissolved oxygen tolerances of the Comal Springs riffle beetle. Final Report. Prepared for the Edwards Aquifer Authority.

Nowlin, W.H., B. Schwartz, T. Hardy, and R. Gibson. 2014. Determination of limitations of Comal Springs riffle beetle plastron use during low-flow study. Final Report. Prepared for the Edwards Aquifer Authority.

Passos, M.I.S., J.L. Nessimian, and L.F.M. Dorville. 2003. Life strategies in an elmid (Insecta: Coleoptera: Elmidae) community from a first-order stream in the Atlantic Forest, southeastern Brazil. Acta Limnologica Brasiliensia 15:29-36.

Payne, S., N. Pence, and C. Furl. 2019. The Edwards Aquifer Habitat Conservation Plan: its planning and implementation. Pages 199-206 *in* J.M. Sharp, R.T. Green Jr., and G.M. Schindel, editors. The Edwards Aquifer: the past, present, and future of vital water resource: Geological Society of America Memoir 215. The Geological Society of America.

Phillips, E.C. 1997a. Life cycle, growth, survival, and production of *Macronychus glabratus* (Coleoptera: Elmidae) in northwest Arkansas and southeast Texas streams. Invertebrate Biology 116:134-141.

Phillips, E.C. 1997b. Life history and energetics of *Ancyrinyx variegata* (Coleoptera: Elmidae) in northwest Arkansas and southeast Texas. Ecology and Population Biology 90:54-61.

Phillips, E.C. 2017. Life history, growth, survival, and secondary production of Ancyronyx variegatus (Germar, 1824) (Coleoptera: Elmidae) in Elk Creek, Erie County, Pennsylvania, USA. The Coleopterists Bulletin 71:627-630.

Recon Environmental, Hicks & Company, Zara Environmental, and BIO-WEST. 2012. Edwards Aquifer Recovery Implementation Program Habitat Conservation Plan. Prepared for the Edwards Aquifer Recovery Implementation Program.

Short, A.E., and D.S. White. 2019. Aquatic Coleoptera. An introduction to the aquatic insects of North America. Pages 791-908 *in* R.W. Merritt, K.W. Cummins, and M.B. Berg, editors. An Introduction to the Aquatic Insects of North America. Kendall Hunt Publishing Company, Dubuque, Iowa.

Thorpe, W.H., and D.J. Crisp. 1949. Studies on plastron respiration: IV. Plastron respiration in the Coleoptera. Journal of Experimental Biology 26:219-260.

U.S. Fish and Wildlife Service. 1996. San Marcos and Comal Springs and associated aquatic ecosystems (revised) recovery plan. Report. Prepared for Region 2 U.S. Fish and Wildlife Service, Albuquerque, New Mexico.

U.S. Fish and Wildlife Service. 1997. Endangered and threatened wildlife and plants; final rule to list three aquatic invertebrates in Comal and Hays Counties, TX, as endangered. Federal Register 62:243(18 December 1997):66295–66304.

U.S. Fish and Wildlife Service. 2007. Endangered and threatened wildlife and plants: designation of critical habitat for the Comal Springs dryopid beetle, Comal Springs riffle beetle, and Peck's cave amphipod. Federal Register 72:136(17 July 2007):39248–39283.

U.S. Fish and Wildlife Service. 2013. Endangered and threatened wildlife and plants; revised critical habitat for the Comal Springs dryopid beetle, Comal Springs riffle beetle, and Peck's cave amphipod. Federal Register 78:205(23 October 2013):63100–63127.

U.S. Fish and Wildlife Service. 2017. Implementation of the refugia program under the Edwards Aquifer Habitat Conservation Plan. Annual Report. Prepared for the Edwards Aquifer Authority.

Walters, A.W., and D.M. Post. 2011. How low can you go? Impacts of a low-flow disturbance on aquatic insect communities. Ecological Applications 21:163-174.

White, D.S. 1978. Life cycle of the riffle beetle, *Stenelmis sexlineata* (Elmidae). Annals of the Entomological Society of America 71:121-125.

Microbiome of Comal Springs Riffle Beetle

USFWS Final report F20AC11680

Period of Performance: 09/01/2020 -12/31/2022

Dr. Camila Carlos-Shanley, PI
Dr. Katherine D. Bockrath
Desiree M. Moore
Samuel Tye

Table of Contents

BACKGROUND....3

OBJECTIVES....3

METHODS....4

DESCRIPTION OF THE SPECIMENS....4
STAPHYLOCOCCUS EXPOSURE....4
DNA ISOLATION AND SEQUENCING....6
METAGENOMIC ANALYSIS....7

RESULTS AND DISCUSSION....7

OBJECTIVE 1.....7
OBJECTIVE 2.....8
OBJECTIVE 3.....10

CONCLUSION....10

TABLES AND FIGURES....12

REFERENCES....19

Background

In the past decade, several studies have described the insect microbiome's crucial digestive/nutritional role, including the production of enzymes to digest recalcitrant material (Engel & Moran, 2013; Douglas 2015). In addition to dietary functions, several studies have demonstrated that both environmental and host-associated microbes can play an important role in insect development and survival (Coon et al., 2014).

Little is known about the microbes associated with *Heterelmis comalensis*, also known as the Comal Spring riffle beetle (CSRB), and their roles in the beetle's health, nutrition, and development. We recently published the first characterization of bacterial communities associated with wild and captive CSRB using amplicon sequencing of the 16S rRNA gene where the microbiome of captive beetles (from the SMARC facility) was found to be more diverse than wild beetles, and bacteria such as *Staphylococcus* spp. were more abundant in SMARC beetles (Mays et a., 2021). However, the methodology utilized to characterize the bacterial communities in the previous study had some limitations (biases introduced by PCR primers), and the study was conducted only with adult CSRB. Therefore, we proposed using a more comprehensive methodology (shotgun metagenomic sequencing) to characterize the microbiome of the CSRB at both larval and adult stages.

Objectives

Several studies show that captivity drastically changes the microbiome of several species (McKenzie et al., 2017; Bahrndorff et al., 2016). However, it is still unknown what are the potential consequences of microbiome shifts for animal conservation and captive breeding. In this study, we proposed:

Objective 1. Determine if Staphylococcus exposure impacts survival and/or pupation of CSRB larvae

Objective 2. To compare the microbiome of CSRB (larva and adult) across different habitats (Comal Spring, SMARC, and Uvalde facility) using shotgun metagenomics.

Objective 3. To compare the microbiome of CSRB larva after different bacterial challenges (2021 *Staphylococcus* exposure experiment) using shotgun metagenomics.

Methods

Description of the specimens

Forty specimens of CSRB were sequenced in this study. All specimens were collected by USFWS personnel and received in 95% ethanol. Table 1 details all the information from each sample. In summary, we sequenced:

- Four adults and four F1 larvae from the Uvalde facility.
- Three adults and six F1 larvae from the SMARC facility.
- Seven adults and six larvae from Comal Springs.
- Nine larvae after the 2021 exposure experiment (three from each treatment group).
- One larva for which no origin information was found.

Staphylococcus Exposure

We examined the survival of late-instar CSRB larvae exposed to a high level of *Staphylococcus* sp*p.* against two control groups. We included negative and positive control groups to examine the effects of no bacteria added and general increased bacterial load by adding harmless bacteria (*Bacillus subtilis* SID-166) instead of *Staphylococcus*. Because there was a risk that captive reared CSRB larvae at the SMARC were already exposed to *Staphylococcus* spp., SMARC staff collected wild CSRB larvae from cotton lures and woody debris at Landa Lake and immediately transferred them to the Freeman Aquatic Building (FAB) at Texas State University (TSU) campus. All equipment was disinfected with 70% ethanol and staff wore gloves disinfected with 70% ethanol during collection to prevent contamination of wild larvae. Larvae were acclimated at TSU for at least two weeks prior to the start of the experiment. Ten larvae were sacrificed before each experimental trial to confirm the absence of *Staphylococcus* in their gut, prior to exposure. Thirty larvae were used for each treatment and each treatment consisted of two trials (i.e., 15 larvae per treatment

per replicate; n = 90).

Food items were conditioned at the FAB to prevent contamination from SMARC water or staff. For the first trial, Sycamore leaves were collected from the areas surrounding Spring Runs 1-3 using the same precautions as larvae collections (i.e., disinfection, gloves). The leaves were immediately transferred to the FAB and placed in a conditioning container to develop biofilm. After having trouble recovering experimental larvae on the leaves in the first trial (see Results section), cloth was used for the second trial. We cut 200 thread count 60% cotton, 40% polyester blend cloth into approximately 9.5 cm x 24.5 cm pieces, then washed the cloth pieces to remove any contaminants from the manufacturing process and soaked them in 70% ethanol for disinfection. After all cloth pieces dried, they began conditioning in a container at the FAB to develop biofilm. All leaves and cloth conditioned for 30-45 days before being used in a trial.

Forty-five cylindrical containers (Figure 1) were constructed to hold larvae for the duration of the bacteria exposure. Containers were 16 mL and 4.5 cm height x 4.3 cm inner diameter. We outfitted the container lids with inflow hoses and outflow barbs (Figure 1) to prevent cross contamination among containers and treatments. We also placed 250 µm nylon screening between the lid and jar to prevent larvae escape. Dr. Carlos-Shanley and her student Samuel Tye cultured *S. Aureus 278* and *Bacillus* sp. for the *Staphylococcus* and positive control treatments, respectively. The jars were separated into 3 groups of 15 according to their treatment designation. 300 mL of 0.8% agarose (Sigma-Aldrich CAS: 9012-36-6) was prepared using the aquifer water that is pumped into the FAB lab and sterilized by autoclaving. The sterilized agarose was split into three containers and inoculated. The first treatment was a control treatment with no bacteria added and the second and third treatments were inoculated with their respective bacteria. All cultures grew in brain heart infusion (BHI) broth then washed in sterile spring water prior to use. An overnight 18-hour culture of *B. subtilis* 166 was used in the positive control treatment and a 32-hour culture of *S. aureus* 278 was used in the staph treatment. All jars with solution were stored in a 4°C refrigerator overnight until use.

We randomly placed larvae in containers with small amounts of food items to

examine individual survival. Larvae holding conditions at the FAB were as similar to wild conditions as possible and survival and pupation was monitored for 45 days. Dividers between treatments prevented any contamination from splashing. Shade cloth placed over all containers provided a dark environment for the larvae. We cleaned and disinfected (70% ethanol or autoclaving) all equipment and supplies after each trial to ensure residual bacteria did not contaminate the next trial.Larvae were checked daily for the first trial and weekly during the second trial to reduce potential escape. Flow was checked daily for both trials. The negative control containers were checked first, then the positive control containers, and the *Staphylococcus* containers last to prevent contamination.

Ten larvae from each treatment group from each trial was test using genetic sequencing to determine if the *Staphylococcus* larvae contain *S. aureus 278*. in their gut and confirm that the larvae in the control groups were not contaminated with *Staphylococcus*. The number of larvae sacrificed for testing varied based on the number of mortalities collected during the trial (i.e., the number of collected mortalities plus the number of sacrificed larvae = 10 for each treatment). All living larvae that were not sacrificed were relocated to the SMARC and placed in holding tubes by treatment type and trial for extended monitoring. SMARC staff conducted an inventory of all holding tubes monthly to assess long term survival, pupation, and eclosion of treatment groups.

Kaplan-Meier survival curves (Goel et al. 2010) were used to conduct a survival analysis, examining treatment and tank effects. Only the survival data collected during the exposure experiment were used to create the survival curves. Data recorded after larvae were transferred to the SMARC were not included. Once the curves were created, we tested the null hypotheses that survival was not affected by the tank in which larvae were held or by adding *S. aureus* 278 or *B. subtilis* 166. using the log-rank test comparing the survival curves. The analyses were conducted in the “survival” package (Therneau 2020) in the program R 4.0.3.

DNA isolation and sequencing

For all specimens collected from 2020, photographs of each sample were taken and labeled before DNA extraction. Single specimens were homogenized using MP

Biomedicals™ Yellow Zirconium Oxide Beads. Metagenomic DNA was isolated from the homogenates using the QIAmp BIOstic Bacteremia DNA kit with the addition of Zymo Spike-in Control II and quantified with a Qubit-4 fluorometer. The DNA of the specimens collected in 2019 was isolated, according to Mays et al. (2021). All samples with DNA concentration within the acceptable range (≥ 2.0 ng/µl) were sent for sequencing at the Microbial Genomics Sequencing Center (Pittsburgh, PA). Library preparation and sequencing were performed using the Illumina tagmentation protocol and the NextSeq Illumina platform (2 × 150 bp).

Metagenomic analysis

Sequence reads were filtered and trimmed using the default settings of fastp (Chen et al., 2018). Filtered reads were taxonomically classified using the Kaiju software using the proGenomes v3 database (2021-03-02). Taxa counts were normalized as reads per million (RPM). Statistical analyses were performed in the MicrobiomeAnalyst platform, taxa with median abundance < 100 RPM were removed from the analysis. Bowtie2 was used to align and quantify metagenomic reads to the *Staphylococcus sciuri* S00278 and *Bacillus* sp. S00166 reference genomes (GenBank assembly accession numbers: GCA_014204615.1 and GCA_014204475.1 respectively).

Results and Discussion

Objective 1. Determine if Staphylococcus exposure impacts the survival and/or pupation of CSRB larvae.

The first trial proceeded successfully but no larvae pupated, and some larvae went missing (Table 2). Missing larvae were not assumed to be alive or dead, because there is no way of knowing if they escaped or died. At least one larva had escaped to the lip of their container and was crushed in the threads when the lid was removed or replaced on the container. Some larvae that survived the first trial (i.e., two positive control and three *staphylococcus*) were sacrificed for *Staphylococcus* infection testing by genetic sequencing.

Long-term monitoring for the first trial lasted three months, at which point all

larvae were dead (Table 2). Several instances of low or no flow occurred during long-term monitoring at the SMARC. Low- and no-flow events occurred due to calcification and debris buildup associated with the partially recirculating system in which the larvae were held. Additionally, the tube screens had to be cleaned every day to maintain or resume appropriate flow conditions. No larvae from the first trial pupated.

The second trial proceeded successfully with one minor setback associated with calcium debris reducing flow, and no pupation occurred (Table 2). Significant calcium deposits were found on and cleaned from the screens of containers in Tank 2. The deposits decreased flow to some containers and notes were made to account for those differences. No larvae went missing during this trial. Some larvae that survived the second trial (i.e., three positive control and one *staphylococcus*) were sacrificed for *Staphylococcus* infection testing by genetic sequencing.

Long-term monitoring for the second trial resulted in four pupation events. No low- or no-flow events occurred during the long-term monitoring for trial two, but a high-flow event occurred within the first month the larvae were at the SMARC. Several mortalities occurred during the first month (Table 2), but the state of several dead larvae (crushed against the outflow screen) indicated the high-flow event might have contributed to some of those mortalities. One adult Comal Springs riffle beetle was found in each of the negative control and staph tubes after their first month at the SMARC. At the second monthly check, two additional larvae had pupated and eclosed in the positive control tube and no other larvae were alive. The staph tube was retired at the two-month check because the adult that remained was found dead.

Treatment affected the probability of survival of larvae over time, but the tank in which they were held did not. The negative control survival curve was statistically different than the positive control ($\chi^2 = 9.8$, $p = 0.002$; Figure 2). However, the *Staphylococcus* survival curve was not statistically different ($\alpha = 0.05$) from the negative control ($\chi^2 = 2.9$, $p = 0.09$) or positive control ($\chi^2 = 2.9$, $p = 0.09$) groups (Figure 2). Survival in Tank 1 was not different from survival in Tank 2 ($\chi^2 = 1.7$, $p = 0.2$).

Objective 2. To compare the microbiome of CSRB (larva and adult) across different habitats (Comal Spring, SMARC, and Uvalde facility) using shotgun metagenomics.

For this objective, we compared the microbiome composition of four adult samples from the Uvalde facility (B21, B22, B24, and B25), and four adults from Comal Springs (WB2, WB3, WB4, and WB5). We excluded from this analysis the six adult specimens used in Mays et al. (2021) because their DNA was extracted using a different protocol. We also compared the microbiome composition of four larvae samples from the Uvalde facility (L21, L23, L24, and L25), six larvae samples from the SMARC facility (LF11, LF12, LF13, LF14, LF15, and LF16), and four larvae from Comal Springs (WL3, WL4, WL4, and WL5).

Overall, we found the difference between the microbiome of adults and larvae, regardless of location, was statistically significant (PERMANOVA, Bray-Curtis dissimilarity, $F=9.14$, $p<0.0001$) (Figure 3). The microbiomes of Uvalde adults did not differ significantly from those of the adults from the Comal Springs (PERMANOVA, Bray-Curtis dissimilarity, $F=2.27$, $p=0.0594$). We found the difference between the microbiomes of Uvalde larva and larva from the Comal Springs was not statistically significant (PERMANOVA, Bray-Curtis dissimilarity, $F=1.274$, $p=0.1868$). The difference between the microbiomes of Uvalde larva and SMARC larva was statistically significant (PERMANOVA, Bray-Curtis dissimilarity, $F=3.679$, $p=0.0099$). And finally, we found SMARC larvae and larvae from the Comal Springs had different microbiomes (PERMANOVA, Bray-Curtis dissimilarity, $F=3.504$, $p=0.0016$).

We found 29 bacterial species to be differentially abundant among the three larval habitats (Table 3). For example, *Acinetobacter johnsonii* was more abundant in larvae from the Comal Springs, *Thiothrix eikelboomii* was less abundant in SMARC larvae, and an unclassified Mycobacterium species was more abundant in SMARC larvae (Figure 4). Although we could speculate about the potential roles of these bacteria in the CSRB microbiome based on the scarce literature available for these species, experiments are needed to determine their potential ecological interactions with CSRB (mutualists, pathogenic, etc.).

Together these results indicate that environmental and breeding conditions in the SMARC facility could significantly impact the larval microbiome, when compared with the Uvalde facility. At the moment, there is no indication that differences in the

microbiome impacts larval production at either facility. Although the captive cultivated microbiome of CSRB in Refugia may not be permanent and may change post reintroduction (Chong et al. 2019), captive cultivated microbiomes may impact the survival of reintroduced individuals. The microbiome should be assessed, and its impact considered when developing reintroduction plans (Zhu et al. 2021).

Objective 3. To compare the microbiome of CSRB larva after different bacterial challenges (2021 Staphylococcus exposure experiment) using shotgun metagenomics.

In this objective, we compared the microbiome composition of three larvae from the non-inoculated control (NIC) treatment (UN2, UN3, and UN4), three larvae from the *Staphylococcus sciuri* S00278 treatment (ST1, ST3, and ST5), and three larvae from the *Bacillus* sp. S00166 treatment (BA1, BA3 and BA4). The overall composition of microbiome of the larvae across the three exposure treatments was not statistically different: NIC vs. *Bacillus* (PERMANOVA, Bray-Curtis dissimilarity, F=0.8794, p=0.6977); NIC vs. *Staphylococcus* (PERMANOVA, Bray-Curtis dissimilarity, F=0.9078, p=0.6031); *Bacillus* vs. *Staphylococcus* (PERMANOVA, Bray-Curtis dissimilarity, F=1.128, p=0.4044). metagenomeSeq analysis of the mapped reads that align to the *Staphylococcus sciuri* S00278 and *Bacillus* sp. S00166 reference genomes showed no statistically significant difference across treatments.

Conclusion

Mays et al. (2021) found that the *Mammaliicoccus sciuri* S00278, formerly known as *Staphylococcus sciuri* S00278 (Madhaiyan et al., 2020), was more abundant in adult beetles from the SMARC facility than in adult beetles from Comal Springs. Therefore, we choose this bacterium for exposure experiments aiming to assess its impact on larval survival. Although the presence of *Staphylococcus* was suspected to impact survival, this study found no statistically significant difference between the staph-exposed larvae survival and that of the two non-staph groups. Although survival was not statistically different for staph-exposed larvae, there are potential biological implications. It might be more appropriate to use a value of 0.1 for α here, because type II error

(accepting a false null hypothesis) have larger consequences than type I error (rejecting a true null hypothesis) in this case. If an α value is set to a higher level (0.1), the staph treatment larvae would have statistically significantly lower survival than the *Bacillus* group and higher survival than the negative control group. Because CSRB is endangered, it might be advantageous to interpret the results of this study more cautiously (Martínez-Abraín 2008) and consider the biological relevance to the organism. Any decrease in survival of an endangered and sensitive species could result in harm to the Refugia population.

Interestingly, our results show that the microbiomes of larvae and adults, regardless of location, are different, which could explain why we observed a small impact of exposure treatments with bacteria isolated from adults. Future CSRB microbiome studies should take in consideration differences between developmental stages.

Tables and Figures

Table 1. Description of *Heterelmis comalensis* specimens sequenced in this study.

Sample name	Description	Stage	Habitat/Group	Collection date
B21	adult Uvalde facility - specimen 1	adult	Uvalde	Apr-21
B22	adult Uvalde facility - specimen 2	adult	Uvalde	Apr-21
B25	adult Uvalde facility - specimen 3	adult	Uvalde	Apr-21
B24	adult Uvalde facility - specimen 4	adult	Uvalde	Apr-21
CJ12	adult from SMARC (Mays et al. 2021) - specimen 12	adult	SMARC	May-19
CJ7	adult from SMARC (Mays et al. 2021) - specimen 7	adult	SMARC	May-19
CJ8	adult from SMARC (Mays et al. 2021) - specimen 8	adult	SMARC	May-19
WB2	adult from Spring Run - specimen 2	adult	Comal_Springs	Dec-20
WB3	adult from Spring Run - specimen 3	adult	Comal_Springs	Dec-20
WB4	adult from Spring Run - specimen 4	adult	Comal_Springs	Dec-20
WB5	adult from Spring Run - specimen 5	adult	Comal_Springs	Dec-20
WI6	adult from Comal Springs (Mays et al. 2021) - specimen 6	adult	Comal_Springs	Feb-19
WM4	adult from Comal Springs (Mays et al. 2021) - specimen 4	adult	Comal_Springs	Feb-19
WM8	adult from Comal Springs (Mays et al. 2021) - specimen 8	adult	Comal_Springs	Feb-19
Bex6	wild larva before 2021 exposure experiment - specimen 6	larva	Comal_Springs	Apr-21
Bex8	wild larva before 2021 exposure experiment - specimen 8	larva	Comal_Springs	Apr-21
L21	F1 larva Uvalde facility - specimen 1	larva	Uvalde	Apr-21
L23	F1 larva Uvalde facility - specimen 3	larva	Uvalde	Apr-21
L24	F1 larva Uvalde facility - specimen 4	larva	Uvalde	Apr-21
L25	F1 larva Uvalde facility - specimen 5	larva	Uvalde	Apr-21
LF11	F1 larva SMARC - specimen 1	larva	SMARC	Dec-20
LF12	F1 larva SMARC - specimen 2	larva	SMARC	Dec-20
LF13	F1 larva SMARC - specimen 3	larva	SMARC	Dec-20
LF14	F1 larva SMARC - specimen 4	larva	SMARC	Dec-20
LF15	F1 larva SMARC - specimen 5	larva	SMARC	Dec-20
LF16	F1 larva SMARC - specimen 6	larva	SMARC	Dec-20
WL2	larva - specimen 2	larva	Unknown	Dec-20

WL3	larva from Spring Run - specimen 3	larva	Comal_Springs	Dec-20
WL4	larva from Spring Run - specimen 4	larva	Comal_Springs	Dec-20
WL5	larva from Spring Run - specimen 5	larva	Comal_Springs	Dec-20
WL6	larva from Spring Run - specimen 6	larva	Comal_Springs	Dec-20
BA1	larva after Bacillus sp. exposure - specimen 1	larva	2021 exposure experiment – *Bacillus* sp. S00166 group	Jun-21
BA3	larva after Bacillus sp. exposure - specimen 2	larva	2021 exposure experiment - *Bacillus* sp. S00166 group	Jun-21
BA4	larva after Bacillus sp. exposure - specimen 3	larva	2021 exposure experiment - *Bacillus* sp. S00166 group	Jun-21
ST1	larva after Staphylococcus sp. exposure - specimen 1	larva	2021 exposure experiment - *Staphylococcus* sp. S00278 group	Jun-21
ST3	larva after Staphylococcus sp. exposure - specimen 2	larva	2021 exposure experiment - *Staphylococcus* sp. S00278 group	Jun-21
ST5	larva after Staphylococcus sp. exposure - specimen 3	larva	2021 exposure experiment - *Staphylococcus* sp. S00278 group	Jun-21
UN2	larva after Non-inoculated control - specimen 1	larva	2021 exposure experiment - Non-inoculated control group	Jun-21
UN3	larva after Non-inoculated control - specimen 2	larva	2021 exposure experiment - Non-inoculated control group	Jun-21
UN5	larva after Non-inoculated control - specimen 3	larva	2021 exposure experiment - Non-inoculated control group	Jun-21

Table 2. Survival results from the two trials of the Comal Springs riffle beetle *Heterelmis comalensis* exposure to *Staphylococcus* research project. The total is the number of larvae included in that treatment of that trial. Unknown indicates the number of larvae that were lost or escaped and cannot be included in analyses. We calculated the percent dead and alive at the end of the trial out of the total minus the number of unknown larvae. The number of larvae transferred to the SMARC accounts for the larvae sacrificed for testing by Dr. Camila Carlos-Shanley at Texas State University. Asterisks indicate individuals that pupated and eclosed. The number of larvae alive in each treatment 1-, 2-, and 3-months post transfer is reported, where NA indicates that inventory has not yet occurred.

	Negative control 1	*Bacillus* 1	Staph exposed 1	Negative control 2	*Bacillus* 2	Staph exposed 2
Total	14	15	15	15	15	15
Unknown	2	6	6	0	0	0
Dead	6 (50%)	3 (33%)	4 (44%)	12 (80%)	3 (20%)	8 (53%)
Alive	6 (50%)	6 (67%)	5 (56%)	3 (20%)	12 (80%)	7 (47%)
Transferred	6	4	2	3	9	6
Alive 1-month	3	2	2	2 + 1*	5	1*
Alive 2-month	1	2	1	1*	2*	0
Alive 3-month	0	0	0	0	2*	0

Table 3. Differentially abundant microbial species across CSRB larvae from different habitat identified using metagenomeSeq. P-values indicate how significantly different the identified taxa is from the whole dataset. The False Discovery Rate is the expected fraction on non-differentially abundant taxa, or the rate of falsely identified significant taxa. A low P-value and False Discovery Rate show greater confidence in taxonomic identity of the genetic data.

Species	P-values	False Discovery Rate
Acinetobacter johnsonii	1.58E-06	0.003212
Acinetobacter sp. ANC 4204	1.04E-05	0.015919
Acinetobacter tjernbergiae	5.25E-05	0.028693
Candidatus Acetothermia JdFR 52	9.29E-05	0.037626
Candidatus Dadabacteria RIFCSPHIGHO2 12 FUL 53 21	3.51E-05	0.028693
Candidatus Sungbacteria RIFCSPLOWO2 01 FULL 47 32	4.94E-05	0.028693
Cronobacter sakazakii	0.000116	0.042713
Cryobacterium arcticum	7.56E-07	0.003212
Deinococcus actinosclerus	1.78E-06	0.003212
Eremococcus coleocola	2.98E-08	0.000319
Gammaproteobacteria bacterium Ga0077536	1.41E-05	0.018808
Lentilactobacillus curieae	9.49E-05	0.037626
Methanocalculus sp. 52 23	7.23E-05	0.03093
Mycobacterium sp. UNC267MFSha1 1M11	1.63E-06	0.003212
Mycobacterium syngnathidarum	6.17E-05	0.028693
Mycolicibacterium peregrinum	1.80E-06	0.003212
Nocardia sp. Root136	2.16E-05	0.021839
Pantoea sp. IMH	4.12E-05	0.028693
Pantoea sp. PSNIH2	1.88E-05	0.021839
Parcubacteria group bacterium GW2011 GWA2 38 13b	2.53E-05	0.022587
Paucilactobacillus oligofermentans	6.88E-05	0.030681
Porphyromonas endodontalis	4.18E-05	0.028693
Pseudomonas sp. ML96	4.79E-05	0.028693
Pseudomonas sp. BMS12	6.15E-05	0.028693
Psychrobacter pasteurii	5.59E-05	0.028693
Thioalkalivibrio halophilus	4.37E-05	0.028693
Thiothrix eikelboomii	5.86E-05	0.028693
Tsukamurella pseudospumae	0.000111	0.042246
Tsukamurella tyrosinosolvens	2.25E-05	0.021839

Figure 1. An empty container from the Comal Springs riffle beetle *Heterelmis comalensis* exposure to *Staphylococcus* research project (left) and four containers operating during the project (right).

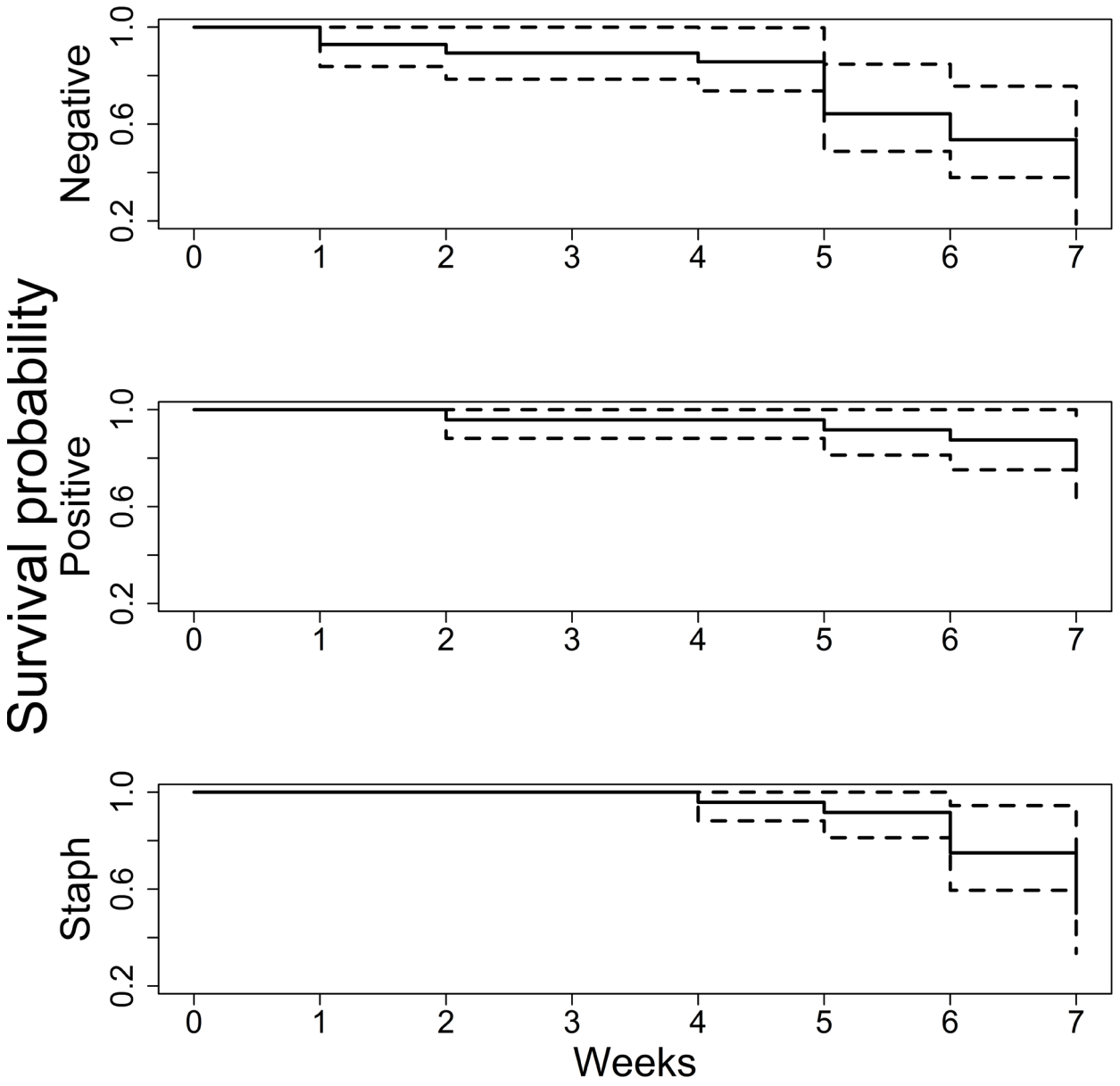


Figure 2. Kaplan–Meier survival curves developed for Comal Springs riffle beetle larvae exposed to *Staphylococcus aureus 278* (staph), *Bacillus subtilis* 166(positive), and no bacteria (negative). All groups were held in the same conditions except agarose in their containers contained the bacteria for their respective treatments. We show the survival probability with 95% confidence intervals (dashed lines) over time (weeks) where mortality occurred 1–7 weeks post exposure.

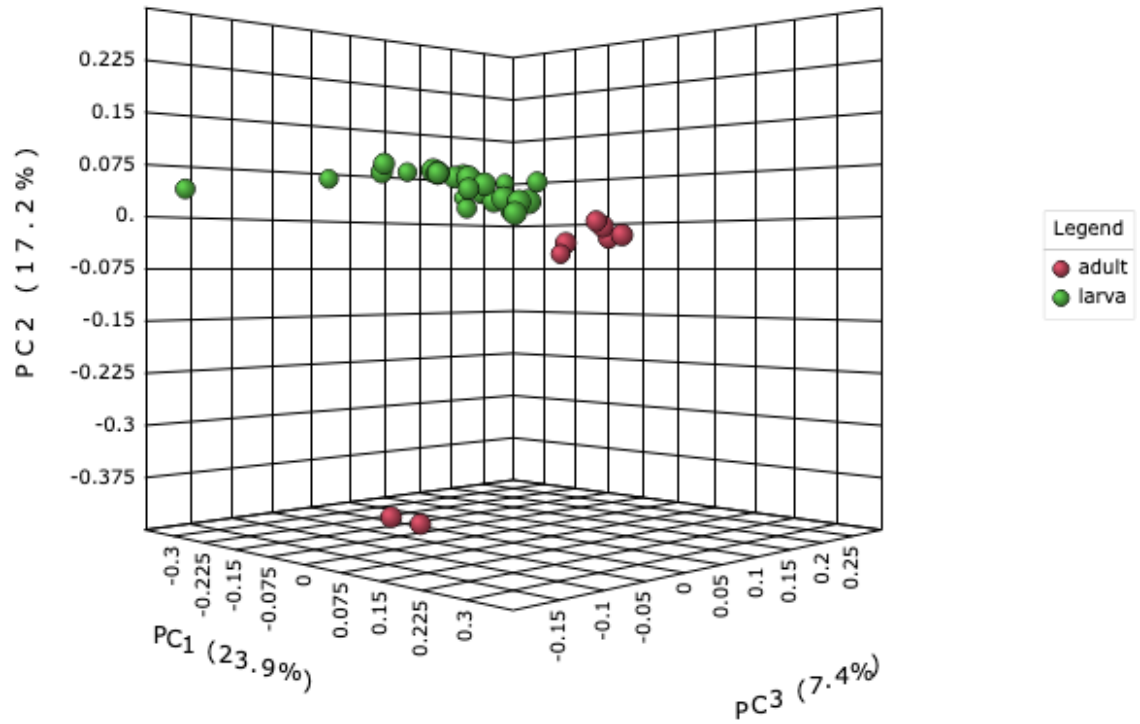


Figure 3. Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity index of CSRB microbiomes (at the species level). Adult samples are represented by red circles and Larval samples are represented by green circles.

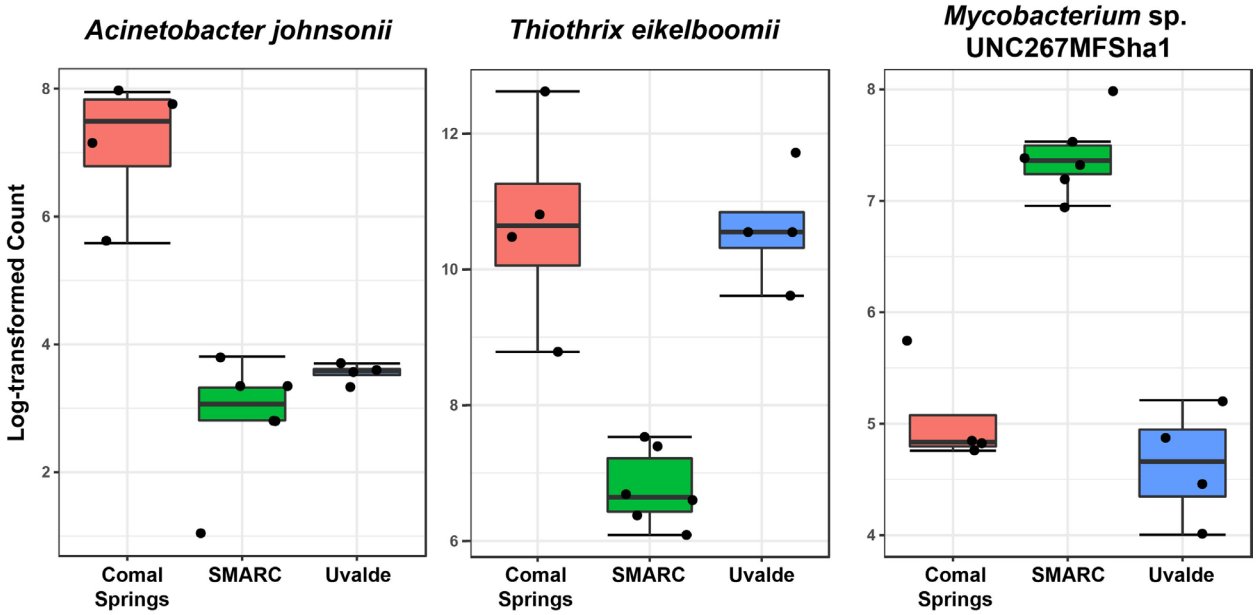


Figure 4. Boxplot of relative abundance (log scale) of selected microbial species across CSRB larvae from different habitat.

References

Bahrndorff, S., T. Alemu, T. Alemneh, and J. Lund Nielsen. 2016. The Microbiome of Animals: Implications for Conservation Biology. International Journal of Genomics 2016:5304028.

Chen, S., Y. Zhou, Y. Chen, and J. Gu. 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34(17):i884-i890.

Chong. R., G., C. E., Fox, S., Wise, P., Barrs, V. R., Hogg, C. J., Belov, K. 2019. Looking like the locals - gut microbiome changes post-release in an endangered species. Animal Microbiome 1(8):1-10.

Coon, K. L., K. J. Vogel, M. R. Brown, and M. R. Strand. 2014. Mosquitoes rely on their gut microbiota for development. Molecular Ecology 23(11):2727-2739.

Douglas, A. E. 2015. Multiorganismal insects: diversity and function of resident microorganisms. Annu Rev Entomol 60:17-34.

Engel, P., and N. A. Moran. 2013. The gut microbiota of insects – diversity in structure and function. FEMS Microbiology Reviews 37(5):699-735.

Madhaiyan, M., J. S. Wirth, and V. S. Saravanan. 2020. Phylogenomic analyses of the Staphylococcaceae family suggest the reclassification of five species within the genus Staphylococcus as heterotypic synonyms, the promotion of five subspecies to novel species, the taxonomic reassignment of five *Staphylococcus* species to Mammaliicoccus gen. nov., and the formal assignment of Nosocomiicoccus to the family Staphylococcaceae. International Journal of Systematic and Evolutionary Microbiology 70(11):5926-5936.

Martínez-Abraín, A. 2008. Statistical significance and biological relevance: a call for a more cautious interpretation of results in ecology. Acta Oecologica 34:9-11.

Mays, Z., A. Hunter, L. G. Campbell, and C. Carlos-Shanley. 2021. The effects of captivity on the microbiome of the endangered Comal Springs riffle beetle (*Heterelmis comalensis*). FEMS Microbiology Letters 368(17).

McKenzie, V. J., and coauthors. 2017. The Effects of Captivity on the Mammalian Gut Microbiome. Integrative and Comparative Biology 57(4):690-704.

Menzel, P., K. L. Ng, and A. Krogh. 2016. Fast and sensitive taxonomic classification for metagenomics with Kaiju. Nature Communications 7(1):11257.

Zhu, L., Wang, Jianjun, Bahrndorff, Simon. 2021. Editorial: The wildlife gut microbiome and its implcations for conservation biology. Frontiers in Microbiology 12(6).

Increasing Comal Springs Riffle Beetle (*Heterelmis comalensis*) F1 Adult Production

2023 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Desiree M. Moore, Dr. Katie Bockrath, and Israel Prewitt

San Marcos Aquatic Resources Center

U.S. Fish and Wildlife Service

Table of Contents

Background **3**

Objectives **3**

Methods **3**

Density: 3

Wild biofilm and cultivated biofilm 4

Results and Discussion **5**

Tables and Figures **7**

References **7**

Background

We contracted an outside partner, BIO-WEST Incorporated, in 2019 and 2020 to investigate ways to increase Comal Springs riffle beetle (*Heterelmis comalensis*; CSRB) pupation rates. Dr. Ely Kosnicki led the research for BIO-WEST. Some success was found among flow-through tubes that contained 20 larvae each (Kosnicki, 2020), with the highest success rate for pupation/eclosion being 60%. The purpose of this study was to build on previous work towards improved F1 CSRB propagation rates.

Objectives

The overarching goal of this study was to increase production of CSRB at the SMARC and Uvalde National Fish Hatchery (UNFH). This goal was to be accomplished with three objectives:

1) *Completed in 2021: Determine if a tube design modified as a small rectangular flow-through box can maintain or improve upon measured pupation/eclosion rates. The results of this study can be found in Kosnicki et al. 2022.*
2) *Completed in 2022: Determine if higher densities of larvae in flow-through tubes can maintain or improve previously measured pupation/eclosion rates.*
3) *Completed in 2022: Based on objective 2, determine if providing wild-cultivated biofilm (on leaves, wood, and cloth) to larvae will improve pupation/eclosion rates.*

Methods

Density: This study investigated the survivorship and pupation of larvae housed in flow-through tubes at target densities of 20, 30, and 40 individuals. Larvae used for this experiment were captively produced by wild-caught CSRB adults collected in 2021 and held in flow-through tubes at the SMARC. The first five larvae and every fourth larva thereafter were measured to determine if the larvae were in the late-instar stage. The head-capsule widths were recorded for measured individuals and all other individuals were determined to be of similar size by the selector. Three replicates were used to test each of the density treatments. Tubes contained conditioned cloth, wood, and leaves, and chilled well water flowed through each tube at a discharge between 10 – 15 mL/sec.

Treatment tubes were inventoried once larvae reached the age of expected pupation. The number of adults, pupae, and larvae (dead and living) was recorded at each inventory event. The time larvae spent in their experimental tubes depended on the approximate age of the larvae when the tubes were launched. Tubes continued to be inventoried at one-month intervals thereafter until no larvae remained. Replicates were staggered according to larvae availability.

We used a chi-square goodness-of-fit test to determine if pupation/eclosion frequencies differed among density treatments. We did not include CSRB that went missing in the analysis because pupation and eclosion could not be confirmed. The null hypothesis was that pupation/eclosion frequency was the same at the three densities. The alternate hypothesis was that the pupation/eclosion rate was statistically different for CSRB larvae held at different densities.

Wild biofilm and cultivated biofilm: This study compared the pupation success of CSRB larvae that were provided biofilms cultivated at the SMARC versus biofilms cultivated in the wild. Biofilm was allowed to cultivate on leaf packs, cloth, and wood in the Comal Springs system close to a spring source in Spring Run 3. Cloth and leaf packs were collected after one month of conditioning, and wooden dowels were collected after three months of conditioning in the spring water. Resource materials from the field were isolated in a recirculating system using Comal Springs water at the SMARC. Invertebrates were removed from resource materials before they were used in an experiment. Fresh Comal Springs water taken at a spring source was added to the system as needed to replenish evaporated water.

The pupation success of larvae reared on wild-cultivated biofilm or captive-cultivated biofilm and kept in flow-through tubes at the SMARC was compared. Three wild-biofilm replicates were conducted with a density of 30 to compare pupation success to the medium density study described in the previous section. Each tube contained the same resource materials as the density trials, but the materials were conditioned in spring water at the Comal Springs. This design allowed us to compare the wild-cultivated biofilm treatment to the tubes in the density experiment and minimize the number of larvae needed. Although the wild biofilm was cultivated in a system with only Comal Springs water, the flow-through tubes were supplied with SMARC water. It is not feasible to collect enough Comal Springs water to supply the flow-through tubes with Comal Springs water for several months, especially under the drought conditions during this study. The tubes were inventoried after three months and monthly thereafter until no larvae remained. CSRB that went missing were not included in the analysis because pupation and eclosion could not be confirmed. The number of adults, pupae, and larvae (alive and dead) was recorded at each inventory event.

A chi-square goodness-of-fit test was used to determine if pupation/eclosion frequencies differ between biofilm treatments. The null hypothesis was pupation/eclosion frequency was the same with wild-cultured biofilm as with SMARC-cultured biofilm. The alternate hypothesis was the pupation/eclosion rate was statistically different for CSRB larvae reared on wild-cultivated biofilm compared to SMARC-cultivated biofilm. Due to several larvae going missing in the study, this hypothesis was also tested with a variable for the number of missing larvae added to determine if that changed the results.

Results and Discussion

Density: The tubes housing the density experiment were successfully checked monthly until no larvae remained. Some larvae went unaccounted for in each replicate of each treatment (Table 1). These larvae were considered missing and were not used for the analysis. It is possible the missing larvae were mortalities and the carcasses were consumed or degraded before the following inventory. It is also possible these larvae escaped the tube, burrowed too deep into a dowel, or escaped into some other hidden area that is hard to observe. For a conservative approach, missing individuals were not considered part of the study.

There was no indication that density was related to the pupation/eclosion rate of Comal Springs riffle beetle larvae. The pupation/eclosion rate was not statistically different among density treatments ($\chi^2 = 0.56$, $P = 0.75$). These results indicate that it is not harmful to house larvae at densities up to 40 individuals per tube. This doubles the number of CSRB larvae that the SMARC can hold with the same number of tubes and the same volume of water. Using these results, SMARC staff can successfully house more CSRB larvae than before with similar pupation/eclosion rates.

Wild biofilm and cultivated biofilm: This study found no evidence that providing wild-cultivated biofilm would be beneficial to captive-reared CSRB larvae pupation. The larvae provided with wild biofilm pupated at a lower rate ($\chi^2 = 7.72$, $P = 0.005$) than their counterparts provided with SMARC-cultivated biofilm (Table 1). Fewer of the larvae in the wild-biofilm group went missing, which could have biased these results (i.e., more larvae counted as not pupated). However, the results did not change when the variable for number of missing larvae was added. The density study was used to inform larval densities to be used in the wild-biofilm study, thus the wild-biofilm tubes were set up after the density study was concluded. As a result, older larvae were used in the wild-biofilm tubes. It is difficult to determine the health of these larvae and it is possible the older larvae were already near the end of their life and had a limited ability to pupate. Additionally, the larvae might have already been adapted to SMARC biofilm from their time in the brooding tubes, and the wild cultivated biofilm may have changed over time with SMARC water flowing through the tubes. Unfortunately, it is not practical to provide wild water (i.e., collected from spring runs) to tubes in the refugia setting and there is no evidence that providing wild biofilm to CSRB larvae would improve pupation rates.

Tables and Figures

Table 1. Percent Comal Springs riffle beetle larvae pupated, not pupated, and missing in each replicate of the three density treatments and the wild-cultivated biofilm treatment.

Replicate	Pupated	Not pupated	Missing
SMARC biofilm – density 20			
1	35%	25%	40%
2	60%	15%	25%
3	25%	25%	50%
Mean	40%	22%	38%
SMARC biofilm – density 30			
1	23%	20%	57%
2	50%	23%	27%
3	33%	37%	30%
Mean	35%	27%	38%
SMARC biofilm – density 40			
1	28%	28%	45%
2	38%	33%	30%
3	40%	8%	53%
Mean	35%	23%	43%
Wild biofilm – density 30			
1	23%	63%	13%
2	40%	50%	10%
3	20%	67%	13%
Mean	28%	60%	12%

References

Kosnicki, E. 2020. Increasing pupation success in the Comal Springs riffle beetle in a captive setting. Final Report to the Edwards Aquifer Authority. San Marcos, TX.

Kosnicki, E., D. Moore, and K. Bockrath. 2022. Increasing Comal Springs riffle beetle (*Heterelmis comalensis*) F1 production at the refugia level. Interim Report to the Edwards Aquifer Authority. San Marcos, TX.

Historical Fountain Darter (Etheostoma fonticola) Tissue Archive

2022 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Shawn Moore

Table of Contents

Background 3

Objectives 3

Methods 3

Results 6

Discussion 7

Tables and Figures 9

References 14

Background

Fountain darters have been housed at the San Marcos Aquatic Resources Center (SMARC) and Uvalde National Fish Hatchery (UNFH) since the early 1990s. Prior to the Edwards Aquifer habitat conservation plan (EAHCP) these populations were studied to learn more about fountain darter reproduction and life history. Several hundred preserved fountain darters have accumulated over decades of care and research. Preserved mortalities were previously stored in flame resistant cabinets, on open shelves indoors, and in outdoor chemical sheds. These preserved samples can be valuable for assessing differences in historical sex ratios, physical condition, parasitic infections or disease state, and genetic variation. In this study, we aimed to inventory and catalog the preserved fountain darters samples, take body measurements, and assess the suitability of the preserved tissue for future genetic studies.

Objectives

The fountain darter sample archive enables more efficient mortality storage and tracking, provides a record of fountain darter health and variation, and can further inform the collection and propagation strategies.

Methods

Catalog and preparation of tissue samples –

All preserved mortalities were catalog into a tissue archive using a Survey123 form. Data recorded for each sample include species, sex, length, life stage, date of collection, location of collection, date of entry, original method of preservation, position in the cryofreeze (shelf and rack), position in box, and unique cryovial ID. Variation in

the notation of collection locations and tanks made it difficult to discern the exact population individuals originated from. The most accurate origin for each sample was determined using preserved specimen labels and available data logs for daily care, collections, and mortalities. All available information from the original label was recorded on the cryovial that each sample was transferred into. The samples were sexed using observation of external characteristics such as breeding coloration and ovipositor; internal anatomy was examined secondarily to confirm sex when necessary. Nearly all samples were measured for length, only severely degraded mortalities which could not be handled were excluded. Variation in total body length within and among locations was assessed using an AMOVA. Once transferred to a cryovial mortalities are stored in a -80 °C freezer.

DNA extraction and quantification –

A subset of the preserved tissue samples was selected for DNA extraction. This subset represented the distribution of size, sex, collection location, and original method of preservation of the whole collection of preserved tissue samples. The left pectoral fin was removed, weighed, and used for tissue lysis and DNA extraction. If the pectoral fin was insufficient in mass (07 – 21.3 mg) additional tissue was taken from the operculum or caudal fin until the minimum mass was met.

DNA extractions were carried out using a Qiagen DNEasy DNA extraction kit. Tissues preserved in formalin for more than two years or stored outside were significantly degraded. These tissues are highly acidic, which inhibits lysis and extraction reagents. Tissues preserved in formalin for less than two years and stored in ideal conditions were minimally degraded and transferring these samples to cryovials filled with 95% ethanol was sufficient to buffer the tissue in preparation for extractions. To neutralize formalin preserved tissue, the sample was soaked in excess Phosphate Buffered Saline (PBS) for five to seven days with frequent PBS changes for the first three days (Joshi et al., 2013). In trial PBS soaks and extractions, we found a 2:1 ratio of solution volume to tissue mass to be sufficient excess. This process enables the tissues to lyse more

completely and react fully to extraction reagents, producing DNA extractions with a higher concentration of genetic material. Each round of extractions included a negative control to test for contamination. All DNA extractions were quantified using a Qubit fluorometer standardized with a broad range DNA assay.

Polymerase chain reaction and gel electrophoresis-

Polymerase chain reaction (PCR) was used to examine the quality of the genetic material extracted. Bio-Rad CFX Opus 96 real-time PCR thermocyclers and a DreamTaq PCR reaction were used to amplify Cytochome Oxidase Subunit I (COI) for all DNA extractions (Fulmer 1996). Each PCR plate well was filled with 50 μL of solution containing 25 μL of DreamTaq green PCR master mix, 2 μL of 10mM of each forward and reverse primer, 16 μL of nuclease-free water, and 20 ng of DNA. In the final well of each plate DNA, free water was substituted for DNA as a negative control. Once inserted into the thermocycler, PCR reactions were initially heated to 95 °C for three minutes then repeated cycled through three stages: 94 °C for thirty seconds, 40 °C for thirty seconds, and 72 °C for one minute, thirty-four times. The PCR reactions went through a final extension of 72 °C for 7 minutes, then cooled at 4 °C until removed from the thermocycler and placed in the -80 °C freezer.

Amplified PCR products were visualized using a 1.5% agarose gel. All gels were prepared using 200 mL of 1x Tris-borate-EDTA (TBE) buffer, 3.0 grams of UltraPure™ agarose powder, and 7 μL of SYBR™ Safe DNA gel stain. A 100 bp ladder and BlueJuice gel loading buffer were prepared using manufacturer specifications and used to confirm PCR product length. The first gel was run at 90 volts for 90 minutes while adjusting protocol, later gels were run at 200 volts for 55 minutes. All amplifications were observed using a UV transilluminator.

Results

Demographic analysis –

Approximately 1,050 out of over 1,300 total fountain darter tissue samples had complete data records for sample population and collection date (Figure 1). Nearly 282 samples were confidently sexed (Table 1). There is a skew for availability of more recent mortalities, and a lack of data from 2014 through 2016. Samples from 2002 were mostly preserved in formalin while most samples after 2002 were preserved in ethanol (Table 2). Median total body length varied among preserved fountain darters from different collection locations and across years (Table 3, Figures 2 and 3).

Genetic analysis –

The concentration of DNA from tissue samples was consistent with the preservation solution and sample storage conditions. Overall, samples preserve in ethanol produced higher DNA concentrations than those preserved in formalin (Figure 4). Sample storage conditions had a significant impact on DNA extractions. Samples stored in ethanol in ideal conditions (i.e., climate controlled) experienced the least amount of degradation, and extractions from these tissues were the most concentrated. Tissues preserved in ethanol and stored in extreme conditions experienced some degradation and these samples produced inconsistent extractions (Figure 5 and 6).

The quality of PCR products also followed these predictions, as illustrated by the percent of successful PCR reactions (Table 4). Successful PCR reactions showed that the extracted DNA was intact and suitable for genetic analysis. Tissues preserved in ethanol and stored in climate-controlled conditions produced the most consistent high-quality products (Figure 6). Tissue preserved in ethanol and exposed to extreme conditions produced inconsistent results. These samples produced adequate concentrations of DNA extractions to carry out a PCR reaction, but environmental exposure caused significant degradation of the genetic material, resulting in

unsuccessful PCR reactions (Figures 5 and 6). There was an insignificant number of formalin preserved tissues exposed to extreme conditions, and these samples did not produce quality products. Formalin preserved tissues stored in climate-controlled conditions produced inconsistent results. Overall, DNA extracted from formalin preserved tissues were fragmented and unable to produce enough PCR product to be visualized through electrophoresis, but the products of some formalin preserved samples were visualized clearly (Figures 5 and 6). This demonstrates that soaking samples in PBS prior to DNA extraction can be used to neutralize tissue preserved in formalin for extended periods of time and enable the extraction of intact genetic material.

Discussion

Average sample length and variation in length was significantly different across sampling locations (Table 3, Figure 2) and collection year (Table 3, Figure 3). This unexpected variation within populations that may warrant additional investigation into how this variation is represented in the refugia, if it corelates to genetic variation, or if this length variation is tied to environmental fluctuations from year to year. Variation in total body length within and across populations has not been, to our knowledge, explicitly measured, thus it is unclear if the length variation observed is outside of normal ranges.

The extractions from the mortalities preserved in ethanol and stored outside were the most variable (Figure 4) and less than 12% of extractions produced quality PCR products (Table 4). The genetic value of these mortalities is low. Future DNA extractions and genetic analysis should prioritize recently preserved mortalities from all populations. Tissue samples from these specimens produce the highest quality and most consistent extractions. Recent mortalities are representative of present populations health and will bolster the tissue archive as a genetic database.

There are approximately 600 preserved fountain darters from 2002-2013 held at SMARC that have not been cataloged. These samples are preserved in ethanol and formalin and were previously stored in an outdoor chemical shed; they are now stored in climate-controlled conditions. The mortalities preserved in formalin are exclusively from 2002 and likely do not contain intact DNA. There are approximately 200 or more preserved fountain darters from 2022 held at SMARC that have not been cataloged.

The fountain darter tissue archive will be used to inform research, captive husbandry and collection strategies by correlating darter health and variation with habitat changes and disease occurrences. For instance, Comal Springs fountain darters have consistently tested positive for Large Mouth Bass Virus, until very recently. By looking at historical samples, we may be able to track the presence/absence and overall frequency of specific disease occurrences over time in the Comal and San Marcos Rivers, which can inform collection strategies and reintroduction plans.

Tables and Figures

Table 1. Fountain Darter Samples by sex and collection year.

Year	Sex	Number of Samples
2002	F	102
	M	83
2009	M	2
2010	F	7
	M	9
2011	F	7
	M	7
2012	F	4
2013	F	4
	M	19
2015	F	4
	M	7
2016	F	1
2017	F	1
	M	1
2018	F	24
Total		282

Table 2. Samples by preservation method by year.

Year	Preservation Method	Number of Samples
2002	Formalin	194
2008	Ethanol	1
2009	Ethanol	22
2010	Ethanol	26
2011	Ethanol	27
2012	Ethanol	20
2013	Ethanol	41
2015	Ethanol	5
	Formalin	15
2016	Ethanol	1
2017	Formalin	24
2018	Ethanol	90
	Formalin	49
2019	Ethanol	454
	Formalin	10
2020	Ethanol	213
	Formalin	4
2021	Ethanol	65
2022	Ethanol	1
Total		1,262

Table 3. AMOVA of total body lengths by year and collection location. Significant differences in total body length are evident for both Year and Collection Location.

	Df	Sum Sq.	Mean Sq	F-value	P-value	Significance Code
Year	1	1138	1138.1	47.887	2.44E-11	***
Collection Location	7	992	141.7	5.964	1.53E-06	***
Residuals	323	7677	23.8			

Significance Codes: P<0.001 = ***, P<0.01 = **, P<0.1 = *, P<0.5 = ., P<1 = ns

Table 4. A table showing the percent of PCR products that reached the target length in gel electrophoreses.

Preservation Solution and Storage Conditions	% Samples with Successful PCR Amplifications
Ethanol, Outside	11.19
Formalin, Outside	0
Formalin, Inside	37.5
Ethanol, Inside	87.74

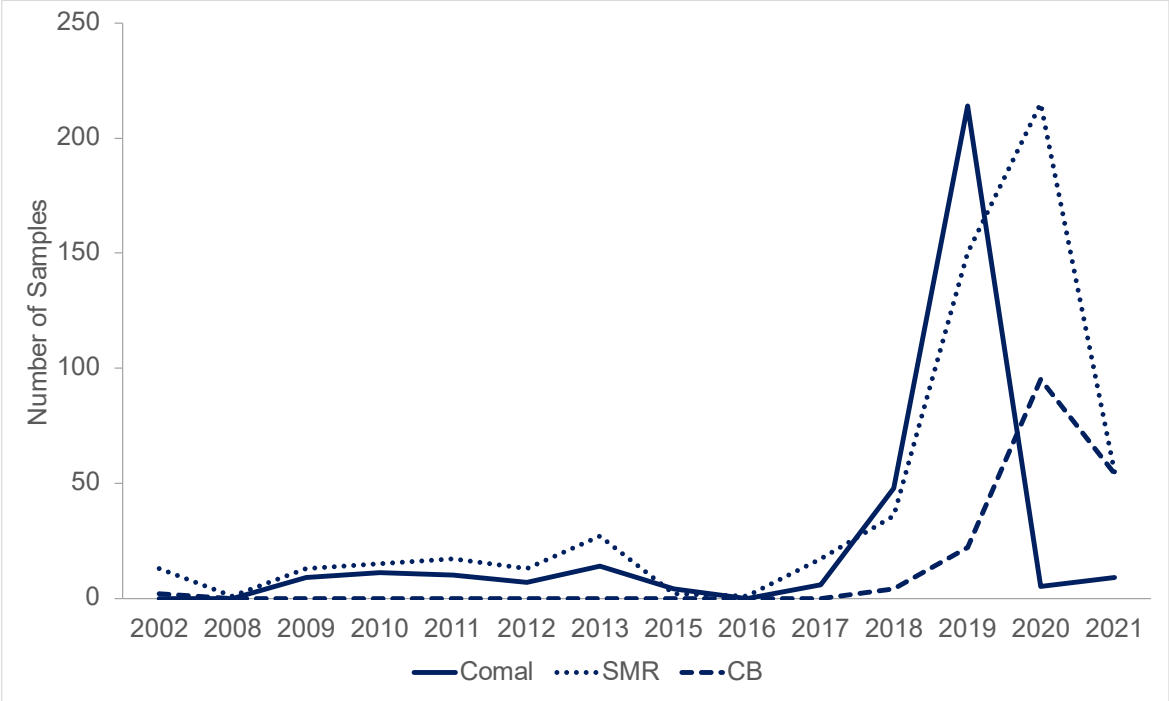


Figure 1. A line graph showing the number of samples archived from different populations over time. SMR – San Marcos River, CB – Captive Bred.

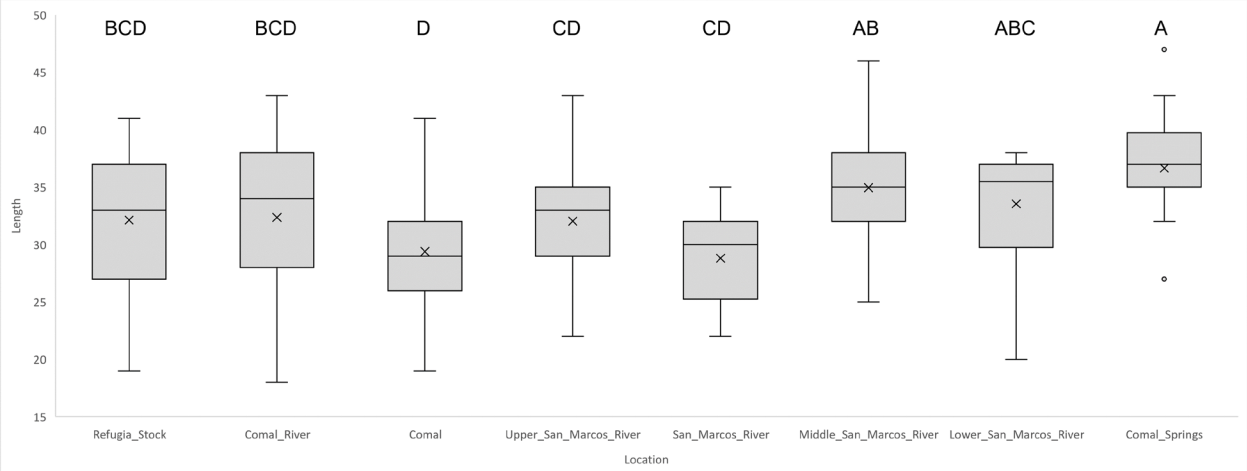


Figure 2. Total length distribution in mm of fountain darters from different collection locations. Mean is represented by the x within each box and the median length is represented by the horizontal bar in each box. Letters above each box represent significant relationships between locations. Locations that share letters are more similar to locations that do not.

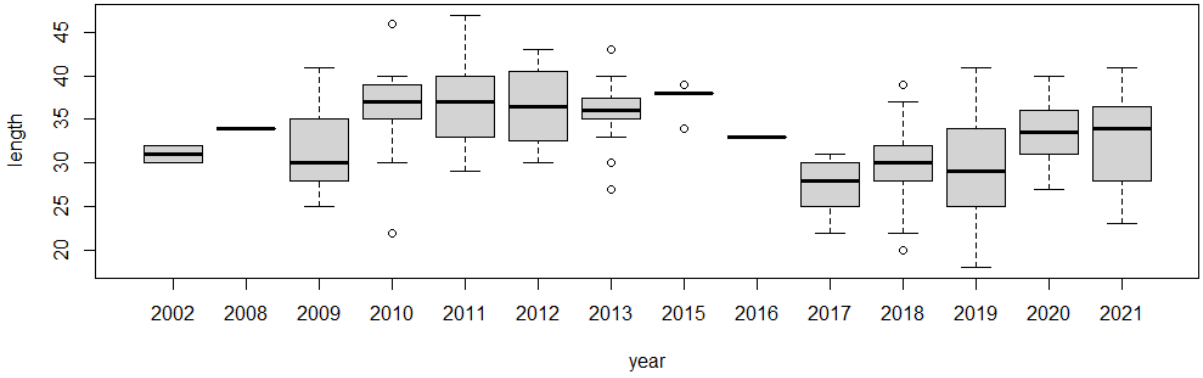


Figure 3. Length by collection year. Median length is represented by the horizontal bar within each box.

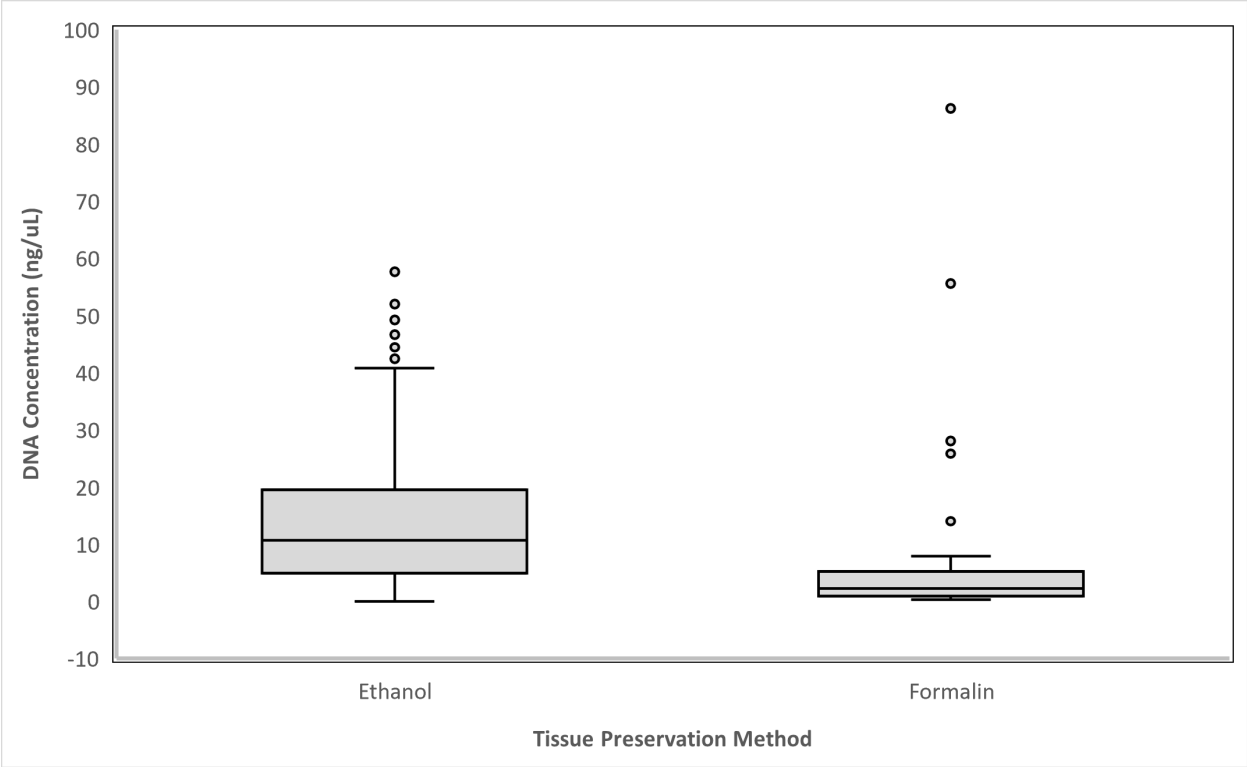


Figure 4. Distirbution of DNA concentrations post extraction from two different tissue preservation methods, 95% ethanol and 10% formalin. DNA concentration in ng/uL is listed on the y-axis while perservation method is listed on the x-axis. The horizontal bar within each box is the median DNA concntration.

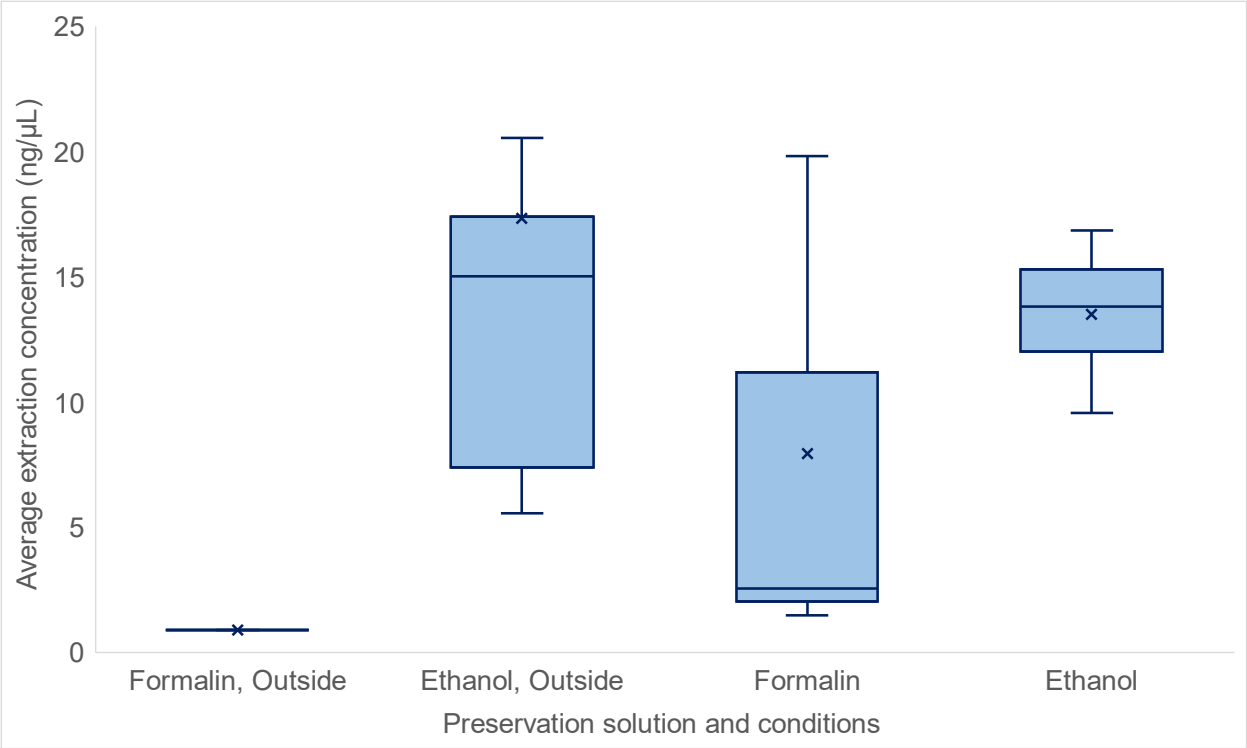


Figure 5. Distribution of DNA extraction concentrations for samples preserved in either ethanol or formalin and stored either inside or outside. Horizontal bars in the box represent the median concentration while the "x" represents the mean.

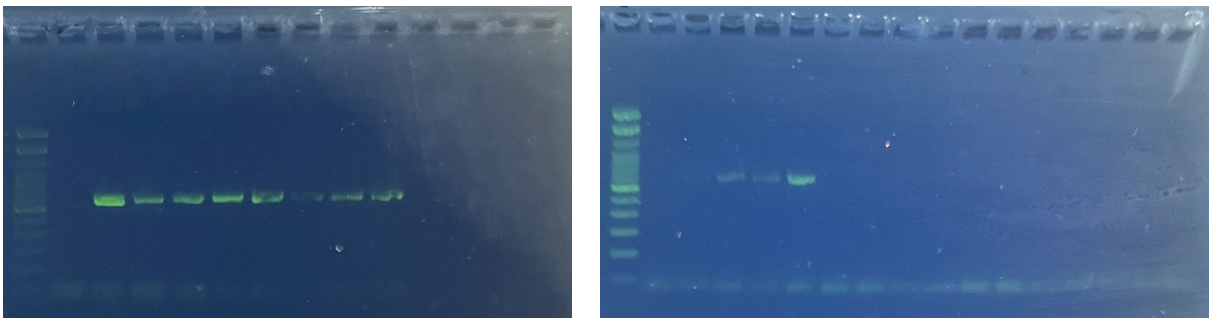


Figure 6. Left, resulting gel electrophoresis of samples preserved in ethanol and stored in climate-controlled conditions. The gel was run at 90 volts before the 200-volt protocol was determined to produce more clear banding. All wells show banding at the target 600 bp length, except for the negative control in the first well to the left of the ladder. Right, resulting electrophoresis of samples preserved in ethanol and exposed to extreme conditions. One well produced a clear band at the target length and four wells show faint bands at the target length. The negative control did not produce banding.

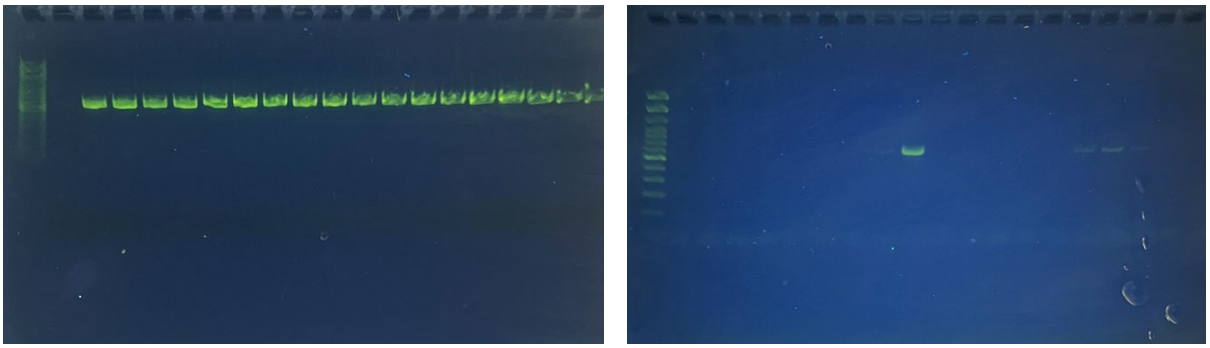


Figure 6. Left, resulting gel electrophoresis of samples preserved in formalin and stored in climate-controlled conditions. Eight samples out of twelve show clear banding at the 600 bp target length; the negative control produced no visible banding. Right, the two furthest right wells hold products of samples preserved in formalin and exposed to extreme conditions which did not produce visible banding. The other wells contain products of samples stored in formalin in climate-controlled conditions. One bright band and two faint bands are visible at the target length; eleven samples produced no banding. The negative control did not produce a visible band.

References

Britton, D., Campbell, L., and Anderson, K. 2021. Implementation of the Edwards Aquifer refugia program under the Edwards Aquifer habitat conservation plan. USFWS SMARC and UNFH Annual Report 2020.

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. 1996. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3(5), 294-299.

Joshi, B. D., Mishra, S., Sujeet, K. S., Goyal, S. P. 2013. An effective method for extraction and polymerase chain reaction (PCR) amplification of DNA from formalin preserved tissue samples of snow leopard. African Journal of Biotechnology. 12(22):3399-3404

Olsen, J.B., Kinziger, A.P., Wenburg, J.K. et al. 2016. Genetic diversity and divergence in the fountain darter (*Etheostoma fonticola*): Implications for conservation of an endangered species. Conserv Genet 17, 1393–1404.

Qiagen. 2020. DNeasy® Blood & Tissue Handbook. Qiagen, 30-33, 48.

ThermoScientific. 2016. DreamTaq™ Green PCR master mix (2X). Thermo Fisher Scientific Inc.

Evaluating the Effects of P-Chip Tags on Small-bodied Salamanders (*Eurycea* spp.)

2022 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Desiree M. Moore and Dr. Katie Bockrath

San Marcos Aquatic Resources Center

U.S. Fish and Wildlife Service

Table of Contents

Background **3**
Objectives **5**
Methods **5**
Results **6**
Discussion ..... Error! Bookmark not defined.
Tables and Figures **9**
References **14**

Background

The information gained through reliable tagging methods is important and aids in the research, conservation, and management of wildlife species. In captive (i.e., refugia) populations, tagging can be used to identify individuals and track associated information such as sex, age, and growth. Individual identification can reduce the number of enclosures needed in a refugia because organisms do not need to be separated based on collection location or year collected and therefore, individual enclosures are not needed to track individual-specific data for genetic or research purposes. Tags associated with low retention or readability can result in lost or incorrect information, ort ags with low readability may be confused with others or undetected completely. Mortality associated with tagging varies by species (Musselman et al. 2017) but is common in small-bodied organisms (Black et al. 2010, Kimball and Mace 2020) and should especially be avoided in at-risk species. Selecting appropriate tagging methods will depend on the taxon and objective of the project.

Visible implant elastomer (VIE) tags are currently used in the three salamander species (i.e., San Marcos salamander, Comal springs salamander, and Texas Blind salamander) held in refugia at the San Marcos Aquatic Resources Center (SMARC). VIE tags were found to be the best tagging method for these species when compared to passive integrated transponder (PIT) and visible implant alpha (VIA) tags (Campbell and Moon, 2020). Misidentification of VIE tags is becoming more of a problem for the refugia because many tags are older, and readability decreases over time. Additionally, there were many user errors identified during a recent inventory of Texas blind salamanders (*Eurycea rathbuni*). As biotechnicians were determining which individuals were still in the refugia, many tag codes were either not identified in the database, given to more than one individual, or identified as mortalities in the database. These errors are common when using VIE tags because it is easy to accidentally inject the incorrect color, write down incorrect information accidentally, or misidentify colors. VIE tags also do not provide individual identification unless several tags are used together, and using several VIE tags is more invasive for the organism. Additionally, SMARC is approaching the limit for unique VIE combinations to accurately identify individual salamanders. Unique VIE codes will be exhausted within the next 2-3 years, limiting research and tracking

applications. P-Chip microtransponders (hereafter p-Chips) may be a beneficial alternative.

The design of p-Chips may provide low mortality, high tag retention and readability, individual identification, and fewer user errors compared to the current VIE tagging method for salamanders held in the SMARC refugia. P-Chips are small microtransponder tags (500 µm x 500 µm x 100 µm) with photocells powered and read by a handheld wand that emits a laser. The wand is connected to a device (e.g., computer) with PharmaSeq's p-Chip Reader software to record the unique 9-digit code (PharmaSeq Inc., Princeton, NJ). The wound from injecting a p-Chip is smaller than injecting PIT or VIA tags, potentially resulting to less trauma and lower chances of infection. Furthermore, p-Chips are lightweight and may be less likely to be pushed back out of the wound from salamander movement (i.e., flexing muscles) because of their small size. P-Chips are less prone to human reading and recording error because they are read by a laser and codes are directly recorded into a database by the connected device (Pharmaseq Inc. 2020, Moore and Brewer 2021). P-Chips have not been evaluated in any salamander species but have been associated with high survival and retention in other small-bodied aquatic species (Chen et al. 2013; Faggion et al. 2020; Moore and Brewer 2021) and provide individual identification.

Tagging methods associated with higher survival and retention compared to other tagging methods are especially important for small-bodied salamanders like the San Marcos salamander (*Eurycea nana*) and Comal Springs salamander (*Eurycea pterophila*). Due to their small size and weight, p-Chips may cause less trauma and produce lower mortality rates in San Marcos salamanders than other tags that provide individual identification (i.e., passive integrated transponder, visible implant alphanumeric, and complex VIE tags). Additionally, p-Chips may exhibit higher tag retention compared to the simple VIE tags (in which one is inserted on each side of the salamander) currently used at the SMARC.

Tag retention and readability may improve with the use of p-Chips compared to our current use of VIE tags. Low mortality, high tag retention and readability, and individual identification can lead to improved conclusions in research and better-informed conservation and management decisions. Examining p-Chip microtransponders in the Comal Springs salamanders will provide the information

needed to assess the efficacy of a novel tagging strategy in small-bodied salamanders for use in the refugia.

Objectives

Our objective is to examine the survival and tag retention associated with p-Chip microtransponder tags in Texas blind and Comal Springs salamanders at the San Marcos Aquatic Resources Center.

Methods

SMARC staff examined the effects of tagging with p-Chips on two salamanders held in refugia, Texas blind salamander and Comal Springs salamander. Comal Springs salamander is not listed as a sensitive species and was used in leu of San Marcos salamanders to reduce potential negative effects to the refugia population. Texas blind salamanders have lower rejection rates than small-bodied salamanders for VIE tags and served as a comparative control. When possible, captive bred (F1) salamanders were used to minimize possible harm to the refugia standing stock population. Two control groups were used for each species to compare to the survival of tagged salamanders. The first group is a sham control, where salamanders are handled the same as tagged salamanders (i.e., anesthetized and punctured with a needle), except no tag was placed (hereafter sham). The second group is an untreated control, where salamanders are placed in a tank without handling (hereafter control). Using a sham and control allowed us to distinguish the effects of the handling process from the effects of the tag itself (Jepsen et al. 2015).

All salamanders were anesthetized by immersion in 0.5 g/L tricaine methanesulfonate (MS- 222) solution buffered with sodium bicarbonate. Anesthetized salamanders were placed in a clear plastic bag for easier handling. SMARC staff measured the weight and snout-to-vent length of each salamander, identified their sex by candling if possible, and performed the appropriate treatments (e.g., tag injection, puncture with no tag) before randomly assigning them to tanks with similar size classes.

P-Chips were injected subcutaneously into the base of the tail just posterior to

the left hindlimb of each salamander in the tagged groups. Taggers followed the manufacturer guidelines (PharmaSeq Inc 2020) using a 0.8-mm-diameter hypodermic needle. P-Chips were scanned with the laser reader after placement to record the tag number. Sham salamanders were treated the same as tagged salamanders, except no tag was placed (e.g., anesthetized, punctured with a needle). Control salamanders were measured and placed in experimental tanks with no further handling.

Salamanders were placed in flow-through aquaculture tanks after recovery for monitoring. Salamanders were held in a recovery tank until they were able to right themselves and swim properly. Salamanders were then placed in flow-through refugia tanks. All tagging equipment was disinfected after each salamander. SMARC staff monitored salamander tag retention, negative effects, and novice tag readability over six-eight months. Mortality was monitored daily for the duration of the study period. Each salamander was inspected for negative effects from tagging (e.g., infection, difficulty swimming) on the day they were tagged and daily thereafter. Tag loss was recorded in the event of a mortality and assessed weekly by scanning all tagged individuals. Novice readers scanned a subset of the salamanders (at least 20%) monthly to determine any tag readability differences between expert and novice operators. A new novice reader was selected each month to ensure novice readers did not gain experience between monthly reads. Tags were considered readable if the tag number was recorded by the laser reader. All salamanders were measured at the end of the study to compare net growth among treatments.

Kaplan-Meier time-at-event curves were created to visualize survival and tag retention temporally (Goel et al. 2010). These curves estimate the probability of the event (i.e., mortality, tag loss) over time. Staff used days since tagging and weeks since tagging as the time increments for the survival and tag retention curves, respectively. Log-rank tests were used to compare curves for each applicable treatment and examine any effects of salamander size on survival and tag retention. Our null hypothesis was that survival does not differ by treatment and tag retention does not differ by species.

Growth was examined by comparing the average length of salamanders at the beginning and end of the study. The length and weight of salamanders were closely correlated (0.93), meaning it was necessary to examine only one of these metrics. Less handling is required to measure the length of a salamander than the weight. Therefore, staff chose to only record the lengths of salamanders at the conclusion of the study. SMARC staff used two-sample t-tests to compare the change in mean length measurements in the tagged group and sham group to the control group. The null hypothesis was that the mean length did not differ statistically among treatments.

Results

Due to limited availability of salamanders, the sample size varied among treatments (Table 1). The tagged groups were the most numerous for each species to ensure the ability to conduct analyses examining tag retention. Salamander size was similar among treatments for each species (Table 1).

Our results indicated that tagging did not negatively affect survival for Comal Springs and Texas blind salamanders. One mortality occurred among Comal Springs salamanders which was part of the control group (Table 2; Figure 1). One mortality occurred among the Texas blind salamanders which was part of the tagged group (Table 2; Figure 2). The difference between survival curves was not statistically significant for the Comal Springs salamanders ($\chi^2 = 2.3$, $P = 0.3$) or Texas blind salamanders ($\chi^2 = 1.1$, $P = 0.6$).

Tag retention and readability did not differ between the two salamander species. One tag in a Comal Springs salamander was either lost or shifted to the point it could not be read (Figure 3). No tags were lost in Texas blind salamanders. However, tag retention was not significantly different between species ($\chi^2 = 1.1$, $P = 0.6$). All novice tag readers were able to accurately read all tags throughout the study.

Salamander growth did not appear to be affected by treatment and multiple clutches of eggs were laid by tagged salamanders of both species throughout the study. The change in length was not statistically different from the control group for tagged ($P = 0.53$) or sham ($P = 0.94$) Comal Springs salamanders. Similarly, the Texas blind salamander growth was not statistically different in the tagged ($P = 0.64$)

or sham (P = 0.99) groups when compared to the control group. All Texas blind salamander groups had longer average lengths at the end of the study than at the beginning (Table 1). For the Comal Springs salamander groups, only the sham had a longer average length at the end of the study. The tagged and control Comal Springs salamanders had shorter average lengths at the end of the study than the beginning (~1 mm). Staff noted that the highest amounts of growth were seen in the younger Texas blind salamanders. Additionally, most of the egg clutches produced by tagged salamanders in this study were laid within a month of tagging, when tagging stress is typically most apparent.

Discussion

Tagging methods that provide individual identification are important for maintaining a captive assurance population and reintroducing threatened and endangered species. P-Chips provided a high survival (97-100%) and tag retention (98-100%) without inhibiting growth in aquatic salamanders. Additionally, p-Chips provided an improved readability rate and higher number of individual identification codes than the VIE tags previously used at the SMARC.

Results from this study suggest P-Chips are a better tagging alternative to VIE tags for the Edwards Aquifer Refugia Program. Previous tagging efforts found that VIE tags are associated with high survival (95-96%) and retention (100%) in Comal Springs and Texas blind salamanders (Moon et al. 2022). However, the readability of VIE tags decreased over the year of that study and have been observed to continue to decrease thereafter. Because these salamanders live several years in captivity, it is particularly important for readability to be consistent over time. Although these tags were only monitored over six-eight months, the readability did not decrease at all during that time and was 100% for all novice readers. The VIE tag codes required multiple tags be placed in each salamander for identification. Because a single p-Chip is used, there are fewer needle wounds which should lessen stress and reduce opportunity for infection. Only two VIE tags could be placed in Comal Springs salamanders because of their small body size, reducing the ability for unique identification. P-Chips eliminate this problem because only one tag is needed.

SMARC staff did not observe any negative effects from tagging in either salamander species. Growth was not different among groups, and salamander behavior appeared normal in all treatment groups. Texas blind salamanders grew more than Comal Springs salamanders, but staff expected that result because all the Comal Springs salamanders were adults. It is possible many of the Comal Springs salamanders and larger Texas blind salamanders were already at or near their size limits. Some of the Comal Springs salamanders appeared to actually shrink during the study (e.g., shorter mean length at the end of the study; Table 1). Due to the small difference in length (i.e., ~1 mm) and because multiple people measured these salamanders, it is suspected that this is simply variation caused by measurement error. All treatment groups of each species produced eggs over the course of the study. Although egg production was not analyzed for this study, there did not appear to be a difference in production among treatments. Additionally, staff suspect the handling stress might have triggered some of the spawning events. Multiple groups that had never produced eggs before laid eggs within the next month, with most of those occurring within the first two weeks.

Tables and Figures

Table 1. Sample sizes, mean snout-to-vent lengths (SVL; ±SD), weights (±SD), and ranges for each treatment group. The final mean SVL was obtained at the conclusion of the study.

Species	Treatment	n	Mean SVL (mm) ± SD	SVL (mm) range	Mean weight (g) ± SD	Weight (g) range	Final mean SVL (mm) ± SD	Final SVL (mm) range
Comal Springs	Control	34	32.62 ± 2.17	29 - 37	0.63 ± 0.12	0.5 - 0.9	31.68 ± 1.96	28 - 36
	Sham	34	33.03 ± 2.05	30 - 39	0.65 ± 0.15	0.4 - 1.1	33.23 ± 1.83	30 - 38
	Tagged	43	33.02 ± 2.44	29 - 39	0.67 ± 0.15	0.4 - 1.1	32.34 ± 1.73	28 - 36
Texas blind	Control	20	48.60 ± 12.14	28 - 64	3.16 ± 1.91	0.5 - 6.3	49.70 ± 10.55	32 - 66
	Sham	20	47.90 ± 11.80	26 - 64	3.11 ± 1.90	0.4 - 6.9	49.55 ± 10.36	31 - 64
	Tagged	38	50.63 ± 10.05	26 - 67	3.38 ± 1.66	0.4 - 8.1	51.25 ± 9.40	28 - 68

Table 2. Percent survival and percent tag retention over the duration of the study in each treatment group. Tag retention in control and sham salamanders was not applicable (-).

Species	Treatment	n	Survival (%)	Retention (%)
Comal Springs	Control	34	97%	-
	Sham	34	100%	-
	Tagged	43	100%	98%
Texas blind	Control	20	100%	-
	Sham	20	100%	-
	Tagged	38	97%	100%

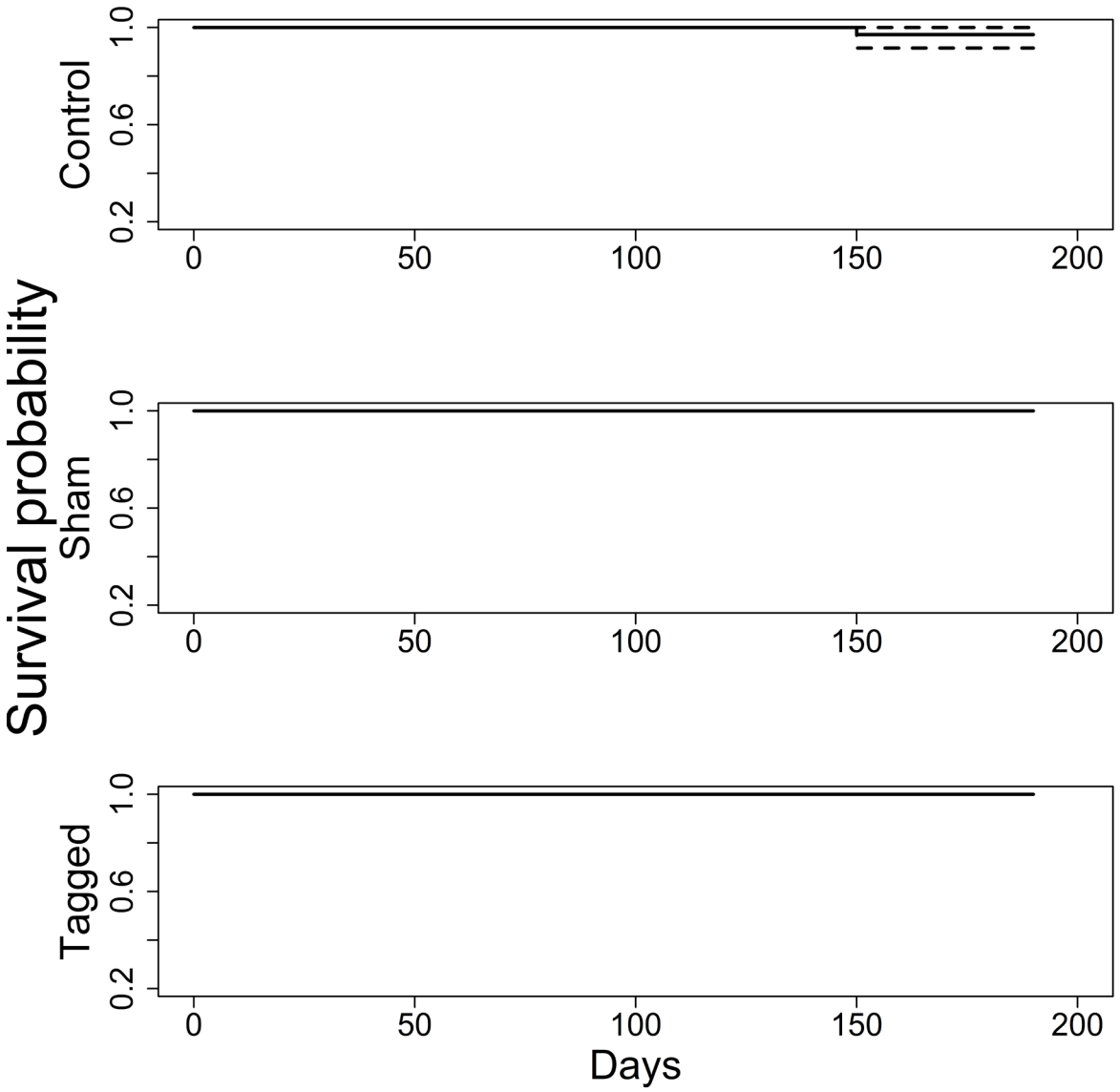


Figure 1. Kaplan–Meier time-at-event curves for survival developed for Comal Springs salamander treatment groups. The control group was handled the same as the tagged group (i.e., anesthetized and measured) except no puncture or tag was placed. The sham group was handled the same as the tagged group (i.e., anesthetized, measured, and punctured with a needle) except no tag was placed. The tagged group was tagged subcutaneously at the base of the tail near the left hindlimb with p-Chips. The curves show the survival probability with 95% confidence intervals (dashed lines) over time where one mortality occurred in the control group 150 days post-tagging.

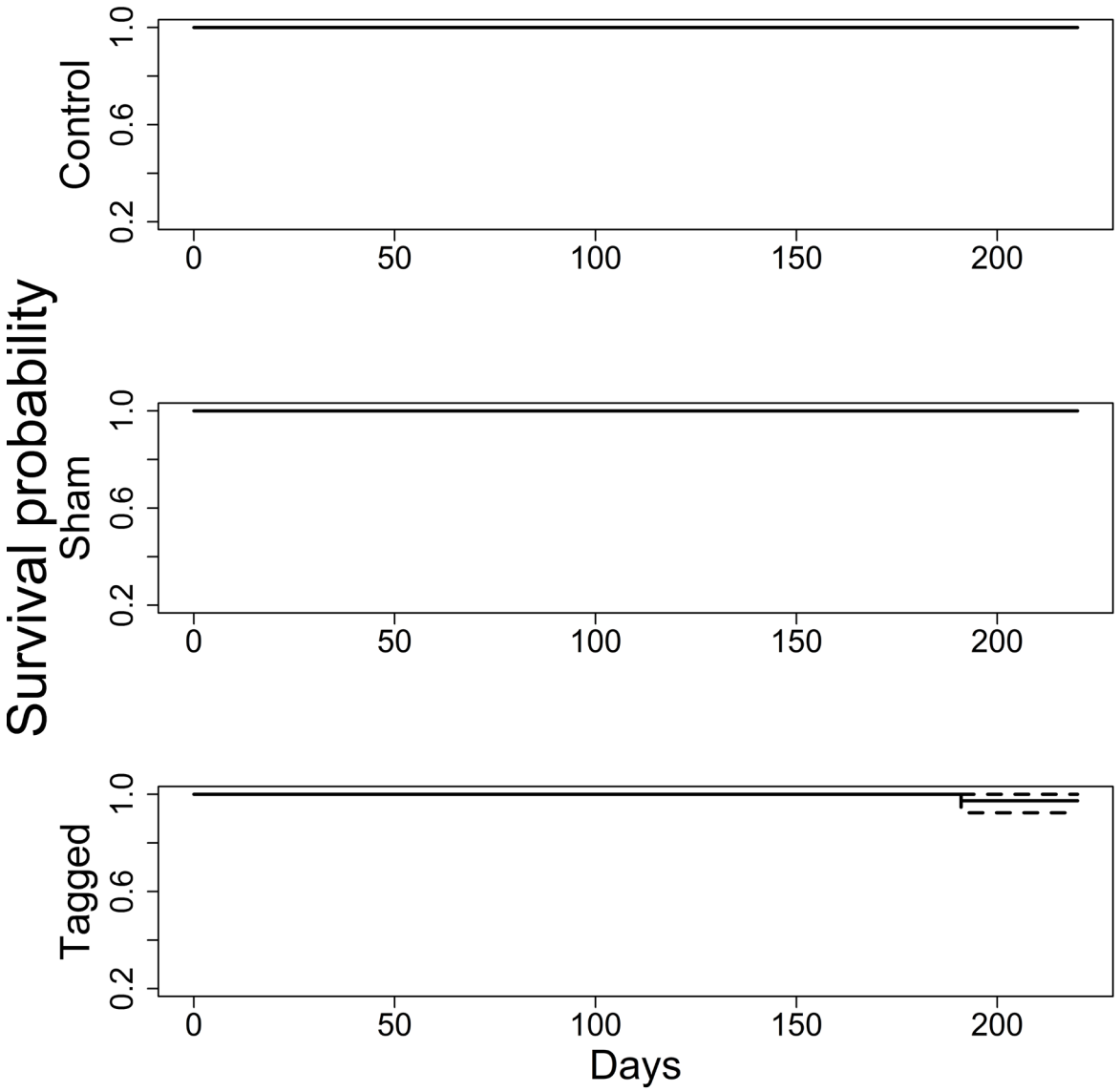


Figure 2. Kaplan–Meier time-at-event curves for survival developed for Texas blind salamander treatment groups. The control group was handled the same as the tagged group (i.e., anesthetized and measured) except no puncture or tag was placed. The sham group was handled the same as the tagged group (i.e., anesthetized, measured, and punctured with a needle) except no tag was placed. The tagged group was tagged subcutaneously at the base of the tail near the left hindlimb with p-Chips. The curves show the survival probability with 95% confidence intervals (dashed lines) over time where one mortality occurred in the tagged group 191 days post-tagging.

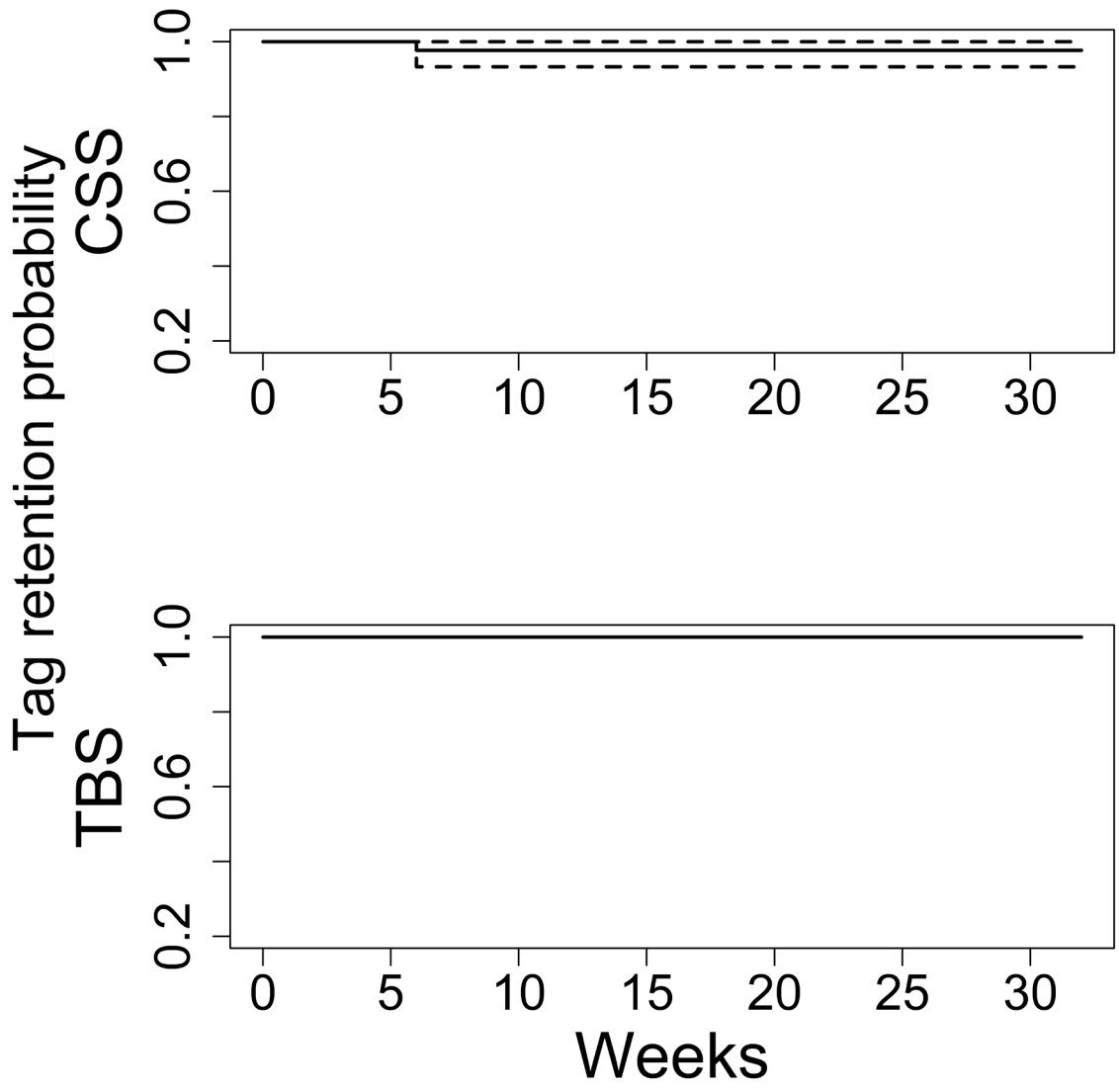


Figure 3. Kaplan–Meier time-at-event curves for tag retention developed for p-Chip tagged Comal Springs salamanders (CSS) and Texas blind salamanders (TBS). The salamanders were tagged subcutaneously at the base of the tail near the left hindlimb with p-Chips. The curves show the tag retention probability with 95% confidence intervals (dashed lines) over time where one tag loss occurred in the Comal Springs salamanders 6 weeks post-tagging.

References

Black, T. R., S. S. Herleth-King, and H. T. Mattingly. 2010. Efficacy of internal PIT tagging of small-bodied crayfish for ecological study. Southeastern Naturalist 9:257-266.

Campbell, L., and L. Moon. 2020. Evaluating three different long-term tagging methods in aquatic salamander species. Report for the Edwards Aquifer Authority from the Edwards Aquifer Refugia Program. San Marcos, TX.

Chen, C. H., E. Durand, J. Wang, L. I. Zon, and K. D. Poss. 2013. Zebraflash transgenic linesfor in vivo bioluminescence imaging of stem cells and regeneration in adult Zebrafish. Development 140(24):4988–4997.

Edwards Aquifer Refugia Program. 2020. Salamander sedation via MS-222 (tricaine methanesulfonate) standard operating procedure. San Marcos Aquatic Resources Center, San Marcos, TX.

Faggion, S., P. Sanchez, M. Vandeputte, F. Clota, A. Vergnet, M. O. Blanc, and F. Allal. 2020. Evaluation of a European Sea Bass (*Dicentrarchus labrax* L.) post-larval tagging method with ultra-small RFID tags. Aquaculture 520:734945.

Goel, M., P. Khanna, and J. Kishore. 2010. Understanding survival analysis: Kaplan-Meier estimate. International Journal of Ayurveda Research 1(4):274–278.

Kimball, M. E., and M. M. Mace. 2020. Survival, growth, and tag retention in estuarine fishes implanted with passive integrated transponder (PIT) tags. Estuaries and Coasts 43(1):151-160.

Moore, D. M., and S. K. Brewer. 2021. Evaluation of VIE, PIT, and p-Chip tagging methods in a small-bodied minnow species. North American Journal of Fisheries Management 41(4):1066-1078. https://doi.org/10.1002/nafm.10607.

Musselman, W. C., T. A. Worthington, J. Mouser, and D.M. Williams. 2017. Passive integrated transponder tags: review of studies on warmwater fishes with notes on additional species. Journal of Fish and Wildlife Management 8(2):353-364.

PharmaSeq Inc. 2020. Zebrafish p-Chip implantation protocol. PharmaSeq, Inc. MonmouthJunction, NJ.

Treatment Trials for *Batrachochytrium dendrobatidis* Infections in Aquatic Salamanders

2023 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Dr. Katie Bockrath and Desiree M. Moore

San Marcos Aquatic Resources Center
U.S. Fish and Wildlife Service

Table of Contents

Background 3

Objectives 3

Methods 3

Results 5

Discussion 6

Tables and Figures 7

References 8

Background

Batrachochytrium dendrobatidis (Bd) is a deadly fungal infection for amphibians. It is associated with high mortality in frogs and terrestrial salamanders (Scheele 2020) and is found on the skin/epidermal tissue of aquatic salamanders (Chatfield 2012). Depending on the strain and environmental conditions, Bd shows large variation in its physical presentation in amphibian infections (Retallick 2007). Aquatic salamanders associated with the Edwards Aquifer region routinely test positive for Bd, yet Bd does not present with the same signs and extent of clinical disease or lesions as frogs, toads, and terrestrial salamanders. Through observation, Texas blind salamanders (*Eurycea rathuni*) appear to be mostly unaffected by Bd infections with only a few individuals displaying clinical signs through lesions and degradation of foot tissue. Observationally, San Marcos salamanders (*Eurycea nana*) may be more impacted by Bd infections. Potential clinical presentation in San Marcos salamanders may include ruptured abdomens and capillaries in the head and tail. Bd treatments are established for terrestrial amphibians and include itraconazole dips (Brannelly 2012) and extended exposure to increased temperatures (Chatfield 2011). Itraconazole treatments have been tested in a few aquatic salamander species with some reported success (Del Valle 2019). Here, we aim to test the efficacy of approved medication for Chytrid treatment in terrestrial amphibians on San Marcos salamanders with and without Bd infections. The goal is to develop a Bd prophylactic treatment for aquatic salamanders entering the San Marcos Aquatic Resources Center Edwards Aquifer Refugia.

Objectives

To test the efficacy of itraconazole and dosing strategies to treat Bd infections in aquatic salamanders.

Methods

As many F1 San Marcos salamanders as possible were used for these trials, but some individuals from the refugia population (i.e., wildstock) were used to supplement the F1 salamanders. San Marcos salamanders were not tested for Bd prior to the start of the pilot and treatment trials due to time constraints. Instead, salamanders that shared a tank were randomly and evenly distributed among treatments under the assumption that approximately equal numbers of Bd-positive and Bd-negative salamanders would be assigned to each treatment. Because a wide range of doses have been used to treat Chytrid in the literature, it is unclear what concentration of itraconazole should be used for San Marcos salamanders. Prior to starting the treatment trials, a small pilot study following the same protocol outlined below was conducted to determine tolerable dose.

Pilot Study: Three dose treatments (high, mid, low), as prescribed by a veterinarian, were tested using three San Marcos salamanders for each dose. All individuals were monitored for immediate and long-term effects of exposure over the course of 2 weeks. The high dose group

received the dose at 0.01% itraconazole, the mid dose group received 0.005% itraconazole, and the low dose group received 0.0025% itraconazole. Salamanders were swabbed before and after treatments for Bd testing. No ill effects resulted from any treatment group, but the results did not show change in Bd detections. Therefore, the high dose of 0.01% (hereafter low treatment) and a higher dose of 0.025% (hereafter high treatment) were selected for the full-scale treatment trial.

Treatment Trials: An equal number of F1 and wildstock salamanders were included in each treatment to control for differences between generations. Sex was recorded for each salamander when possible, and the presence of eggs in females was noted. The snout-to-vent length (SVL) of each salamander was also measured. All salamanders were held in individual 0.5-gal tanks on a shared flow-through system to prevent cross transmission of Bd during treatment while providing similar water quality across individuals. Three treatment groups were established where two of the three groups test the efficacy of itraconazole at two different concentrations (low and high), while the third is a no-treatment control group. Each treatment group consisted of 24 randomly selected salamanders randomly assigned to tanks for a total of 72 salamanders in the study. Three trial runs of the study were conducted due to limited space. Each trial run contained at least eight salamanders in each treatment group. However, the number of salamanders varies by trial run because any mortalities or missing salamanders were replaced in subsequent trial runs to maintain the sample size of 24 per treatment.

The itraconazole treatment groups followed a modified Del Valle and Eisthen (2019) protocol using the itraconazole concentrations determined in the pilot study. Salamanders were submerged in at least 30 mL of their respective dose treatments for ten minutes every 24 hours for 10 days. All salamanders were swabbed for Bd infection testing at treatment days 0, 5, and 10. Salamanders were swabbed again for Bd testing 10 days after the treatment was completed. All swabs were stored at a minimum of -20˚ C until analysis. The no-treatment control salamanders were housed in the same set-up configuration, handled the same as the treatment groups, and tested for Bd at the same frequency but were not exposed to itraconazole. Salamanders in all treatment groups were observed between and after treatments for latent effects of drug exposure and potential mortalities.

Bd Testing: Swabs were removed from cold storage and allowed to reach room temperature before undergoing DNA extractions using the Qiagen DNeasy Blood and Tissue Kit (Brennelly et al. 2020, Qiagen ID: 69516). Sunfish (*Lepomis* sp.) tissue was used as a positive extraction control and sterile water was used as a negative extraction control. Extraction controls were included in all extraction reactions. DNA extracts were stored at -80˚ C until further analysis.

DNA extractions were tested for the presence of Bd in quadruplicate using a real-time (or quantitative) Polymerase Chain Reaction (qPCR) following Boyle et al. 2004. Two 5-point standard curves, two Bd positive controls, and four Bd negative controls were run along with the DNA extractions to provide quality assurance and quality control for accurate data

interpretation. qPCR reactions were carried out on a BIORAD Opus 96 real-time thermocycler using the following methods described in Boyle et al. 2004. Data was visualized and analyzed using the BIORAD CFX Maestro software vs 2.3. The baseline threshold for determining a positive detection was auto calculated in the Maestro software using the standard curves. Any qPCR amplification crossing this threshold between 15 and 41 cycles was considered a positive detection. Differences in Bd positive status and copy number values before, during, and after treatment were compared within and between treatment groups.

Results

Bd Treatments: Some salamanders died or went missing over the course of their treatment. Two female salamanders were found dead in their tanks with visible hemorrhages in the abdomen after three days of treatment. One was in the low treatment group and the other was in the high treatment group. Additionally, two no-treatment control salamanders were found dead after escaping and falling from their tanks. One no-treatment control salamander and one low treatment salamander went missing from their tank and presumably escaped into the drain in the room. Salamanders were found in the drain but were not returned to the study in case of mistaken identity and effects of being in the drain. All these salamanders were replaced in subsequent trials to maintain a sufficient sample size. One incident occurred where four no-treatment control salamanders were accidentally placed in the high treatment solution, and one was accidentally placed in the low treatment solution for a minute or less. The salamanders were then placed in water without any medication and this mistake was noted to account for any strange results in the study. There were many occasions where a salamander escaped to the floor and returned to their tank after no visible damage was seen. This is unsurprising as these salamanders are well known for successfully escaping their tanks.

No evidence of harm apart from lethargy were observed in salamanders and one salamander laid a clutch of eggs in the week after their treatments. No lesions or other physical damage were noted for any salamanders during the study other than the two that died with abdominal hemorrhaging, which is consistent with what is seen in female San Marcos salamanders in the general refugia population. Additionally, a no-treatment control salamander laid a clutch of eggs in the week following their treatment, indicating the handling stress was not harmful for salamanders in the study.

Bd Testing: The Bd treatments using itraconazole do not appear to impact the Bd status in aquatic salamanders. Salamanders, regardless of treatment group or dosage, may test positive prior to treatment, test negative post treatment, and test positive again 10 days post treatment while in isolation. Additionally, salamanders may test negative prior to treatment and test positive post treatment (Table 1).

All treatments combined, 30% of salamanders who underwent treatments tested positive for Bd prior to treatment. Most salamanders exhibited to change in Bd status after treatment (47%). Only 24% of salamanders were positive pre-treatment and tested negative

post-treatment. The remaining 29% of salamanders were negative pre-treatment and tested positive for Bd post-treatment (Table 2).

qPCR QA/QC controls ran as expected (R2 = >95% and E-Value between 0-120%, positive controls amplifying and negative controls failing to amplify). Most positive samples crossed the threshold late in the reaction and at a lower copy number than the lowest standard curve value (10 copies), suggesting Bd is in low abundance on San Marcos salamanders.

Discussion

The high and low dose itraconazole treatment did not have a noticeable effect on San Marcos salamander Bd status, which may be due to the overall low concentration of itraconazole administered. Previous literature used a significantly higher dose than what was administered in this study, suggesting that the vet approve dosage was simply too low to eliminate Bd in Bd positive individuals, or, at minimum, prevent individuals from testing positive for Bd.

Throughout the study, we tried to have the same person swab salamanders at each treatment step, but this was not always possible. Variation in swabbing pressure and technique can impact the collection of Bd from the salamander's skin. If the swabbing is not aggressive enough to transfer Bd from the skin to the swab, Bd would not be detected even if the salamander has an active Bd infection.

In this study, we did not test the water in which the salamanders are held for Bd. The sporadic Bd detections may have been Bd detections from the water the salamanders were in and not indicative of Bd infections on the skin of the salamander itself. Because the salamanders exhibited no ill effects associated with exposure to the drug, additional studies using much higher doses of itraconazole and additional investigation on occurrence of Bd in well water are still promising. Unfortunately, at this time, there is no evidence gathered from this study suggesting itraconazole should be used as an effective approach to treat Bd infections in aquatic salamanders.

Tables and Figures

Table 1. Bd status of San Marcos Salamanders pre and post Itraconazole Bd treatment across three doses (high, low, none). Only Trial 1 is shown. In the Pre-treatment and post-treatment columns, a "1" indicates positive Bd status and a "0" indicates negative Bd status. Change overall after treatment was no change post-treatment (none), improvement or no bd detected post-treatment (improve) and decline or Bd detected post treatment (Decline).

Dose	Pre-treatment	Post-treatment	Change
High	0	0	None
High	0	1	Decline
High	0	1	Decline
High	1	0	Improve
High	1	1	None
Low	0	0	None
Low	0	0	None
Low	0	1	Decline
Low	0	1	Decline
Low	0	1	Decline
Low	1	0	Improve
None	0	0	None
None	0	0	None
None	0	0	None
None	0	0	None
None	1	0	Improve
None	1	0	Improve

Table 2. Percent of San Marcos Bd condition post-treatment. Bd status post itraconazole treatment (low and high doses combined) either improved (no Bd detection post treatment), declined (Bd negative pre-treatment, but Bd positive post treatment), or No Change (same Bd status pre- and post-treatment).

Condition Post Treatment	Percent
Improved	24
Declined	29
No Change	47

References

Boyle, D. G., D. B. Boyle, V. Olsen, A. T. Morgan, and A. D. Hyatt. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms 60:141-148.

Brannelly, L. A., C. L. Richards-Zawacki, and A. P. Pessier. 2012. Clinical trials with itraconazole as a treatment for chytrid fungal infections in amphibians. Diseases of Aquatic Organisms 101:95-104.

Brannelly, L. A., Wetzel, D. P., West, M., Richards-Zawack, C. L. (2020). Optimized Batrachochytrium dendrobatidis DNA extraction of swab samples results in imperfect detection particularlywhen infection intensities are low. Deisease of Aquatic Organisms, 139(2020), 233-243.

Chatfield, M. W. H., P. Moler, C. L. Richards-Zawacki. 2012. The amphibian Chytrid fungus, *Batrachochytrium dendrobatidis*, in fully aquatic salamanders from Southeastern North America. PLOS ONE 7(9):e44821.

Chatfield, M. W. H., and C. L. Richards-Zawacki. 2011. Elevated temperature as a treatment for *Batrachochytrium dendrobatidis* infection in captive frogs. Diseases of Aquatic Organisms 94:235-238.

Del Valle, J. M., and H. L. Eisthen. 2019. Treatment of Chytridiomycosis in laboratory axolotls (*Ambystomoa mexicanum*) and rough-skinned newts (*Taricha granulosa*). Comparative Medicine 69(3):204-211.

Retallick, R. W. R., and V. Miera. 2007. Strain differences in the amphibian chytrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. Diseases of Aquatic Organisms 75:201-207.

Scheele, B. C., F. Pasmans, L. F. Skerratt, L. Berger, et al. 2020. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. Science 363:1459-1463.

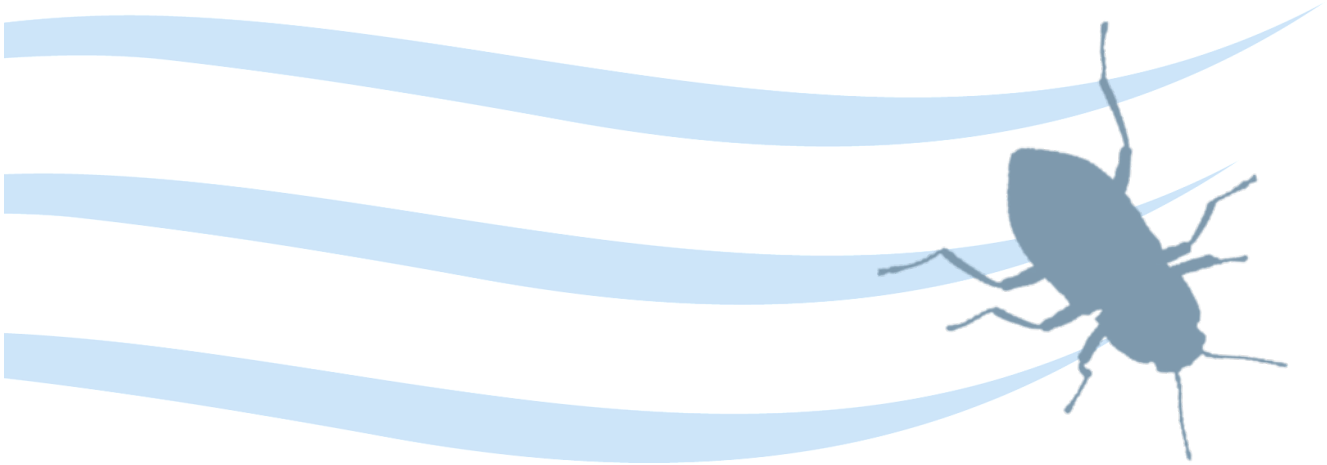
Standish, I., E. Leis, N. Schmitz, J. Credico, S. Erickson, J. Bailey, J. Kerby, K. Phillips, and T. Lewis. 2018. Optimizing, validating, and field testing a multiplex qPCR for the detection of amphibian pathogens. Diseases of Aquatic Organisms 129:1-13.

Takahara, T., T. Minamoto, and H. Doi. 2013. Using environmental DNA to estimate the distribution of an invasive fish species in ponds. PLoS One 8(2):e56584.

Genetic Assessment of the Comal Springs Riffle Beetle in Landa Lake – Interim Report

2022 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program



Prepared by Dr. Katie Bockrath and Desiree M. Moore

San Marcos Aquatic Resources Center

U.S. Fish and Wildlife Service

Table of Contents

Background **3**
Objectives **3**
Methods and Results **4**

Field Collections 4
Lab Work 5

Background

Landa Lake in New Braunfels, TX is the prominent recreational feature of Landa Park. It is surrounded by residential housing and has been heavily modified with the addition of paved river edges. Despite its recreational use, endemic groundwater species persist at the spring upwellings located across the lake. In addition to habitat destruction and heavy recreational usage, groundwater species are sensitive to fluctuations in environmental conditions. Drought events and water usage from several large cities put immense pressure on ground water availability in the Edwards Aquifer and can lead to low flow and high temperature conditions. The Edwards Aquifer Refugia Program serves to develop a functional refugia for endemic species dependent on flow from the Edwards Aquifer. If the habitat was drastically altered and becomes uninhabitable, these endemic species will be brought into the Refugia until they can be reintroduced. To ensure the population is accurately reflected in the Refugia, it is critical to understand how genetic variation is distributed across a species range. This information informs where individuals should be collected and the minimum number of individuals required to ensure the Refugia population accurately reflects the wild.

Gonzales (2008) and Colman (2021) show distinct genetic clustering among riffle beetle species across central Texas, as expected. Their data also show genetic separation between the Comal Springs and San Marcos Springs populations of Comal Springs riffle beetle. When assessed at a finer scale, both Gonzales (2008) and Colman (2021) show distinct clustering of subpopulations across Landa Lake with one of the studies suggesting distinct genetic lineages among spring runs (Colman 2021). Here, we aimed to assess the genetic diversity of the Comal Springs riffle beetle found in spring upwellings across Landa Lake. The genetic data gathered will inform future Refugia collection needs by ensuring the total genetic diversity of this population is reflected in the Refugia.

Objectives

Assess the population-level genetic diversity of the Comal Springs riffle beetle across Landa Lake and estimate effective population size to inform Refugia collection and Comal Springs riffle beetle conservation needs.

Methods and Results

Field Collections

Spring openings across Landa Lake were identified for candidate locations to set poly-cotton lures in 2023. Collections were postponed until 2023 due to drought conditions causing spring flows to decrease to below 130 cfs in 2022.

Figure 1. General locations for each spring run reach in the Comal Springs system in Comal County, Texas where the Comal Springs riffle beetle has been collected (Bosse, Tuff & Brown 1988; Barr 1993; Coleman, Gibson, and Norris, 2022, pers. comm.).

Table 1. Candidate sites for setting poly-cotton lures. Sites include biomonitoring sites and new locations not used for biomonitoring.

Site	Number of Spring Openings
Spring Run 1	10
Spring Run 3	10
Spring Run 4	8
Western Shoreline	8
Spring Island	27

Lab Work

F1 mortalities of larval and adult Comal Springs riffle beetles reserved from other ongoing research efforts were preserved in 95% ethanol for downstream DNA extraction. DNA was extracted using a Qiagen DNeasy Blood and Tissue kit. A negative extraction control was included with each reaction set.

Works Cited

Colman, W. (2021). Genetic Diversity of the Comal springs riffle beetle (Hterelmis comalensis). T. T. G. I. Forum. The 2021 Texas Groundwater Invertebrate Forum, The 2021 Texas Groundwater Invertebrate Forum.

Gonzales, T. K. (2008). Conservation genetics of the comal springs riffle beetle (heterelmis comalensis) populations in Central Texas, with examination of molecular and morphological variation in Heterelmis sp. throughout Texas. https://digital.library.txstate.edu/handle/10877/3118, Texas State University. **Master of Science**.

January 2022 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dr. Katie Bockrath, Adam Daw, Desiree Moore, and Jennifer Whitt

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

On January 8, 2022, Dr. Katie Bockrath, Adam Daw, Tommy Funk, and Braden West placed traps for Texas blind salamanders at Rattlesnake Cave and well in San Marcos, TX. The traps were checked on the 13, 18 and removed on the 21 (Figure 1). No Texas blind salamanders were captured.

On January 13, 2022, Mr. West and Jennifer Whitt joined PhD student Will Coleman (Texas State University, Dr. Chris Nice lab), Randy Gibson (SMARC), and Amelia Hunter (Ecological Services, Austin TX) on a sampling event for Mr. Coleman's current genetics research project at Spring Runs 1, 2, and 3 in Landa Park and Spring Island in New Braunfels, TX (Figures 2 and 3). The Edwards Aquifer Refugia Program (EARP) collected 152 adult Comal Springs riffle beetles as bycatch from the event. The Comal Springs riffle beetles collected were retained for the refugia population at the Uvalde National Fish Hatchery (UNFH).

Husbandry

On January 14 and 18, EARP staff met with Sarah Valdez and Sarah Mock from the Edwards Aquifer Authority (EAA) to go over husbandry care for some of the threatened and endangered species of the Edwards Aquifer (Figures 4 and 5). Ms. Valdez and Ms. Mock oversee the aquarium displays that will showcase San Marcos fountain darters and Texas blind salamanders at the new EAA Education Outreach Center (EOC) in San Antonio, TX.

Uvalde

Mr. Daw finished the initial modification to tank RE15 in the UNFH refugia. Mr. Daw added a mechanical filtration system that will allow the system to run for extended periods without well water input. Mr. Daw also added a monitor and control system that will dose CO2 into the water to maintain a constant system pH. Several sensors were added to the system to record water flow, air pressure, and water depth to monitor the proper functioning of the tank. If conditions deviate from the defined settings, the system alarms will activate and turn on/off equipment as needed.

To prevent further rusting of the wall in the invertebrate room at the UNFH refugia, Ms. Whitt removed the peeling paint and rust. Ms. Whitt then treated the area and prepped it for painting.

Ben Thomas performed an overhaul on tank RE13 in the refugia. Mr. Thomas used a razor to remove the old separation barriers and then modified them to fit more securely in the tank.

SMARC

Mr. Daw, Mr. Funk, and Mr. West started consolidating tanks in the SMARC quarantine building to make room for new quarantine systems.

Mr. Daw started building the first of two new invertebrate tank racks in the refugia room at the SMARC.

Mr. Daw started the construction of the new filter system that will go on one of the Texas wild rice tanks at the SMARC to reduce algae growth in the tanks and system maintenance.

Mr. Funk plumbed and assembled a new tank system for holding refugia blackworms.

Ruben Tovar (University of Texas), Brittany Dobbins (Texas State University), and Adam Walle (Texas State University) set up an incubator at the SMARC to house salamander eggs during development. Mr. Funk and Mr. West trained the visiting researchers in the EARP biosecurity protocols and the care of salamander eggs. The eggs are used in Mr. Tovar's Ph.D. research on the evolution of eye development.

Animal Health

A Texas blind salamander was observed in refugia tank ER6 at the SMARC to have abnormal growths on its body and was euthanized on January 17 due to its poor condition. The following week, two more individuals in that tank showed similar symptoms. The EARP team moved all of the salamanders from tank ER6 to the quarantine room during which two more affected salamanders were found. The four salamanders that showed symptoms were separated from the larger population into four smaller individual tanks. After receiving approval, two of the symptomatic salamanders were shipped to the Southwestern Fish Health Unit in Dexter, NM for disease analysis.

Task 2 Research

Comal Springs Riffle Beetle Pupation

Dr. Bockrath, Ms. Moore, and Israel Prewitt (BIO-WEST) confirmed flow was within the adequate range for all tubes weekly.

Ms. Moore and Mr. West completed construction of the second flow-through system, which will be used to house the tubes for Phase III of the project.

Ms. Moore prepared dowels for wild biofilm development. Mr. Prewitt placed the prepared dowels in Spring Run 3 for conditioning for Phase III of the project.

Comal Springs Riffle Beetle Exposure to Staphylococcus

Ms. Moore recorded the monthly inventory of Comal Springs riffle beetles at the SMARC from Trial 2 (Table 2). The living adults were placed back in their tube for monitoring. These adults are not included in the refugia census.

Captive Habitat for San Marcos Salamanders

The third replicate trial of this experiment is ongoing. Mr. Thomas and Ms. Whitt continued conducting daily checks for egg presence. One female salamander was transferred from the experimental system to a hospital tank due to swelling near the mandible. The salamander was replaced by a new female of similar size. No oviposition has occurred thus far.

Ms. Moore continued compiling reports, manuscripts, and records for the San Marcos salamander Refugia Handbook.

Comal Springs Riffle Beetle Population Genetics

This project will assess the genetic diversity of Comal Springs riffle beetles across Landa Lake and estimate effective population size to inform collections and Comal Springs riffle beetle conservation needs.

Dr. Bockrath and Ms. Moore began purchasing and gathering the equipment and supplies for this project.

Batrachochytrium dendrobatidis *(Bd) Infection Treatment in Aquatic Salamanders*

This year, SMARC staff are testing the efficacy of medication (itraconazole) and dosing strategies to treat Bd infections in aquatic salamanders.

Dr. Bockrath and Mr. Daw met with Dr. Trista Becker (Southwestern Native Aquatic Resources and Recovery Center) to discuss the protocols for testing treatments on salamanders.

Dr. Bockrath and Ms. Moore began purchasing and gathering the equipment and supplies for this project.

*Comal Springs Riffle Beetle (*Heterelmis comalensis*) Refugia Manual*

The objective of this project is to write a Comal Springs riffle beetle Refugia Handbook that details the knowledge of husbandry and captive propagation methods for CSRB.

Ms. Moore began compiling reports, manuscripts, and records pertaining to Comal Springs riffle beetles.

P-Chip Tag Effects on Eurycea *spp.*

This study will examine the survival and tag retention associated with p-Chip microtransponder tags in Texas blind and Comal Springs salamanders.

Dr. Bockrath and Ms. Moore began purchasing and gathering the equipment and supplies for this project.

*Catalog of Historical Fountain Darter (*Etheostoma fonticola*) Tissue*

The goal of this project is to inventory and catalog the fountain darter tissue samples that have been preserved and kept on station since the 1990s. The database will include length and sex data, as available. An archived tissue database and catalog/preservation standard operation procedure (SOP) will be generated, and a subset of the tissue samples will be prepared for DNA extraction to test the tissues' suitability for future genetics assays.

Dr. Bockrath sent the Student Conservation Association (SCA) intern position announcement to local universities to increase visibility and encourage quality applicants. She also reviewed applications as they were submitted.

Additional Accomplishments

All SMARC staff and Mr. Daw finished moving office furniture to make way for genetics lab furniture. Two fume hoods were set up in the genetics lab for upcoming genetics projects.

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

Dr. Bockrath, Mr. Daw, Ms. Moore, and Ms. Whitt contributed to the monthly report.

Dr. Bockrath, Mr. Daw, and Ms. Moore continued to revise end-of-year reports, proposals, and project plan.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss updates, research progress and plans, collection plans, standard operating procedure development, and species collection datasheet modifications.

Dr. Bockrath, Mr. Daw, and Dr. Scott Walker interviewed potential Student Conservation Association (SCA) interns for husbandry at the UNFH. The selected intern is scheduled to start on April 4 for a 16-week internship.

Dr. Bockrath, Mr. Daw, Mr. Funk, and Mr. West conducted interviews for applicants to the SCA intern positions at the SMARC.

EAPR Staff virtually attended the Texas Conservation Symposium

Table 1. January's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility housed. NT indicates that species were not targeted for collection this month. NA indicates that inventory was not conducted this month.

Species	SMARC Jan kept	UNFH Jan kept	Released	Total collected	SMARC Jan incorporated	UNFH Jan incorporated	SMARC Jan mortalities	UNFH Jan mortalities	SMARC Jan census	UNFH Jan census
Fountain darter: San Marcos	NT	NT	--	--	0	0	38	5	377	478
Fountain darter: Comal	NT	NT	--	--	0	0	0	0	125	35
Comal Springs riffle beetle	NT	152	67	219	0	6	NA	NA	NA	38
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	0	0	0
Peck's cave amphipod	NT	NT	--	--	0	55	11	10	110	198
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	0	0	3	2	189	68
San Marcos salamander	NT	NT	--	--	0	0	3	2	158	197
Comal Springs salamander	NT	NT	--	--	0	0	0	1	114	64
Texas wild rice plants	NT	NT	--	--	0	13	0	0	191	182

Table 2. Survival results from the two trials of the Comal Springs riffle beetle exposure to* Staphylococcus *research project (2021). "Total" is the number of larvae included in that treatment of that trial. "Unknown" is the number of larvae that were lost or escaped and cannot be included in analyses. The percent dead and alive are the total, minus the number of unknown larvae. Asterisks indicate individuals that pupated and eclosed. The number of larvae alive in each treatment at 1-, 2-, and 3-months post-transfer is reported.

	Negative control 1	Positive control 1	Staph exposed 1	Negative control 2	Positive control 2	Staph exposed 2
Total	14	15	15	15	15	15
Unknown	2	6	6	0	0	0
Dead	6 (50%)	3 (33%)	4 (44%)	12 (80%)	3 (20%)	8 (53%)
Alive	6 (50%)	6 (67%)	5 (56%)	3 (20%)	12 (80%)	7 (47%)
Transferred	6	4	2	3	9	6
Alive 1-month	3	2	2	2 + 1*	5	1*
Alive 2-month	1	2	1	1*	2*	0
Alive 3-month	0	0	0	0	2*	0

Summary of January Activities

January 8-21, 2022 – Traps for Texas blind salamanders were set at Rattlesnake Cave and well on January 8 in San Marcos, TX. The traps were checked on January 13, 18 and removed on 21.

January 13-14, 2022 – EARP staff virtually attended relevant presentations at the Texas Conservation Symposium.

January 13, 2022 – Collected Comal Springs riffle beetles from Spring Run 1, 2, and 3 at Landa Park and Spring Island, New Braunfels, TX.

January 14, 2022 – Sarah Valdez and Sarah Mock came to the UNFH for a tour and training.

January 18, 2022 – Sarah Valdez and Sarah Mock came to the SMARC for a tour and training.

January 20, 2022 – Dr. Bockrath and Mr. Daw met with Damon Childs, Dr. Chad Furl, and Kristy Kallus to discuss 2021 end of year budget expenditures.

January 20, 2022 – Dr. Bockrath and Mrs. Moore met with Mr. Prewitt (BIO-WEST) to set timelines for the 2022 CSRB pupation Phase II and Phase III efforts

January 31, 2022 – Two symptomatic salamanders were shipped to the Southwestern Fish Health Unit in Dexter, NM for disease analysis.

Pictures

Figure 1. Tommy Funk exiting Rattlesnake Cave, San Marcos, Texas. Photo credit: Desiree Moore, USFWS

Figure 3. Braden West and Will Coleman collecting Comal Springs riffle beetles at Spring Run 3, New Braunfels, Texas. Photo credit: JL Whitt, USFWS

Figure 3. Will Coleman, Randy Gibson, Amelia Hunter, and Braden West at Spring Island, New Braunfels, Texas. Photo credit: JL Whitt, USFWS

Figure 4. Sarah Mock, Ben Thomas, Sarah Valdez, Jennifer Whitt, and Adam Daw during the tour at the Uvalde National Fish Hatchery. Photo credit: Sarah Valdez, EAA

Figure 5. Adam Daw, Sarah Valdez, Desiree Moore, Sarah Mock, Tommy Funk, Braden West, and Dr. Katie Bockrath during the tour at the San Marcos Aquatic Resources Center. Photo credit: Sarah Valdez, EAA

February 2022 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desiree Moore and Jennifer Whitt

Dr. Katie Bockrath and Adam Daw

With contributions from Tommy Funk, Ben Thomas, and Braden West

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

On February 1, 2022, Adam Daw, Tommy Funk, Braden West, and Jennifer Whitt collected and retained 11 Comal Springs dryopid beetles from woody debris near spring upwellings around Spring Island in New Braunfels, TX (Figure 1). The Comal Springs dryopid beetles were retained for the refugia at the Uvalde National Fish Hatchery (UNFH).

On February 9-22, 2022, Funk and West deployed baited minnow traps at Johnson's Well and Primer's Fissure in San Marcos, Texas to collect Texas blind salamanders. Funk and West checked the traps two to three times per week, capturing six Texas blind salamanders at Johnson's Well and five at Primer's Fissure. Four of the six salamanders at Johnson's Well and two of the five at Primer's Fissure were retained for the refugia at the San Marcos Aquatic Resource Center (SMARC). One of the salamanders at Primer's Fissure was clipped and released on February 11, recaptured and released on the February 18, re-caught and retained on the February 22. The traps were removed on the final salamander check on February 22, 2022.

On February 28, 2022, Funk, West, and Desiree Moore collected 119 Texas wild rice tillers from 10 stands in Section B of the San Marcos River in San Marcos, TX (Figure 2). Tillers were collected from areas recently identified by the Edward Aquifer Refugia Program (EARP) staff to contain rice plants with alleles that are unrepresented or uncommon in the refugia population based on the most recent genetic assessment. The plants were retained for the refugia population at the SMARC. A second set of tillers with unique alleles was collected and retained for the refugia at the UNFH.

Husbandry

Uvalde

Daw and Whitt calked and painted the wall of the invertebrate room to protect it from rusting (Figure 3).

Whitt transferred 25 San Marcos fountain darters to the modified refugia tank RE15.

Ben Thomas continued overhaul efforts in February. Thomas used a razor to remove the silicone holding the old tank dividers from RE14 and then modified the dividers to achieve a closer fit.

Whitt started the annual repotting of the Texas wild rice.

Whitt finished the elastomer tagging identification for the Texas blind salamanders that were transferred from the SMARC to the UNFH last year.

SMARC

Funk and West worked with support technicians from Aqua Logic, Inc. to diagnose and devise a fix to the continued malfunction issues of the heater/chiller units servicing Texas wild rice tanks at the SMARC. West repurposed sensor cables to bypass a broken sensor in the unit. After the fix was in place, the unit functioned properly with no errors.

West reconstructed the Texas wild rice quarantine system at the SMARC. West replaced the broken pump and added a heater to the system.

Funk acquired up-to-date tank location information of all visible-implant-elastomer tagged Texas blind salamanders at the SMARC. He then compiled a spreadsheet of known life history data of the captive refugia population.

Daw, West, and Dr. Katie Bockrath worked with representatives from OTT HydroMet to restore the remote water quality sensing capabilities at the SMARC.

Daw, Funk, and West assisted the SMARC Facility Operations Specialist, Juan Martinez, in the removal of the large tanks no longer being used from the Edwards Aquifer Authority (EAA) quarantine building.

Animal Health

Thomas and Whitt swabbed the Comal Springs salamanders in quarantine and two salamanders that were removed from the San Marcos salamander habitat project at the UNFH. West and Moore swabbed the Texas blind salamanders in quarantine at the SMARC (Figure 4). The samples will be sent to the San Diego Zoo in San Diego, CA to test for Bd/Bsal. The preliminary analysis of the histology from the two Texas blind salamanders sent to USFWS Southwestern Fish Health Unit was that they had steatitis/pancreatitis and were positive for Bd. The specific cause of the steatitis is unknown. Potential causes of steatitis/pancreatitis are being evaluated, including diet and husbandry practices. Since the initial outbreak in tank ER6, after which all individuals in the tank were moved to the quarantine room, no new individuals have shown symptoms in the SMARC refugia.

Task 2 Research

Comal Springs Riffle Beetle Pupation

Dr. Bockrath, Moore, and Israel Prewitt (BIO-WEST) confirmed flow was within the adequate range for all tubes weekly.

Dr. Bockrath met with a Nikon representative to download the software needed for measuring larvae for Phases I and II.

West prepared a heater/chiller unit for the new flow-through system that will be used for Phase II. West monitored the system daily to confirm the temperature stayed in an acceptable range for Comal Springs riffle beetle. Moore prepared the flow bar for the Phase II tubes.

Comal Springs Riffle Beetle Exposure to Staphylococcus

No work was performed related to this project this month.

Captive Propagation for San Marcos Salamanders

The third replicate trial of this experiment is ongoing. Thomas and Whitt continued conducting daily checks for egg presence. Two mortalities (one male, one female) were replaced by salamanders of similar size. No oviposition has occurred thus far.

Egg oviposition dates, egg hatching and survival rates, and juvenile salamander survival were recorded for reporting in the propagation manual.

Comal Springs Riffle Beetle Population Genetics

Dr. Bockrath and Moore continued purchasing and gathering the equipment and supplies for this project.

Batrachochytrium dendrobatidis *(Bd) Infection Treatment in Aquatic Salamanders*

Dr. Bockrath and Moore continued purchasing and gathering the equipment and supplies for this project. They also determined a preliminary design and strategic placement of the research system to hold this experiment.

Dr. Bockrath obtained quotes for Thermocyclers and started the purchasing process.

*Comal Springs Riffle Beetle (*Heterelmis comalensis*) Refugia Manual*

Moore continued compiling reports, manuscripts, and records pertaining to Comal Springs riffle beetles.

P-Chip Tag Effects on Eurycea *spp.*

Dr. Bockrath and Moore continued purchasing and gathering the equipment and supplies for this project. They also designed the research systems to hold this experiment.

*Catalog of Historical Fountain Darter (*Etheostoma fonticola*) Tissue*

Dr. Bockrath and Moore reviewed Student Conservation Association (SCA) intern applications and interviewed candidates for this project.

Additional Accomplishments

West cleaned and moved a freezer (-20 degree) to the genetics lab room.

Ruben Tovar continued monitoring and rearing salamander eggs for his research on the evolution of eye development.

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Dr. Bockrath met with EAA staff to discuss amendments to the 2022 work plan.

Dr. Bockrath and Daw finalized and submitted the end-of-year reports, proposals, and project plan.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss updates, research progress and plans, collection plans, standard operating procedure development, and species collection datasheet modifications.

EARP staff virtually attended the USFWS Region 2 Fish and Aquatic Science Symposium. Dr. Bockrath, Daw, and Moore gave presentations about EARP husbandry and research.

Dr. Bockrath, Daw, and West met with Hydromet representatives to discuss billing and functionality of the monitoring system at the SMARC.

Justin Crow (SMARC), West, and Funk hosted Victoria Broderick (Headwaters at the Comal) for a tour of the SMARC.

Crow, Moore, West, and Funk hosted Victor Castillo (Edwards Aquifer Research and Data Center (EARDC), Texas State University) and a group of students working with the EARDC for a tour of the SMARC (Figure 5).

Dr. Bockrath discussed Comal Springs dryopid beetle and Comal Springs riffle beetle research with BIO-WEST. They worked together to get the research and budget proposals submitted through grants.gov.

Dr. Bockrath discussed Comal Springs riffle beetle research with Dr. Shannon Brewer at the Alabama Cooperative Fish and Wildlife Research Unit at Auburn University.

Table 1. February's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility housed. NT indicates that species were not targeted for collection this month. NA indicates that inventory was not conducted this month.

Species	SMARC Feb kept	UNFH Feb kept	Released	Total collected	SMARC Feb incorporated	UNFH Feb incorporated	SMARC Feb mortalities	UNFH Feb mortalities	SMARC Feb census	UNFH Feb census
Fountain darter: San Marcos	NT	NT	--	--	0	0	13	5	364	473
Fountain darter: Comal	NT	NT	--	--	0	0	0	4	125	31
Comal Springs riffle beetle	NT	NT	--	--	0	52	NA	NA	NA	90
Comal Springs dryopid beetle	NT	11	0	11	0	0	0	NA	0	0
Peck's cave amphipod	NT	NT	--	--	0	0	12	3	98	195
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	6	NT	6	11	0	0	3	0	186	68
San Marcos salamander	NT	NT	--	--	0	0	3	2	155	195
Comal Springs salamander	NT	NT	--	--	0	0	0	0	114	64
Texas wild rice plants	10	1	0	11	0	0	2	0	189	182

Summary of February Activities

February 1, 2022 – Collected Comal Springs dryopid beetles from Spring Island in New Braunfels, TX.

February 9-22, 2022 – Traps for Texas blind salamanders were set at Primer's Fissure and Johnson's Well in San Marcos, TX. The traps were checked on February 11, 14, 16, 18 and removed on 22.

February 23-24, 2022 – EARP staff virtually attended the USFWS Region 2 Fish and Aquatic Science Symposium where Dr. Bockrath, Daw, and Moore gave presentations about EARP husbandry and research.

February 23, 2022 – Victoria Broderick from Headwaters at the Comal toured the SMARC.

February 25, 2022 – Victor Castillo and students from the EARDC toured the SMARC.

February 28, 2022 – Collected Texas wild rice from Section B of the San Marcos River in San Marcos, TX.

Pictures

Figure 1. Adam Daw retrieving wood to collect Comal Springs dryopid beetles at Spring Island in New Braunfels, Texas. Photo credit: USFWS

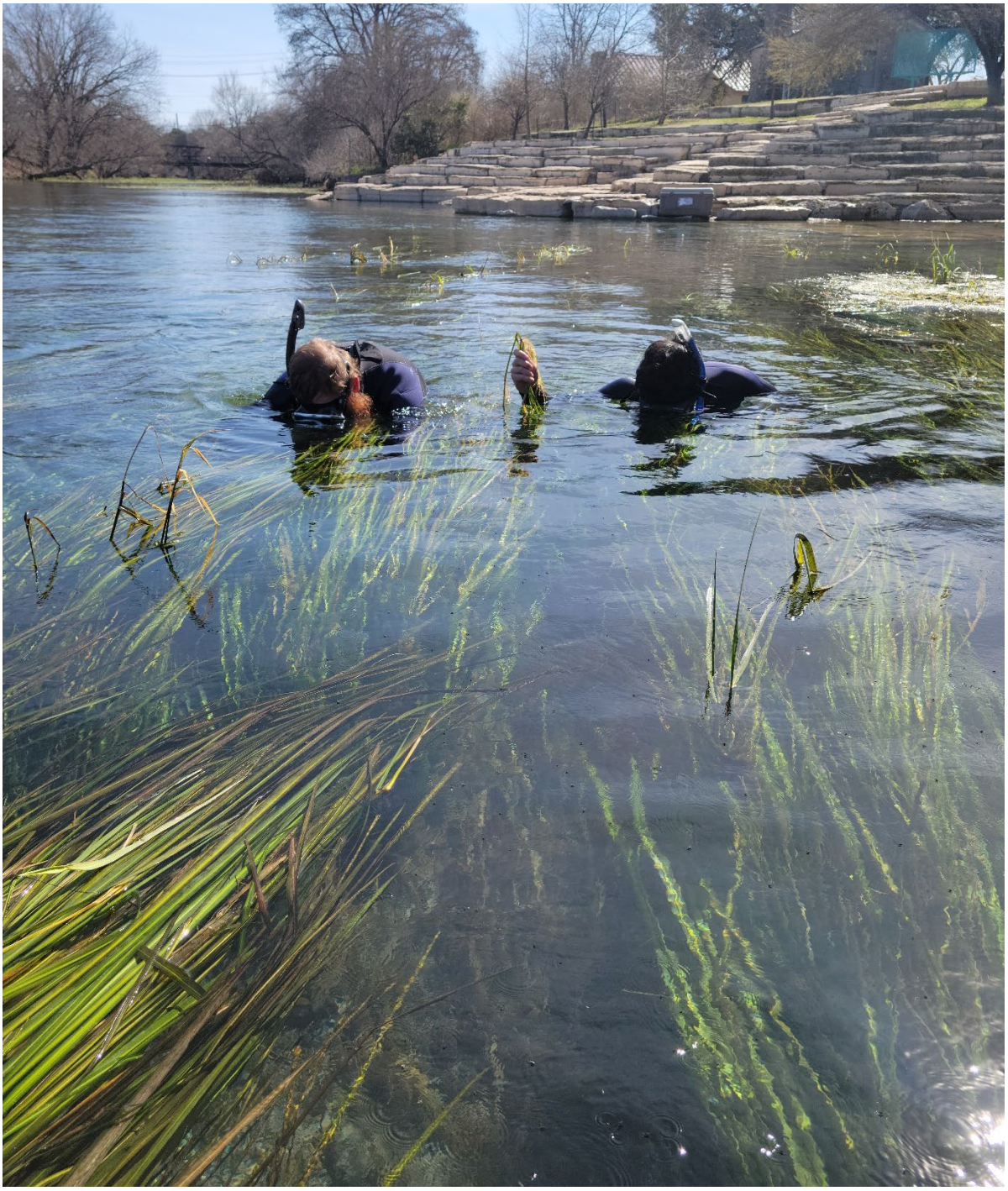


Figure 2. Braden West and Tommy Funk collecting Texas wild rice tillers at Sewell Park in San Marcos, Texas. Photo credit: USFWS

Figure 3. Adam Daw caulking the wall in the invertebrate room at the UNFH in Uvalde, Texas. Photo credit: USFWS

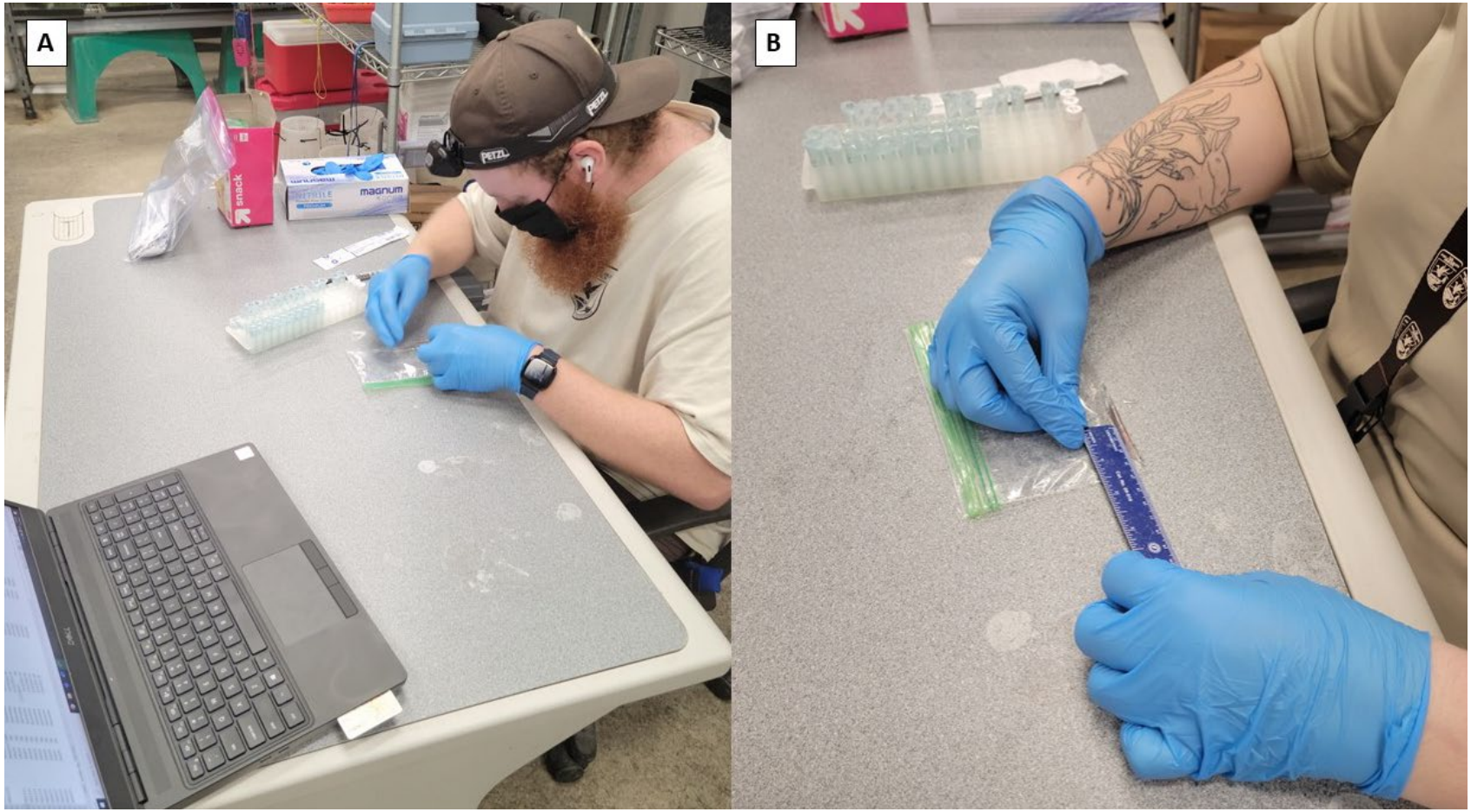


Figure 4. Braden West (A) and Desiree Moore (B) swabbing Texas blind salamanders for Bd/Bsal testing. Photo credit: USFWS

Figure 5. Students working with the EARDC observing Ruben Tovar (University of Texas) handle salamander eggs in the lab during a tour at the SMARC. Photo credit: USFWS

March 2022 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desiree Moore and Jennifer Whitt

Dr. Katie Bockrath and Adam Daw

With contributions from Tommy Funk, Ben Thomas, and Braden West

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

On March 22-23, Tommy Funk used dip nets to collect 12 San Marcos and 13 Comal Springs fountain darters from Spring Lake in San Marcos, TX and Landa Park in New Braunfels, TX, respectively. The 25 fish were sent to the Southwestern Fish Health Unit for parasite enumeration.

On March 28, 2022, Braden West and Jennifer Whitt collected and retained two Comal Springs dryopid beetles from woody debris near spring upwellings around Spring Island in New Braunfels, TX. The Comal Springs dryopid beetles were retained for the refugia at the Uvalde National Fish Hatchery (UNFH).

On March 31, 2022, Funk, West, and Whitt collected 163 Peck's cave amphipods from Spring Island in New Braunfels, TX (Figure 1). The team retained 159 of the Peck's cave amphipods for the refugia at the UNFH.

Husbandry

Uvalde

Ben Thomas and Whitt transferred additional San Marcos fountain darters to the test refugia system, which includes CO_2 dosing, filters, and UV sterilizer. To date, all the fish appear healthy. Preparations were made to start modifying other tanks in the Refugia.

The third and final trial of the San Marcos salamander habitat modification project concluded this month. Whitt transferred the 88 salamanders used in the final trial to two larger tanks in the refugia. To observe trends in propagation across varying sex ratios, Whitt organized the salamanders into tanks at different configurations. Thomas added new vegetation structures to both tanks to provide benthic cover and vertical foliage for the salamanders, and Daw adjusted the timing on the lights from a 12-hour to a 10-hour photoperiod. Salamander eggs were observed in one of the San Marcos salamander tanks within two weeks of the changes. Egg oviposition dates, egg hatching and survival rates, and juvenile salamander survival were recorded for reporting in the propagation manual.

Thomas removed the faulty lighting system above the amphipod cultivation tanks and replaced them with an LED system.

Whitt continued construction of the third rack system in the invertebrate room.

SMARC

Daw continued the construction of a new invertebrate rack and quarantine rack at the San Marcos Aquatic Resources Center (SMARC; Figure 2).

Daw, Funk, Thomas, and West moved a large Texas wild rice raceway tank in the greenhouse at the SMARC to make room for new sand and UV filters that will be added to the recirculating system.

Funk, Desiree Moore, and West worked together to move food storage from the quarantine building to the refugia building to improve biosecurity measures at the SMARC.

Salamander egg oviposition dates, egg hatching and survival rates, and juvenile salamander survival were recorded for reporting in the propagation manual.

Animal Health

The final health report was receeved for three Texas blind salamanders from the SMARC that were sent to the Southwestern Native Aquatic Resources and Recovery Center (SNARRC). Two of the animals had signs of chytrid disease caused by *Batrachychytrium dendrobatidis* (Bd). All three individuals had severe chronic steatitis, which appeared to not be pathogen-associated and likely due to a dietary cause. Dr. Trista Welsh-Becker from SNARRC proscribed a 10-day Itraconazole treatment. At the SMARC, Funk and West began the treatment on a subset of the Texas blind salamanders that displayed similar Bd symptoms. These five individuals were swabbed for Bd before treatment and will be swabbed again on the tenth day of treatment. Swabs will be tested at the SMARC for the presence of Bd pre and post-treatment.

On March 23, 12 San Marcos and 13 Comal Springs fountain darters were shipped to the Southwestern Fish Health Unit at the SNARRC for parasitology examination.

Additional Accomplishments

Daw met with David Pritchard from Texian Geospatial & Asset Solutions, LLC. for a demonstration of an EOA Arrow Gold® TRK GNSS receiver. Daw recorded information about the new global network satellite system (GNSS) to inform discussions on improving the accuracy of GPS locations of Edwards Aquifer Refugia Program (EARP) collection sites (Figure 3).

Dr. Bockrath, Daw, Funk, Moore, and West cleaned out and reorganized the EARP shed at the SMARC. They were able to store equipment more efficiently and create more space for activities.

Task 2 Research

Comal Springs Riffle Beetle Pupation

Dr. Bockrath, Moore, and Israel Prewitt (BIO-WEST) confirmed flow was within the adequate range for all tubes weekly.

Prewitt enumerated the F1 Comal Springs riffle beetle larvae in two of the three breeding tubes and used 90 late-instar larvae to launch the first replicate of Phase II. This replicate consisted of three tubes with varying densities of larvae (20, 30, and 40).

Comal Springs Riffle Beetle Exposure to Staphylococcus

Dr. Camila Carlos-Shanley (Texas State University) purchased and prepared the supplies for extracting DNA from the remaining larvae from the first trial of this experiment.

Captive Propagation for San Marcos Salamanders

Thomas and Whitt continued conducting daily checks for egg presence until the conclusion of this experiment. Thomas and Whitt ended the third replicate trial of this experiment on March 9, No oviposition occurred in this experiment.

Moore continued compiling reports, manuscripts, and records pertaining to the San Marcos salamander.

Comal Springs Riffle Beetle Population Genetics

Dr. Bockrath and Moore continued purchasing and gathering the equipment and supplies for this project. Dr. Bockrath met with Dr. Chris Nice (Texas State University) to discuss historically collected genetic data and DNA analysis methods used for Comal Springs riffle beetle.

Batrachochytrium dendrobatidis *(Bd) Infection Treatment in Aquatic Salamanders*

Dr. Bockrath and Moore continued purchasing and gathering the equipment and supplies for this project. The treatment medication and detection reagents arrived at the SMARC.

Dr. Bockrath, Daw, and Thomas disassembled a prefabricated multi-tank system at the UNFH and transported it to the SMARC to use for this experiment. Dr. Bockrath, Funk, Moore, and West moved furniture and equipment from the quarantine building to create space for the system. Dr. Bockrath cleaned and disinfected tanks for this project.

Dr. Bockrath began internally validating the standard qPCR protocol used to test salamanders for Bd at the SMARC (Figure 4).

*Comal Springs Riffle Beetle (***Heterelmis comalensis***) Refugia Manual*

Moore continued compiling reports, manuscripts, and records relating to the Comal Springs riffle beetle.

P-Chip Tag Effects on **Eurycea** *spp.*

Dr. Bockrath and Moore continued purchasing and gathering the equipment and supplies for this project. They also designed the research systems to hold this experiment.

Moore tagged five Comal Springs salamanders for the pilot study verifying the salamanders can accommodate p-Chips. No mortality or tag loss occurred thus far.

Moore set up two tanks to house Texas blind salamanders in this study. Moore added dividers to the tanks to easily track which salamanders belong to each treatment while allowing control of water quality parameters across treatments. Moore began the process of tagging Texas blind salamanders (Figure 5).

*Catalog of Historical Fountain Darter (***Etheostoma fonticola***) Tissue and DNA Viability Testing*

Dr. Bockrath and Moore selected a Student Conservation Association (SCA) intern for this project. The candidate has accepted the position. Dr. Bockrath has begun preparing the workspace for the SCA intern in the Scope/Dissection lab located in the Admin Office.

Additional Accomplishments

Ruben Tovar (University of Texas) brought 3D printed Texas blind and San Marcos salamander heads to the EARP from the Dr. David Hillis (University of Texas) and Dr. Dana Garcia (Texas State University) labs (Figure 6). The 3D printing plans were taken from the diceCT scans of the heads donated from the SMARC to Tovar for his ongoing research on eye development in aquatic salamanders.

The SMARC Facility Operations Specialist, Juan Martinez, began construction on the EARP building to separate the genetics lab area from the office area. Funk and West assisted with the construction as needed (Figure 7).

Dr. Bockrath, Daw, and Moore discussed potential Comal Springs riffle beetle research with Ruben Tovar (University of Texas) involving diceCT scans of adults and larvae.

Dr. Bockrath discussed Peck's cave amphipod genetics research and future collaboration with Dr. Chris Nice (Texas State University).

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss updates, research progress and plans, collection plans, and standard operating procedure development.

Dr. Bockrath, Daw, Funk, Moore, and West met with Nick Panyard from OTT HydroMet to learn how to run diagnostics and reprogram the settings of the data sondes and the SMARC and UNFH.

Dr. Bockrath, Dr. David Britton, Daw, and Moore met with Kristy Kollaus and Scott Storment of the EAA for a quarterly progress meeting at the SMARC.

Dr. Bockrath, Dr. Britton, Daw, and Dr. Scott Walker (UNFH) met to discuss future staffing strategies for the EARP to increase continuity.

EARP staff met to discuss exclusion designs for the Peck's cave amphipod husbandry project evaluating brooding chamber designs.

Table 1. March's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility housed. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC Mar kept	UNFH Mar kept	Released	Total collected	SMARC Mar incorporated	UNFH Mar incorporated	SMARC Mar mortalities	UNFH Mar mortalities	SMARC Mar census	UNFH Mar census
Fountain darter: San Marcos	NT	NT	--	--	0	0	17	14	347	459
Fountain darter: Comal	NT	NT	--	--	0	0	8	2	117	29
Comal Springs riffle beetle	NT	NT	--	--	0	0	10	11	13	79
Comal Springs dryopid beetle	NT	2	0	2	0	10	NA	0	0	10
Peck's cave amphipod	NT	159	4	163	0	0	4	5	94	190
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	0	0	2	0	184	68
San Marcos salamander	NT	NT	--	--	0	0	6	3	149	192
Comal Springs salamander	NT	NT	--	--	0	0	0	1	114	63
Texas wild rice plants	NT	NT	--	--	10	5	0	1	199	186

Summary of March Activities

March 3, 2022 – EARP staff met with Nick Panyard from OTT HydroMet at the SMARC.

March 9, 2022 – The third replicate trial of the captive propagation for San Marcos salamanders research project concluded.

March 22, 2022 – Dr. Bockrath and Moore met with Dr. Shannon Brewer to discuss a potential tagging study for Comal Springs riffle beetles.

March 22, 2022 – Dr. Bockrath met with Dr. Nice to discuss Comal Springs riffle beetles genetics data and future collaborations.

March 22-23, 2022 – Collected San Marcos and Comal Springs fountain darters from Spring Lake in San Marcos, TX and Landa Park in New Braunfels, TX.

March 23, 2022 – Dr. Bockrath and Dr. Britton meet with Daw and Dr. Walker at UNFH.

March 25, 2022 – Dr. Bockrath and Moore met with EAA staff at the SMARC for the quarterly progress meeting.

March 28, 2022 – Collected Comal Springs dryopid beetles from Spring Island in New Braunfels, TX.

March 29, 2022 – EARP staff met to discuss Peck's cave amphipod brooding chamber designs.

March 31, 2022 – Collected Peck's cave amphipods from Spring Island in New Braunfels, TX.

Figures

Figure 1. Braden West collecting Peck's cave amphipods with a dip net at Spring Island in New Braunfels, Texas. Photo credit: USFWS

Figure 2. Construction of the new invertebrate rack system (left) in the refugia at the SMARC. Photo credit: USFWS

Figure 3. David Pritchard with Texas Geospatial in New Braunfels, TX demonstrating the EOA Arrow Gold® TRK GNSS receiver. Photo credit: USFWS

Figure 4. Dr. Katie Bockrath working in the genetics lab preparing for Bd testing at the SMARC. Photo credit: USFWS

Figure 5. A Texas blind salamander with a p-Chip tag. The p-Chip is circled in black. Photo credit: USFWS

Figure 6. A) Brittany Dobbins (Texas State University), Dr. Katie Bockrath, Braden West, Tommy Funk, Desiree Moore, and Ruben Tovar (University of Texas) at the SMARC. B) The 3D-printed San Marcos salamander and Texas blind salamander heads. Photo credit: USFWS

Figure 7. Modifications to the EARP building begin. Juan Martinez (SMARC) instructing Braden West and Tommy Funk on how to properly create a doorway in a wall. Photo credit: USFWS

April 2022 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desiree Moore and Jennifer Whitt

Dr. Katie Bockrath and Adam Daw

With contributions from Tommy Funk, Ben Thomas, and Braden West

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staff

The husbandry Student Conservation Association (SCA) intern position at the Uvalde National Fish Hatchery (UNFH) was filled April 4 by Mallory Theurer (Figure 1). Theurer received a BS in biological sciences, BA in chemistry, and certificate in environmental science at Florida Atlantic University. Theurer worked as a museum assistant in the division of vertebrate paleontology at the Yale Peabody Museum of Natural History and recently returned from a year of teaching English in South Korea at a private academy.

The husbandry SCA intern position at the San Marcos Aquatic Resources Center (SMARC) was filled April 4 by Eleanor Krellenstein (Figure 2). Krellenstein received a BS in biology at Texas State University. Krellenstein is looking forward to a career in the field of biological sciences.

The research SCA intern position at the SMARC was filled April 11 by Shawn Moore (Figure 2). Shawn Moore is currently earning a BS in wildlife biology at Texas State University. Shawn Moore has a passion for research grounded in conservation and aquatic biology is an area they want to pursue further.

Species Collection

On April 20, Tommy Funk, Krellenstein, Theurer, Ben Thomas, and Braden West collected tillers from nine stands of Texas wild rice in section F of the San Marcos River in San Marcos, Texas (Figure 3). The SMARC retained four of the collected plants for the refugia population, and five plants were retained for the Uvalde National Fish Hatchery (UNFH) refugia population.

Texas wild rice plants identified as genetic duplicates at one station but genetically absent at the other were exchanged between stations on April 20th. Ten plants were moved from the UNFH to the SMARC and five were moved from the SMARC to the UNFH.

On April 26-27, Krellenstein and West coordinated the collection of fountain darters from the BIO-WEST annual bio-monitoring event. The first day, 42 fountain darters were retrieved from the middle section of the San Marcos River in San Marcos, Texas. The second day, 83 darters were retrieved from the lower section of the San Marcos River in San Marcos, Texas. Sixty of the fountain darters were set aside to be shipped to the Southwester Fish Health Unit for routine health assessments.

Husbandry

Uvalde

Thomas and Whitt began training Theurer on all the husbandry (e.g., feeding, biosecurity, water quality) and safety protocols currently used in the refugia program at the UNFH. Thomas focused on teaching Theurer the protocols for the vertebrates, and Whitt focused on teaching Theurer the protocols for the invertebrates and Texas wild rice.

Virginia Lee Montgomery, a multimedia artist from Austin, TX, took video of the Texas blind salamanders in the refugia for an art exhibit in Austin, TX in 2023 (Figure 4).

Theurer and Thomas set up a new system for artemia cultures.

Theurer and Thomas disassembled and cleaned an out-of-production protein skimmer and biofiltering tank to use for cultivating daphnia cultures.

Whitt and Theurer continued the annual repotting of the Texas wild rice.

Theurer assisted Whitt in the inventory of the Comal Spring riffle beetle and the Peck's cave amphipod.

Theurer designed new vertical vegetation for the darter tanks (Figure 5).

Thomas assisted Adam Daw with repairing a break in a water line that supplies water to the quarantine building.

Whitt continued construction of the third rack system in the invertebrate room.

SMARC

Funk and West began training Krellenstein on all husbandry (e.g., feeding, biosecurity, water quality) and safety protocols currently used in the refugia program at the SMARC. Funk and West taught Krellenstein the protocols for the vertebrate and invertebrate species. Funk taught Krellenstein the protocols for the Texas wild rice, disease and algae treatments, and system reporting.

Funk and West installed a higher performing flow meter in the incoming water line that serves the quarantine building. The new probe improved the system monitoring process that aids in early detection of leaks.

West revamped the Texas wild rice quarantine system, replacing the chiller and pump with higher-power models and a standalone heater to improve the system's ability to cover a broader range of temperatures.

Daw finish preliminary construction of one of two new invertebrate racks. West assembled a new, automated culture system. West troubleshot and rebuilt a chiller unit for the new system.

West moved the Comal Springs riffle beetle refugia population and four cohorts of the Peck's cave amphipod refugia population to the new invertebrate system.

Daw started construction of one of four new quarantine racks.

Daw and Funk set up a daphnia culture system to supplement salamander nutrition.

Funk, Krellenstein, and West repotted Texas wild rice plants from rice tank 3. Funk and Krellenstein treated rice tanks 4 and 5 with Microbe-Lift AlgAway 5.4 algaecide.

Funk potted half of the Texas wild rice plants entering quarantine to see if the newly collected rice would retain more biomass during their quarantine period if potted in soil rather than the loose mesh enclosures currently used in the refugia protocols.

Krellenstein assisted Funk in the Texas wild rice and Texas blind salamander inventories at the SMARC.

Animal Health

On April 8, Funk concluded the 10-day treatment of five Texas blind salamanders with Itraconazole. West collected post-treatment skin swabs from each salamander to assess their Bd status after treatment. Swabs were stored at -80 degrees for future testing. There were no mortalities during the treatment. There was little observed improvement in symptoms during the treatment.

Funk moved the rest of the salamanders that were quarantined for steatitis and Bd symptoms back into the refugia after the health report from Dr. Trista Becker (Southwestern Native Aquatic Resources and Recovery Center) indicated the steatitis was not pathogen associated and visible Bd symptoms improved.

Task 2 Research

Comal Springs Riffle Beetle Pupation

Dr. Bockrath, Dr. Ely Kosnicki (BIO-WEST), and Desiree Moore confirmed flow was within the adequate range for all tubes every other week.

Dr. Kosnicki used 180 late-instar larvae to launch the second and third replicates of Phase II. Each of these replicates consisted of three tubes with varying densities of larvae (20, 30, and 40). All replicates are ongoing. Dr. Kosnicki prepared for Phase III of this study investigating the effects of biofilm on captive propagation.

Dr. Kosnicki inventoried two of the three breeding tubes used to generate F1 *Heterelmis comalensis* larvae used in this experiment (Table 1). Due to an initial misidentification in the field, *Microcylloepus* persists in both breeding tubes but do not appear to be interfering with *H. comalensis* propagation and survival.

Table 1. *Heterelmis comalensis* breeding tube inventory.

Life Stage	Breeding Tube 1	Breeding Tube 2
Adult	4	7
Large Larvae	126	122
Small-medium Larvae	31	47
Pupae	0	0
Dead Adults	3	1
Dead Larvae	0	10

Comal Springs Riffle Beetle Exposure to Staphylococcus

Dr. Camila Carlos-Shanley (Texas State University) extracted DNA from the remaining larvae from the first trial of this experiment. There were three larvae from the control group, three from the *Bacillus* group, and two from the *Staphylococcus* group. The samples were sent to the sequencing facility.

Captive Propagation for San Marcos Salamanders

Desiree Moore continued compiling reports, manuscripts, and records pertaining to the San Marcos salamander.

Comal Springs Riffle Beetle Population Genetics

Dr. Bockrath and Moore continued purchasing and gathering the equipment and supplies for this project.

Dr. Bockrath, Dr. Kosnicki, Desiree Moore, and Edmund Oborny (BIO-WEST) met to coordinate field collections between the SMARC and BIO-WEST. They produced a sampling and specimen collection plan that benefits studies by both groups.

Batrachochytrium dendrobatidis *(Bd) Infection Treatment in Aquatic Salamanders*

Dr. Bockrath and Desiree Moore continued purchasing and gathering the equipment and supplies for this project.

Desiree Moore cleaned and disinfected the prefabricated multi-tank system for this project. West plumbed the system to ensure a flow-through design that drains properly. Dr. Bockrath and Desiree Moore assembled the system to prepare for the first trial of this study (Figure 6).

Dr. Bockrath ran the Bd qPCR protocol to confirm the protocol works as expected. Dr. Bockrath ran the marker against a five-point standard curve, in replicate, to establish the assay's limit of detection and limit of quantification. Previously tested samples that tested positive for Bd were identified to further confirm the assay's performance.

*Comal Springs Riffle Beetle (***Heterelmis comalensis***) Refugia Manual*

Dr. Bockrath, Daw, Randy Gibson (SMARC), Dr. Kosnicki, Desiree Moore, and Whitt met to discuss the format of the manual, assignments for each contributor, and potential journals for publication. A general outline of the manual was produced to guide the group. A Teams channel was set up to assist collaboration and communal writing.

P-Chip Tag Effects on **Eurycea** *spp.*

Desiree Moore tagged 38 Texas blind salamanders (Figure 7) and prepared 40 salamanders in the control groups. Funk, Shawn Moore, and West assisted Desiree Moore in scanning tagged Texas blind salamanders weekly to monitor tag retention. No mortality or tag loss occurred thus far.

Desiree Moore and Shawn Moore set up a tank to house Comal Springs salamanders in this study. Desiree Moore added dividers to the tank to easily track which salamanders belong to each treatment while allowing control of water quality parameters across treatments.

*Catalog of Historical Fountain Darter (***Etheostoma fonticola***) Tissue and DNA Viability Testing*

Shawn Moore sorted all the historical specimens from the EARP, separating the fountain darters from other samples. Using a survey123 data form, Shawn Moore recorded all data associated with each specimen. Shawn Moore labeled and stored over 500 samples in vials with unique barcodes.

Additional Accomplishments

Dr. Bockrath, Desiree Moore, and West met with Victoria Broderick from the Headwaters of the Comal to discuss outreach activities for the children at their summer camp.

Dr. Bockrath and Daw met with Dr. Becker to discuss research investigating how largemouth bass virus (LMBV) clinically affects fountain darters, identify ways to non-lethally detect LMBV, and determine LMBV shedding rates.

Juan Martinez (SMARC) and West continued construction on the genetics lab by framing the new office entrance and installing new drywall around the frame (Figure 8).

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss updates, research progress and plans, collection plans, and standard operating procedure development.

Desiree Moore presented information about tagging salamanders with p-Chips to the USFWS Fish Technology Center First Quarterly Meeting.

April 12 and 28, Theurer and Thomas attended career days at the Uvalde Dual Language Academy and Florez Elementary (respectively) in Uvalde, Texas to showcase what the biology staff does to help the threatened and endangered species that are part of the Edwards Aquifer Habitat Conservation Plan (EAHCP) (Figure 9).

April 20, Whitt attended career day at Batesville Elementary in Batesville, Texas to showcase what the biology staff does to help the threatened and endangered species that are part of the EAHCP.

Table 2. April's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility housed. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC Apr kept	UNFH Apr kept	Released	Total collected	Transferred from SMARC to UNFH	Transferred from UNFH to SMARC	SMARC Apr incorporated	UNFH Apr incorporated	SMARC Apr mortalities	UNFH Apr mortalities	SMARC Apr census	UNFH Apr census
Fountain darter: San Marcos	125	NT	0	125	0	0	0	0	8	10	339	449
Fountain darter: Comal	NT	NT	--	--	0	0	0	0	2	0	115	29
Comal Springs riffle beetle	NT	NT	--	--	0	0	0	0	NA	3	13	76
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	2	NA	0	0	12
Peck's cave amphipod	NT	NT	--	--	0	0	0	0	21	9	73	181
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	0	0	0	0	0	0	114	68
San Marcos salamander	NT	NT	--	--	0	0	0	0	4	8	145	186
Comal Springs salamander	NT	NT	--	--	0	0	0	0	0	0	114	63
Texas wild rice plants	4	5	0	9	5	10	0	0	15	1	184	175

Summary of April Activities

April 4, 2022 – Eleanor Krellenstein and Mallory Theurer began their SCA internships at the SMARC and UNFH, respectively.

April 5, 2022 – EARP staff met with Victoria Broderick to discuss outreach opportunities.

April 7, 2022 – Dr. Bockrath and Desiree Moore met with Dr. Kosnicki and Osborny to coordinate Comal Springs riffle beetle samples for genetic analysis.

April 11, 2022 – Shawn Moore began their SCA internship at the SMARC.

April 12, 2022 – Outreach at career day at the Uvalde Dual Language Academy in Uvalde, Texas.

April 14, 2022 – USFWS Fish Technology Center First Quarterly Meeting.

April 20, 2022 – Texas wild rice collection in section F of the San Marcos River in San Marcos, Texas.

April 20, 2022 – Outreach at career day at Batesville Elementary in Batesville, Texas.

April 26-27, 2022 –BIO-WEST annual fountain darter bio-monitoring event in the San Marcos River in San Marcos, Texas.

April 28, 2022 – Outreach at career day at Florez Elementary in Uvalde, Texas.

Figures

Figure 1. Mallory Theurer cleaning the floor in the refugia room at the UNFH. Photo credit: USFWS

Figure 2. Shawn Moore (left) and Eleanor Krellenstein (right) cleaning a Texas wild rice tank at the SMARC. Photo credit: USFWS

Figure 3. Mallory Theurer looking for Texas wild rice tillers in section F of the San Marcos River, San Marcos, TX. Photo credit: USFWS

Figure 4. Virginia Lee Montgomery videoing Texas blind salamanders to the UNFH for an art exhibit. Photo credit: USFWS

Figure 5. Mallory Theurer constructing vertical vegetation for the darter tanks. Photo credit: USFWS

Figure 6. Braden West (left) and Desiree Moore (right) with the newly cleaned and assembled prefabricated multi-tank system at the SMARC. Photo credit: USFWS

Figure 7. Desiree Moore tagging a salamander with a p-Chip. Photo credit: USFWS

Figure 8. Braden West constructing the frame for the new office entrance. Photo credit: USFWS

Figure 9. Ben Thomas presenting at career day at Florez Elementary in Uvalde, TX. Photo credit: USFWS

May 2022 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dr. Katie Bockrath and Adam Daw

With contributions from

Tommy Funk, Desiree Moore, Ben Thomas, Braden West, and Jennifer Whitt

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staff

The Edwards Aquifer Refugia Programs bid congratulations and farewell to Tommy Funk. Funk accepted a job with the US Fish and Wildlife Service at Inks Dam National Fish Hatchery in Burnet, TX.

Species Collection

On May 3, 77 Fountain darters from the Comal River were captured by BIO-WEST as part of their bi-annual survey. EARP staff collected the fountain darters from the BIO-WEST survey for the refugia. The darters were transferred to the SMARC quarantine facility, and 60 individuals were shipped to the Southwestern Fish Health Unit (SFHU) in Dexter, NM for health analysis.

On May 6, Randy Gibson (SMARC) donated twelve Peck's cave amphipods to the refugia. Gibson collected the amphipods via drift net as part of the biannual biomonitoring.

On May 11, Braden West, Funk, Eleanor Krellenstein (Student Conservation Association, SCA), and Mallory Theurer (SCA) set traps for Texas blind salamander at Primer's Fissure and Johnson's Well (Figure 1). Staff checked traps on Monday, Wednesday, and Friday for two weeks. On May 25, staff retrieved the traps from the field. In total eight salamanders were captured and four were released. The remaining four individuals were transferred to the SMARC quarantine.

On May 18, Adam Daw, Theurer, Ben Thomas, and Jennifer Whitt collected Texas wild rice tillers in the San Marcos River in San Marcos, Texas. Ten tillers from one stand in section J and eight tillers from two stands in section K were collected. The Uvalde National Fish Hatchery (UNFH) retained the collected rice plants for the refugia population.

Husbandry

Uvalde

Whitt and Theurer continued the annual repotting of the Texas wild rice. Whitt, Thomas, and Theurer started the semi-annual inventory of the refugia population.

Whitt and Daw continued working on the PCA exclusion set up. The goal is to separate the adult PCA from the offspring after the brooding adult has released their eggs to reduce adult cannibalism of PCA offspring. Whitt assisted Daw in constructing small brooding boxes that allow offspring to separate from the adults and allow staff to visually monitor gravid adults and offspring. The brooding boxes were placed on a full well water system.

To reduce the heat load of the quarantine room during the summer, Thomas consolidated organisms from multiple racks onto a single rack to reduce the number of chillers running in the building.

SMARC

The quarantine period has concluded for Texas wild rice plants collected in April 2022. While the rice was in quarantine, Funk continued to take progress photos to visualize differences in the condition of plants potted in soil and those placed in mesh sleeves. Nine plants received from the Uvalde National Fish Hatchery and four wild collected plants were incorporated into the SMARC refugia population. EARP staff worked to develop plans to improve the condition of Texas wild rice plants held at the SMARC. Discussions included ways to increase flow, incorporate more flow-through systems, and repairing the greenhouse roof. Funk edited the Texas wild rice SOP to include alternative quarantine methods.

Funk designed updated feeding tags and affixed them to all tanks in the refugia and quarantine buildings to assist inexperienced caretakers in correctly feeding animals. Funk kept up with standard husbandry duties, which included siphoning, feeding, preserving mortalities, cleaning tanks and habitat, updating system, oviposition, and collection logs.

Funk and West compiled age, size, origin, and lot information for all salamander and fountain darters held in the refugia and quarantine buildings for the annual chytrid surveillance and fish health inspection to be conducted by Dr. Trista Becker on June 28, 2022. Funk transferred newly incorporated F1 San Marcos salamanders to a permanent tank quarantine.

Funk met with West to ensure optimal transfer and retention of knowledge and documentation following Funk's departure. Funk continued training Krellenstein in many aspects of husbandry at the San Marcos Aquatic Resources Center (SMARC), including inventory, Texas wild rice grooming, and Texas blind salamander minnow trap collections. In Funk's absence, SCA intern Krellenstein has continued to provide excellent care to all organisms in EARP refuge and quarantine populations. Funk worked with West to compile gear checklists for field events, which were then printed and laminated for quick pre-fieldwork reference.

West re-constructed holding systems for EARP invertebrates by installing new bulkheads and flow systems. The new holding systems will improve invertebrate survival while held in captivity. Daw starting construction of one of the four new quarantine racks at the SMARC. The new systems will increase quarantine holding capacity. Daw discussed with West, Dr. Bockrath,

and Moore on future changes to the layout of the SMARC refugia and quarantine rooms to improve useability and increase capacity. To address the high humidity in both the quarantine and refugia rooms a dehumidifier was tested to evaluate the size needed to reduce the humidity to inhibit mold growth and slow equipment degradation. West worked with facilities specialist Juan Martinez to finish construction of a new doorway in the EARP building. Drywall texture was applied to the bare drywall to match existing walls in the laboratory space.

Animal Health

On May 3, staff from the FWS Southwest Fish Health Unit conducted a site visit at the UNFH. While on site they sampled 60 (wild) San Marcos River Fountain darters and 15 Comal River Fountain darters from the refugia population for pathogen analysis. Staff also swabbed salamanders in the refugia population for Bd prevalence.

On May 9, 60 Comal River and 55 San Marcos River Fountain darters caught during the BIO-WEST biomonitoring survey and maintained at the SMARC were shipped to the Southwestern Fish Health Unit in Dexter, NM for health analysis as part of a biannual wild population health inspection.

Task 2 Research

Comal Springs Riffle Beetle Pupation

The density trial is ongoing, and the tubes are waiting to be surveyed in June and July. No additional progress has been made on this project.

Comal Springs Riffle Beetle Exposure to Staphylococcus

Dr. Camila Carlos-Shanley received the sequence data from the sequencing facility has taken a preliminary look at the data. Three larvae from each treatment (*Staphylococcus*, *Bacillus*, and *Uninoculated*) were sequenced. In 2021 exposure trials, the *Staphylococcus* group had the highest mortality while the *Bacillus* exposure group had that highest survivorship. Contrary to expectation, the *Staphylococcus* exposure group did not have a greater abundance of *Staphylococcus* than the other treatment groups, but the *Bacillus* exposure group had a higher abundance of *Chryseobacterium*. Additional analyses are required to make any sound assessments of the data.

Captive Propagation for San Marcos Salamanders

Staff continued to collect relevant literature, thesis reports, and internal reports to inform this report. Dr. Bockrath and D. Moore established an outline, relevant content, and writing deadlines for project completion.

Comal Springs Riffle Beetle Population Genetics

Dr. Bockrath attended the Comal Spring riffle beetle workgroup meeting where Dr. Ely Kosnicki outlined his plan for the population abundance assessment study. Sampling for this genetics study was briefly discussed. Consultation with Dr. Chris Nice and Will Colman (Texas State University) confirmed that a minimum of 4 individuals per lure is required to complete a genetic assessment of the Comal Springs riffle beetle at Landa Lake. Dr. Nice and Colman target 30-40 beetles per spring run, thus 4 beetles per lure would result in the same sample size currently used by Will Colman in his dissertation study.

*Comal Springs Riffle Beetle (***Heterelmis comalensis***) Refugia Manual*

Amelia Hunter was contacted to confirm their participation in the development of the Comal Springs riffle beetle Manual. A Teams channel and OneDrive folder were created to house literature and the draft report. All contributing authors were given access to the Teams channel and the OneDrive folder. Contributors have generated an outline, identified relevant content, established rolls and responsibilities, and developed a schedule for writing and review. Staff continued to collect relevant literature and reports.

Batrachochytrium dendrobatidis *(Bd) Infection Treatment in Aquatic Salamanders*

Dr. Bockrath, D. Moore, and S. Moore measured, sexed, and swabbed nine San Marcos salamanders before being placed in the AHAB unit (Figure 2). These nine F1 San Marcos salamanders will be used in the Bd treatment pilot study to determine itraconazole dosage for the larger Bd treatment study. Habitat was constructed and added to the AHAB tanks.

Materials and reagents for the study have been ordered. Dr. Bockrath is working with Dr. Trista Becker to acquire additional itraconazole for the larger study.

P-Chip Tag Effects on **Eurycea** *spp.*

D. Moore completed tagging the Comal Springs salamanders. All Texas blind and Comal Springs salamanders for this study are tagged and separated into their treatment and control groups (Table 1). Salamanders continue to be scanned for the presence of their p-chips. Thus far, there have been no loss of tags, no mortalities, and all tags were successfully scanned (Figure 3).

Table 1. The number of each species in each treatment in the p-Chip tagging study. Tagged salamanders were injected with a p-Chip subcutaneously at the base of the tail on their left side. Positive control salamanders were treated the same as tagged salamanders (i.e., anesthetized, measured, and punctured with a needle) without a tag being placed. Negative control salamanders were anesthetized and measured only.

Species	Tagged	Positive control	Negative control
Texas blind salamander	38	20	20
Comal Springs salamander	43	34	34

*Catalog of Historical Fountain Darter (***Etheostoma fonticola***) Tissue and DNA Viability Testing*

By the end of May, S. Moore cataloged and archived 1006 fountain darter samples. S. Moore generated a database for these samples that compiles information on the collection location, date of collection, total body length, sex (if it could be determined), Refugia (SMARC or UNFH), if it was wild stock vs Fx, and if used for research. The data was collected using a custom Survey123 form. S. Moore quality control checked the data by cross checking the information on the sample vials with information data collection sheets and refugia inventory trackers. S. Moore drafted a Standard Operating Procedure for archiving and cataloging tissue samples including quality control measures.

Additional Accomplishments

Funk completed a survey in Survey123 for quarantine system checks and a Standard Operating Procedure (SOP) for Visible Implant Elastomer (VIE) tag reading. Funk automated yearly Texas wild rice calendar repotting reminders for the four main EAA (Edwards Aquifer Authority) tanks in the greenhouse.

Dr. Katie Bockrath, Adam Daw, D. Moore, Funk, and Kristy Kollaus (EAA) gave a tour and interview to a news crew from the San Antonio Express on May 13. The subsequent article was published on May 17.

Dr. Bockrath, D. Moore, Funk, and West gave a tour of the EARP building and SMARC to the San Marcos Discovery Center Conservation Crew on May 18th.

D. Moore worked with Dr. Ely Kosnicki (BIO-WEST) to shift the Comal Springs dryopid beetle system over a few feet in the Quarantine building to allow for more efficient use of space and to accommodate new Quarantine racks. D. Moore fixed several leaks in the dryopid system and helped Ely clean and organize all the equipment associated with the system.

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

EARP staff meet weekly to discuss upcoming collections, refugia tasks, and research progress

Dr. Bockrath met with Ed Oborny (BIO-WEST) to discuss the tasks remaining to complete of the Comal Springs riffle beetle propagation project.

Daw and Dr. Bockrath attended the Edwards Aquifer Habitat Conservation Plan Joint Stakeholders meeting on May 19th.

Dr. Bockrath attended the Edwards Aquifer Habitat Conservation Plan Comal Springs work group meeting on May 25th.

Table 2. May's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility housed. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	Transferred from SMARC to UNFH	Transferred from UNFH to SMARC	SMARC incorporated	UNFH incorporated	SMARC mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	--	--	0	0	0	0	17	72*	322	377
Fountain darter: Comal	77	NT	0	77	0	0	0	0	0	15**	117	15
Comal Springs riffle beetle	NT	NT	--	--	0	0	0	0	3	3	10	76
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	2	NA	0	0	12
Peck's cave amphipod	12	NT	0	12	0	0	0	120	7	13	66	288
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0	0	0
Texas blind salamander	4	NT	4	8	0	0	0	0	1	1	178	67
San Marcos salamander	NT	NT	--	--	0	0	0	0	9	3	138	183
Comal Springs salamander	NT	NT	--	--	0	0	0	34	2	0	112	97
Texas wild rice plants	NT	3	0	3	0	0	13	0	1	1	196	184

*60 San Marcos fountain darters from UNFH were transferred to the Southwestern Fish Health Unit for testing in accordance with the annual hatchery inspection.

**15 Comal fountain darters from UNFH were transferred to the Southwestern Fish Health Unit for testing in accordance with the annual hatchery inspection.

Summary of May Activities

May 3 - Staff from the FWS Southwest Fish Health Unit conducted a site visit at the UNFH.

May 6 - Randy Gibson (SMARC) donated twelve Peck's cave amphipods to the refugia.

May 9 - Comal River and San Marcos River Fountain darters were shipped to the Southwestern Fish Health Unit in Dexter, NM for health analysis.

May 11th – Funk, Krellenstein, and Theurer set traps for Texas blind salamanders at Johnson's Well and Primer's Fissure.

May 13th - Dr. Katie Bockrath, Adam Daw, D. Moore, Funk, and Kristy Kollaus gave a tour and interview to a news crew from the San Antonio Express on May 13. The subsequent article was published on May 17.

May 18th - Dr. Bockrath, D. Moore, Funk, and West gave a tour of the EARP building and SMARC to the San Marcos Discovery Center Conservation Crew

May 18th – Daw, Thomas, Whitt, and Theurer collected Texas wild rice tillers

May 18th – Funk's last day as a SMARC employee

May 19th - Daw and Dr. Bockrath attended the Edwards Aquifer Habitat Conservation Plan Joint Stakeholders meeting

May 25th – Staff retrieved the traps set at Johnson's Well and Primer's Fissure

May 25th - Dr. Bockrath attended the Edwards Aquifer Habitat Conservation Plan Comal Springs work group meeting

May 31st – Dr. Bockrath, D. Moore, and S. Moore swabbed, sexed, and measured San Marcos salamanders for the Bd treatment trials

Figures

Figure 1 SCA Interns Eleanor Krellenstein (left) and Mallory Theurer (right) assisting with setting Texas blind salamander traps at Johnson's Well and Primer's Fissure.

Figure 2. Dr. Bockrath (left) and SCA intern Shawn Moore (right) setting up the Bd treatment trials. Dr. Bockrath is making habitat for the AHAB tanks. Shawn Moore and Dr. Bockrath measure, sex, and swab San Marcos salamanders before placing them in the AHAB tanks.

Figure 3. Desiree Moore (left) and Lisa Griego-Lyon (right) scanning the p-Chips tags in Texas blind salamanders.

June 2022 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dr. Katie Bockrath and Adam Daw

With contributions from

Desiree Moore, Ben Thomas, Braden West, and Jennifer Whitt

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

On June 15, Mallory Theurer, Braden West, and Jennifer Whitt collected Comal Springs riffle beetles, Comal Springs dryopid beetles, and Peck's cave amphipods in the Comal River at Spring Island in New Braunfels, TX. The retained invertebrates were transferred to the San Marcos Aquatic Resource Center (SMARC) for incorporation into the refugia population after quarantine.

Husbandry

Uvalde

Whitt and Theurer continued the annual repotting of the Texas wild rice and power washing of the tanks that house the Texas wild rice at the Uvalde National Fish Hatchery (UNFH) (Figure 1). Theurer, Ben Thomas and Whitt finished the bi-annual inventory of the refugia population at the UNFH.

Theurer and Thomas started moving San Marcos fountain darter eggs, laid in the UNFH refugia tanks, into aquaria to increase the number available for distribution to other facilities and research.

Adam Daw replaced the water chiller on the Invertebrate system sump at the UNFH with a larger unit that will allow better water temperature control and evaluation of units produced by a different company. Daw and Whitt continued construction work on the 3rd invertebrate rack at the UNFH.

SMARC

The Peck's cave amphipods and San Marcos River fountain darters collected in April and May 2022 were incorporated into the SMARC EARP refugia populations. Four Texas wild rice isolates, selected based on genetic data, were transferred from the SMARC refugia to the UNFH refugia to maintain similar genetic diversity at both sites. West continued to update and standardize EARP invertebrate culture systems, ensuring conditions are repeatable between tanks.

West constructed wooden stands for the new display aquaria at the EARP refugia building. Display aquaria will house San Marcos salamanders, Fountain darters, and Texas blind salamanders. West and Daw (UNFH) worked to improve the Texas wild rice refugia system at the SMARC by removing the previous water delivery systems and adding sump pumps to each tank to create more laminar flow within refugia tanks. Daw continued construction of the first of

four new quarantine systems at the SMARC. Daw and SMARC refugia staff continued rearranging tanks in the refugia and quarantine rooms to make better use of available space.

Animal Health

The health reports from the 55 wild San Marcos and 59 wild Comal fountain darters collected in April/May during the biannual biomonitoring event were completed. The Comal River fountain darters tested positive for Largemouth bass virus, which has been the case in recent years. Although no adult *Centrocestus formosanus* cycts were observed on the gill arches, immature cycts were observed from the gill arches of four of ten darters. Additionally, Monogenean parasites from eight of ten fish, as well as *Ichthyobodo* from two of ten fish, respectively, were observed on a single, or multiple gill arches of these fish.

The San Marcos fountain darters were negative for Largemouth bass virus. Three San Marcos fountain darters had both immature and adult *Centrocestus formosanus* on their gill arches. In addition, four of ten fish examined had Monogenean parasites, as well as *Ichyobodo* on a single or multiple gill arch.

The Southwest Fish Health Unit (SFHU) conducted an annual animal health inspection of the SMARC; they sampled San Marcos fountain darters in the refugia and swabbed refugia Texas blind salamanders, Comal salamanders and San Marcos salamanders for *Batrachochytrium dendrobatidis (Bd)* testing.

The results from the SFHU annual animal health inspection at the UNFH in May were received. A subsample of salamanders in the refugia population swabbed for *Bd* tested positive.

Task 2 Research

Comal Springs Riffle Beetle Pupation

The first trial of the density experiment was checked this month. The smallest density tube (N=20) was opened and inventoried before the other tubes were assessed. Without breaking up the wood, biofilm materials were inspected. Ten of the original twenty beetles were observed. Six living late instar larvae, one late instar larva carcass, two pupa, and a freshly eclosed adult (Figure 2). It is possible the remaining ten larvae were buried into the wood, thus not observed. All individuals were placed back into the tube to allow the remaining larvae to pupate. Because most of the larvae in the inventoried density tube had not pupated, the other two density tubes for Trial One were not inventoried. All three tubes for Trial One will be re-inventoried in July. Desiree Moore prepared datasheets and equipment needed to complete the tube inventories.

Comal Springs Riffle Beetle Exposure to Staphylococcus

No new activity to report.

Captive Propagation for San Marcos Salamanders

An EndNote library inventorying all relevant literature collected to date was created. Relevant reports and publications available in SMARC annual reports continue to be collected. Reports are now being compiled from different programs at the SMARC and historical records from before the EARP was started.

Comal Springs Riffle Beetle Population Genetics

No updates to report.

*Comal Springs Riffle Beetle (*Heterelmis comalensis*) Refugia Manual*

The funds for BIO-WEST contribution have been approved and released to BIO-WEST. An EndNote library cataloging all relevant literature on Comal Springs riffle beetle captive husbandry and propagation was created

Batrachochytrium dendrobatidis *(Bd) Infection Treatment in Aquatic Salamanders*

Shawn Moore and Desiree Moore tested several designs to securely house salamanders in the AHAB tanks in which the Bd treatment study will be held. A design that prevented salamander escape and reduced salamander stress was finalized. Additional itraconazole medication was sent to the SMARC from Dr. Trista Welsh-Becker (SFHU). DNA was extracted from the swabs that were collected from the nine salamanders that will be used in the dosage trial study.

P-Chip Tag Effects on **Eurycea** *spp.*

D. Moore scanned the p-Chips in all tagged salamanders weekly. No mortality or tag loss has occurred thus far (Table 2).

Randy Gibson (SMARC) scanned a subset of Texas blind salamanders and Comal Springs salamanders as a novice tag reader. All tags were read without the need for assistance.

*Catalog of Historical Fountain Darter (***Etheostoma fonticola***) Tissue and DNA Viability Testing*

The database cataloging fountain darter historical tissues has been quality checked for accuracy and completion. Currently, the database catalogs 1,240 tissue samples (Figure 3). Total body length was collected while archiving each sample and the distribution of total body length by collection location was assessed (Figure 4). Based on the dataset, Comal Springs fountain darters tend to be smaller than San Marcos fountain darters, and captive raised fountain darters (Fx) tend to be the largest. Thus far, the DNA from 18 tissue samples have been extracted. Seventeen of these samples were preserved in ethanol and the DNA extraction was successful. The DNA from one formalin preserved sample was extracted alongside the ethanol preserved samples, but the DNA extraction reaction failed (Table 3). As expected, additional steps are required to remove the formalin from the tissue before moving on to the DNA extraction reactions. PBS solution has been ordered to rehydrate formalin preserved tissue samples for subsequent DNA extraction.

Additional Accomplishments

Desiree Moore taught a p-Chip tagging workshop where several EARP staff members successfully tagged fish, and some staff members also successfully tagged Comal Springs salamanders. Those attending completed a feedback form to assess any areas for improvement in how we teach staff to tag. This feedback will be helpful for teaching future staff members to tag EARP refugia organisms.

On June 14, Dr. Katie Bockrath, Eleanor Krellenstein, Shawn Moore, Mallory Theurer, Braden West, and SMARC staff attended the Headwaters of the Comal camp to educate attending children about our program and the threatened and endangered species in the Edwards Aquifer.

On June 17, Dr. Katie Bockrath and Desiree Moore met with Ruben Tovar (University of Texas at Austin), Ann-Margaret Gonzalez (EAA), and Sarah Valdez (EAA) to discuss the EARP providing Tovar with specimens to create 3D prints to the EAA for outreach and education.

Under Dr. Becker's direction, Eleanor Krellenstein and Desiree Moore began a pilot study assessing the effects of vitamin E supplementation in Texas blind salamanders with signs of

steatitis. Dr. Becker prescribed a weekly supplementation by soaking mysis in the vitamin E treatment and feeding the medicated food to a subset of Texas blind salamanders. Photos of treated and control salamanders before and after four weeks of treatment will be compared to assess the effects of supplementation.

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

EARP staff meet weekly to discuss husbandry duties, collections, and ongoing research.

On June 8, Dr. Katie Bockrath, Chad Furl (EAA), Kristy Kollaus (EAA) met for the second quarterly EAA meeting.

On June 14, Dr. Katie Bockrath, Eleanor Krellenstein, Shawn Moore, Mallory Theurer, Braden West, and Desiree Moore attended the Headwaters of the Comal camp to educate attending children about the EARP and the threatened and endangered species in the Edwards Aquifer.

On June 17, Dr. Katie Bockrath and Desiree Moore met with Ruben Tovar (University of Texas at Austin), Ann-Margaret Gonzalez (EAA), and Sarah Valdez (EAA) to discuss the EARP providing Tovar with specimens to create 3D prints to the EAA for outreach and education.

On June 22, Dr. Katie Bockrath and Desiree Moore met with Ed Osborny (BIO-WEST) and Israel Prewitt (BIO-WEST) to coordinate the schedule of this project for the rest of the calendar.

On June 8, Dr. Katie Bockrath, Chad Furl (EAA), Kristy Kollaus (EAA) met for the second quarterly EAA meeting.

Summary of June Activities

On June 8, Dr. Katie Bockrath, Chad Furl (EAA), Kristy Kollaus (EAA) met for the second quarterly EAA meeting.

On June 14, Dr. Katie Bockrath, Eleanor Krellenstein, Shawn Moore, Mallory Theurer, Braden West, and Desiree Moore attended the Headwaters of the Comal camp to educate attending children about the EARP and the threatened and endangered species in the Edwards Aquifer.

On June 15, Mallory Theurer, Braden West, and Jennifer Whitt collected Comal Springs riffle beetles, Comal Springs dryopid beetles, and Peck's cave amphipods in the Comal River at Spring Island in New Braunfels, TX. The retained invertebrates were transferred to the San Marcos Aquatic Resource Center (SMARC) for incorporation into the refugia population after quarantine.

On June 17, Dr. Katie Bockrath and Desiree Moore met with Ruben Tovar (University of Texas at Austin), Ann-Margaret Gonzalez (EAA), and Sarah Valdez (EAA) to discuss the EARP providing Tovar with specimens to create 3D prints to the EAA for outreach and education.

On June 22, Dr. Katie Bockrath and Desiree Moore met with Ed Osborny (BIO-WEST) and Israel Prewitt (BIO-WEST) to coordinate the schedule of this project for the rest of the calendar

Tables and Figures

Table 1. June's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility housed. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	--	--	33	0	83*	9	263	368
Fountain darter: Comal	NT	NT	--	--	0	0	40	0	77	15
Comal Springs riffle beetle	52	0	--	52	0	0	0	NA	10	76
Comal Springs dryopid beetle	4	0	--	4	0	0	NA	NA	0	12
Peck's cave amphipod	12	0	--	12	6	0	4	26	68	262
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	0	0	0	0	178	67
San Marcos salamander	NT	NT	--	--	0	0	5	1	133	182
Comal Springs salamander	NT	NT	--	--	0	0	0	2	112	95
Texas wild rice plants	NT	NT	--	--	0	3	0	1	196	187

* 60 San Marcos fountain darters sampled from SMARC refugia population for SFHU annual health inspection.

Table 2. The number of each species in each treatment in the p-Chip tagging study. Tagged salamanders were injected with a p-Chip subcutaneously at the base of the tail on their left side. Positive control salamanders were treated the same as tagged salamanders (i.e., anesthetized, measured, and punctured with a needle) without a tag being placed. Negative control salamanders were anesthetized and measured only.

Species	Tagged	Positive control	Negative control
Texas blind salamander	38	20	20
Comal Springs salamander	43	34	34

Table 3. DNA extraction concentrations from preserved fountain darter tissues. Collection locations include Comal Springs (CS), San Marcos River (SMR), Upper San Marcos River (SMR-U), Middle San Marcos River (SMR-M), Lower San Marcos River (SMR-L), and Captive bred individuals (Fx).

Cryovial ID	Collection Year	Collection Location	Sex	Total Length	Tissue Mass (mg)	Extraction Concentration (ng/uL)	Preservative
538262	2021	CS	female	41	5.1	22.8	Ethanol
531202	2015	SMR-M	male	36	3.3	18.3	Ethanol
538029	2002	SMR	male	32	1.3	too low	Formalin
538245	2018	Fx	unknown	35	0.7	4.64	Ethanol
538419	2018	SMR	unknown	31	1.8	5.02	Ethanol
531168	2018	CS	male	32	0.8	4.88	Ethanol
531488	2020	CS	male	36	3.5	1.38	Ethanol
531169	2018	CS	female	30	2.6	6.22	Ethanol
538250	2018	CS	unknown	22	1.6	1.98	Ethanol
531406	2021	Fx	unknown	37	2	2.3	Ethanol
531058	2019	SMR-L	male	29	3.5	9.52	Ethanol
527336	2019	CS	unknown	29	1.9	14.1	Ethanol
527068	2019	CS	female	27	1.8	2.22	Ethanol
538420	2020	SMR-M	unknown	30	1.4	10.6	Ethanol
531414	2019	SMR-U	unknown	23	1.6	7.9	Ethanol
negative	NA	NA	NA	NA	NA	too low	NA

Figure 1. Jennifer Whitt and Mallory Theurer repotting Texas wild rice at the Uvalde National Fish Hatchery. (Photo credit – Adam Daw, USFWS)

Figure 2. Observed developmental stages in the Trial 1 Density 20 propagation tube; a newly eclosed adult (left), pupa (center), and late instar larvae (right).(Photo credit – USFWS)

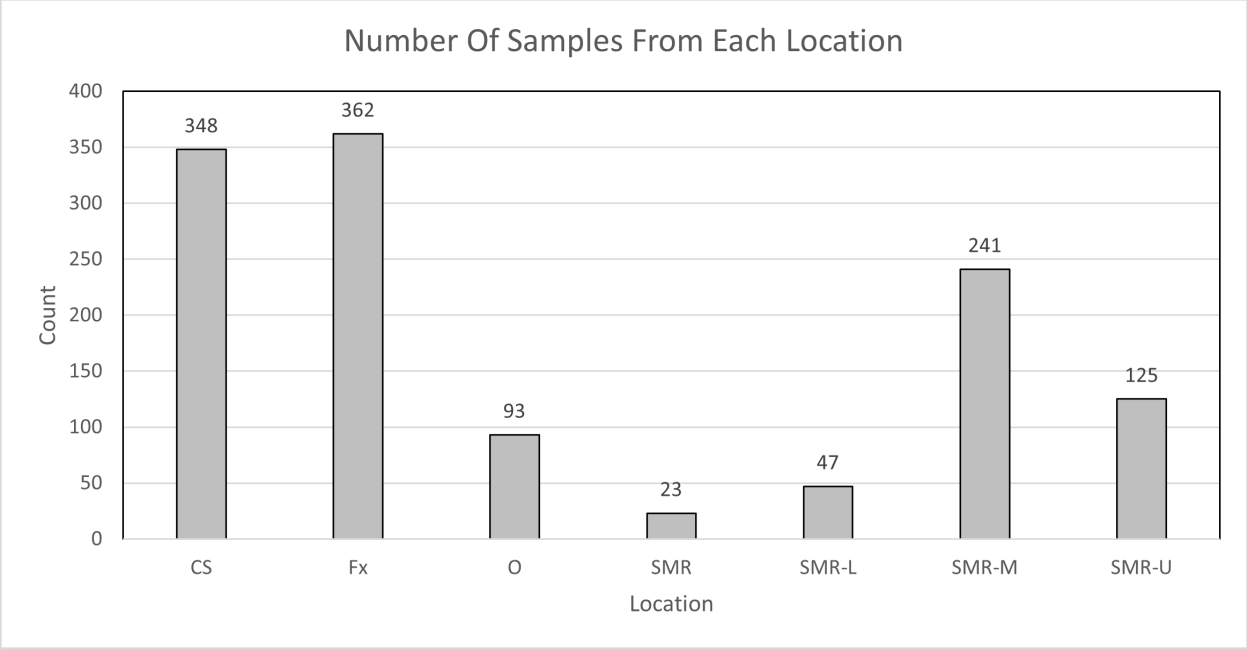


Figure 3. Distribution of preserved fountain darter samples across collection locations. Locations include Comal Springs (CS), Captive propagated individuals (Fx), Unknown location or Other (O), San Marcos River (SMR), Lower San Marcos River (SMR-L), Middle San Marcos River (SMR-M), and Upper San Marcos River (SMR-U). The number of fountain darters from each location is on the y-axis and specified above each bar.

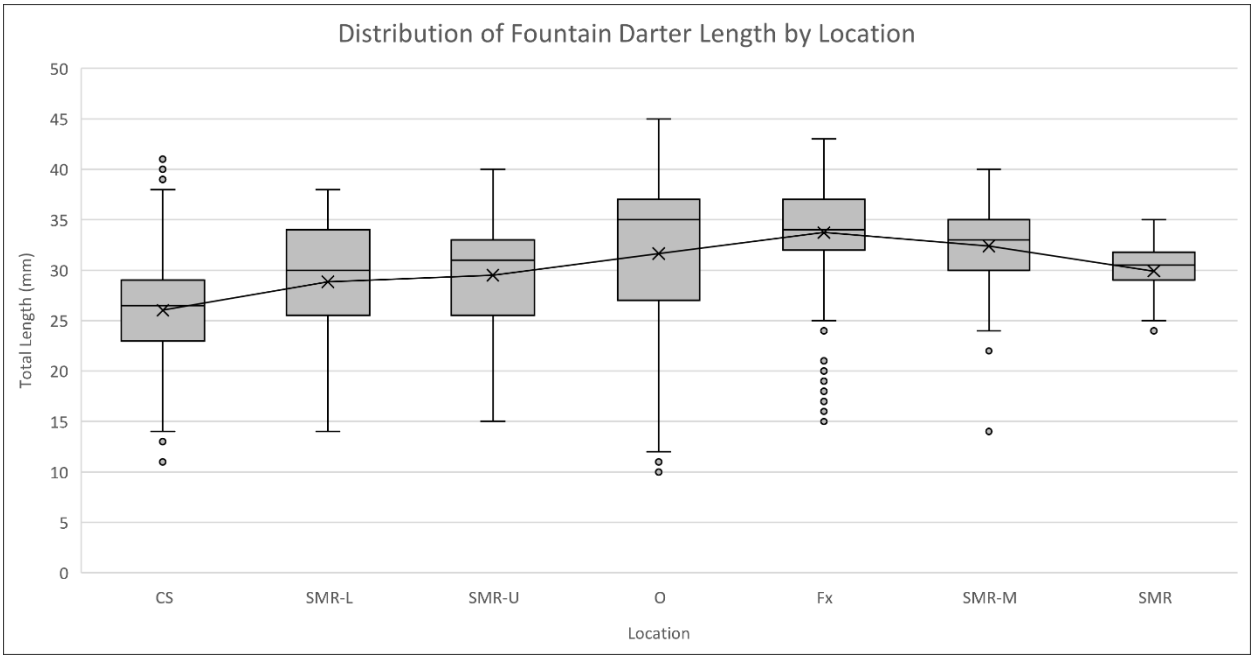


Figure 4. Box and Whisker plot showing the distribution of total body length of fountain darters from collection locations. Locations include Comal Spring (CS), Lower San Marcos River (SMR-L), Upper San Marcos River (SMR-U), Unknown or Other (O), Captive bred individuals (Fx), Middle San Marcos River (SMR-M), and the San Marcos River (SMR). The "x" is the mean total body length, the box is the 1st and 3rd quartile of the data, the horizontal bar in the box is the median.

July 2022 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dr. Katie Bockrath and Adam Daw

With contributions from

Desiree Moore, Ben Thomas, Braden West, and Jennifer Whitt

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staffing

Jennifer Whitt accepted an Aquatic Biologist position with the U.S. Forest Service at the Sierra National Forest. Whitt's last day at the Uvalde National Fish Hatchery (UNFH) was June 29. Mallory Theurer's (SCA Intern) last day at the UNFH was June 22.

Species Collection

On June 20, refugia staff obtained 73 San Marcos River Fountain darters (Figure 1) from BIO-WEST and transferred them to the San Marcos Aquatic Resources Center (SMARC). These were collected as part of a biomonitoring event.

Husbandry

Uvalde

Ben Thomas and Theurer continued collecting San Marcos River Fountain darter eggs from refugia tanks and placing in hatching aquaria. Thomas assisted Whitt in inventorying Comal Springs riffle beetles. Whitt, Theurer, and Thomas continued rotating animals between refugia tanks and acid washing and bleaching tanks. Theurer and Thomas trained Mason Theurer (volunteer) and Nick Yvon (UNFH - Biological Science Technician) on EAA duties. Thomas prepared quarantine racks for use in case refugia organisms salvage is undertaken. Adam Daw continued construction of the invertebrate racks.

SMARC

Braden West worked on new refugia data sheets using Survey 123. Eleanor Krellenstein continued to assist with the Vitamin E treatments of Texas blind salamanders (Figure 2). West, Krellenstein, and D. Moore repotted Texas wild rice in Tank 2. All plant tags were replaced with new tags to ensure readability over time.

Animal Health

West shipped skin swabs from Texas blind salamanders to the San Diego Zoo for *Batrachochytrium dendrobatidis* (Bd) analysis

Task 2 Research

Comal Springs Riffle Beetle Pupation

Israel Prewitt (BIO-WEST) set up Phase III of this study looking at the effects of wild cultivated vs refugia cultivated biofilm on pupation success (Figure 3). Three replicate tubes were wet up with wild cultivated biofilms and 30 late instar larvae were added to each tube. Prewitt checked the status of the Phase II density trial tubes. In all density tubes, not all individuals have been recovered during inventory. Dr. Bockrath and Desiree Moore recorded flow and temperature for all tubes in the Comal Springs riffle beetle pupation study weekly.

	Living			Dead			
Tube	Adults	Pupae	Larvae	Adults	Pupae	Larvae	Total
Density 20	2	0	7	0	0	0	9
Density 30	2	0	3	0	2	3	10
Density 40	4	3	13	1	0	4	25

Comal Springs Riffle Beetle Exposure to Staphylococcus

No updates to report.

Captive Propagation for San Marcos Salamanders

Desiree Moore met with Dr. Bockrath, Dr. David Britton, Krellenstein, and Shawn Moore to assign sections of the San Marcos salamander reproduction handbook. All participants began drafting their sections of the handbook.

Comal Springs Riffle Beetle Population Genetics

No updates to report.

*Comal Springs Riffle Beetle (*Heterelmis comalensis*) Refugia Manual*

Members of the writing group have been working on their respective sections.

Batrachochytrium dendrobatidis *(Bd) Infection Treatment in Aquatic Salamanders*

Dr. Bockrath, D. Moore, West, and Krellenstein concluded the Bd antifungal treatment pilot study (Figure 4). Three antifungal doses were tested (0.05%, 0.001%, and 0.0001%) per the recommendations of Dr. Trista Becker. Three separate groups of F1 San Marcos salamanders were used to test the effects of each dose. Salamanders were held in individual tanks in an AHAB and fed per usual. During treatment, salamanders were removed from the AHAB tanks, placed into individual bags with their dosage for 10 minutes. After treatment, salamanders were moved to individual recovery containers before being placed back into the AHAB. The salamanders were swabbed pre-treatment, post-treatment, and 10 days post-treatment to test for Bd infection and change in Bd infection status after the antifungal treatment. All salamanders behaved normally throughout the pilot treatment and no mortalities or lingering effects were observed due to treatment. The two highest doses will be used in the full Bd treatment study.

P-Chip Tag Effects on **Eurycea** *spp.*

Desiree Moore scanned the p-Chips in all tagged salamanders in the p-Chip tagging study weekly. Alex Klingele (SCA, SMARC) scanned a subset of salamanders as a novice tag reader. All tags were read without the need for assistance. On Comal Springs salamanders tag shifted and the tag could not be scanned. No mortality or tag loss has occurred.

*Catalog of Historical Fountain Darter (***Etheostoma fonticola***) Tissue and DNA Viability Testing*

The oldest fountain darter tissue samples available at SMARC are preserved in formalin. Formalin binds DNA, causing crosslinks. If the proper steps are not taken to remove the formalin prior to extraction, these cross-links cause DNA to become sheared during the DNA extraction process. Student Conservation Association intern, Shawn Moore, successfully extracted DNA from formalin preserved fountain darter tissues by washing the tissues with a PBS solution and gradually rehydrated the tissues prior to DNA extraction. The longer a sample has been stored in formalin, the more difficult it can be to successfully extract DNA. S. Moore has set up a multi factorial approach to determine the optimal PBS soak/wash and rehydration protocol for DNA extraction in the oldest formalin preserved samples.

Additional Accomplishments

- Eleanor Krellenstein and Desiree Moore completed the pilot study assessing the effects of vitamin E supplementation in Texas blind salamanders with signs of steatitis. D. Moore compiled photos of treated and control salamanders before and after four weeks of treatment to be compared to assess the effects of supplementation.
- EARP Staff interviewed new Student Conservation Association (SCA) intern candidates. Richelle Jackson was selected for a position at the SMARC refugia and has accepted the offer. Michelle Nielsen was selected for a position at the UNFH refugia and has accepted the offer. Jackson will join the group August 22nd and Nielsen will start August 28th.

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

On July 21, Dr. Katie Bockrath, Dr. David Britton, Desiree Moore, Chad Furl (EAA), Kristy Kollaus (EAA), Scott Storment (EAA) met to discuss 2023 research projects.

On July 27, D. Moore. Dr. Bockrath, Daw, West, and members of the EAA met to discuss plans for species salvage if the associated triggers are reached due to drought conditions.

Summary of July Activities

- June 20, the SMARC refugia obtained 73 San Marcos River Fountain darters collected during a BIO-WEST biomonitoring event.
- Vitamin E trials to treat steatitis in Texas blind salamanders was completed.
- The Bd pilot study was completed, and all salamanders were not adversely affected by the treatment.
- DNA was successfully extracted from preserved fountain darter tissue.
- Student Conservation Association interns were interviewed and selected.

Tables and Figures

Table 1. July's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility housed. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	73	NT	--	73	0	0	8	14	255	355
Fountain darter: Comal	NT	NT	--	--	0	0	0	0	77	15
Comal Springs riffle beetle	NT	0	--	--	48	0	0	28	58	48
Comal Springs dryopid beetle	NT	0	--	--	2	0	NA	2	2	10
Peck's cave amphipod	NT	0	--	--	11	0	6	3	73	259
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	0	0	1	0	177	67
San Marcos salamander	NT	NT	--	--	0	0	13	1	120	181
Comal Springs salamander	NT	NT	--	--	0	1	0	0	112	96
Texas wild rice plants	NT	NT	--	--	0	2	1	0	195	189

Figure 1. Two male Comal Springs fountain darters displaying their dorsal fins.

Figure 2. Desiree Moore (right) and Eleanor Krellenstein setting up the Vitamin E and Mysis feeding trials to treat steatitis in Texas blind salamanders

Figure 3. Israel Prewitt (BIO-WEST) working on the Comal Springs riffle beetle density and biofilm trials.

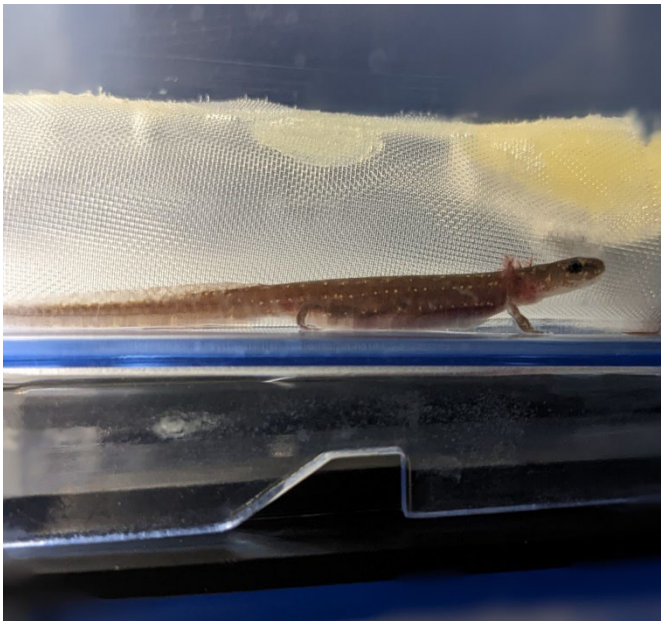


Figure 4. (Left) Dr Katie Bockrath measures itraconazole doses for the Bd treatment pilot study. (Right) San Marcos salamander post Bd treatment.

July 2022 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dr. Katie Bockrath and Adam Daw

With contributions from

Desiree Moore, Ben Thomas, Braden West, and Jennifer Whitt

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staffing

The Edwards Aquifer Refugia Program welcomed two new Student Conservation Association interns to the team. Richelle Jackson is a recent graduate from Texas State and joins the group at the San Marcos Aquatic Resources Center (SMARC). Richelle will focus on maintaining the research organisms and assisting with research tasks, primarily the Bd treatment study. Michelle Emily Nielsen graduated from Humboldt State University and joins the Uvalde National Fish Hatchery (UNFH) team. Hiring actions to bring on permanent positions at both SMARC and UNFH are in progress. The first two positions, on at the SMARC and one at UNFH, will be open for application on September 6 and closes September 19. The positions are posted on USAJOBS.gov.

Species Collection

The Edwards Aquifer Refugia Program conducted more field collections then planned for August in the 2022 Work Plan in response to decreasing spring flows in the San Marcos and Comal rivers.

On August 1, staff collected two Comal springs salamanders and 50 Peck's cave amphipods from the Spring Island area of the Comal River. Organisms were taken to the SMARC.

On August 3, staff collected 60 San Marcos salamanders from the San Marcos River, below the Spring Lake Dam, and retained 29 of them for the UNFH.

On August 3, staff collected 37 Comal fountain darters from Landa Lake and 73 from Spring Island in the Comal River. The fish were taken to the SMARC.

On August 4, staff collected 128 San Marcos fountain darters from Spring Lake in the San Marcos River and brought them to the UNFH.

On August 8, staff set traps at Johnson's Well for Texas blind salamanders. The water level in Primer's Fissure was too low to set traps. Traps were checked on the 5th, 8th, 12th, and removed on the 15th. Two Texas blind salamanders were captured and retained for the SMARC.

On. August 10, staff collected 45 Peck's cave amphipods from the Spring Island area of the Comal River. Organisms were taken back to the SMARC.

On August 11, staff collected 166 Comal fountain darters from the Spring Island area of the Comal River and retained 159 for the SMARC.

On August 11, staff collected Texas wild rice tillers from 10 locations in Section F of the San Marcos River. Tillers were taken to the UNFH.

On August 12, staff collected Texas wild rice tillers from 10 locations in Section B of the San Marcos River. Tillers were taken to the UNFH.

On August 15, staff collected 165 Comal fountain darters from the Spring Island area of the Comal River and retained 158 for the SMARC.

On August 16, staff collected 24 San Marcos fountain darters and 64 San Marcos salamanders, retaining 21, from below Spring Lake Dam in the San Marcos River for the UNFH. Staff collected 87 San Marcos fountain darters from Spring Lake in the San Marcos River and retained them for the SMARC.

On August 18, staff collected 104 Comal fountain darters from the Spring Island area of the Comal River and brought them to the UNFH.

On August 24, staff picked up 95 Comal fountain darters captured by BIO-WEST in the Landa Lake area of the Comal River and brough them to the UNFH.

On August 25, staff picked up 168 Comal fountain darters captured by BIO-WEST in the Old Channel area of the Comal River and brough them to the UNFH.

On August 25, staff collected Texas wild rice tillers from 10 locations in Section B of the San Marcos River. Tillers were taken to the SMARC.

On August 26, staff picked up 11 Comal fountain darters captured by BIO-WEST in the New Channel area of the Comal River and brough them to the UNFH.

Husbandry

Uvalde

Ben Thomas and Adam Daw trained Michelle Emily Nielsen (Emily Nielsen) a new SCA intern to UNFH on all EAA husbandry duties. Thomas and Daw prepared quarantine systems in anticipation of the increased collection activity. Thomas setup a new tank to house recently laid Comal salamander eggs. Daw continued construction of Invertebrate Rack 3.

SMARC

UNFH and SMARC EARP staff moved several refugia thanks from the UNFH to the SMARC refugia room. Braden West constructed two new daphnia culture systems. West finished two new Survey123 data entry forms: one for Texas wild rice collections and the other for disease

treatments. Braden finished converting Peck's cave amphipod and Comal Springs riffle beetle boxes to an updated design. Staff started training Richelle Jackson (SCA intern) on husbandry operations.

Animal Health

EARP staff received results from the Texas blind salamander swabs sent to the San Diego Zoo. Four of the five new wild caught Texas blind salamanders in quarantine were positive for *Batrachochytrium dendrobatidis* (Bd). No salamanders were positive for *Batrachochytrium salamandrivorans* (Bsal).

Ten Comal fountain darters and ten San Marcos fountain darters were shipped to the Southwestern Native Aquatic Resources and Recovery Center's Southwestern Fish Health Unit for parasite analysis. Of the ten Comal fountain darters analyzed, two had *Centrocestus formosanus* and four had monogenetic trematodes. Of the ten San Marcos fountain darters analyzed, one had *Centrocestus formosanus* and six had monogenetic trematodes.

Dr. Bockrath, Eleanor Krellenstein, and West examined the photos of Texas blind salamanders who received the vitamin E treatment and Desiree Moore compiled and analyzed the results. No clear negative or positive effects of vitamin E supplementation in Texas blind salamanders were observed.

Task 2 Research

Comal Springs Riffle Beetle Pupation

Israel Prewitt (BIO-WEST) inventoried Phase II (Density) and Phase III (Biofilm) flowthrough tubes. Dr. Katie Bockrath and Desiree Moore recorded flow and temperature for all tubes in the Comal Springs riffle beetle pupation study weekly. Phases and trials are staggard so no conclusions can be until the study concludes. Larvae are still present in all tubes and the the tubes will continue to be checked monthly until no larvae remain. Pupation and eclosion are occurring in all tubes.

Comal Springs Riffle Beetle Exposure to Staphylococcus

Dr. Camila Carlos-Shanley is analyzing the data.

Captive Propagation for San Marcos Salamanders Handbook

Staff have compiled the relevant reports and publications and are writing the handbook.

Comal Springs Riffle Beetle Population Genetics

No updates to report.

*Comal Springs Riffle Beetle (***Heterelmis comalensis***) Refugia Manual*

A first draft is complete. The authors will meet to discuss the draft and identify any missing information in September.

Batrachochytrium dendrobatidis *(Bd) Infection Treatment in Aquatic Salamanders*

The pilot study is complete. All nine salamanders in the pilot study showed no ill effects during or after treatment, regardless of the dose. Two individuals were positive for Bd prior to treatment and remained positive post treatment. Because this treatment is novel in San Marcos salamander, the initial prescribed doses were conservative. Dr. Bockrath is discussing increasing the dosage with Dr. Trista Becker for use in the full study.

P-Chip Tag Effects on **Eurycea** *spp.*

Texas blind and Comal Springs salamanders were scanned weekly. Richelle Jackson was the novice reader this month (Figure 6). No mortalities have occurred. One Comal Springs salamander's p-Chip either shifted and is now unreadable or it was ejected.

*Catalog of Historical Fountain Darter (***Etheostoma fonticola***) Tissue and DNA Viability Testing*

Shawn Moore worked on extracting DNA from fountain darter tissue samples preserved in formalin and stored in a non-climate-controlled building. These samples are the oldest and potentially the most damaged tissues samples of the collection. Formalin preserved tissue samples were soaked in Phosphate Buffered Solution (PBS) for a week to remove residual formalin and adjust the pH of the tissues to pH 7.0. Post PBS soak, DNA was successfully

extracted from these highly damaged tissues at quantities suitable for DNA analysis. The quality of the DNA will be assessed in September.

Additional Accomplishments

On August 26, Sarah Mock and Clayton Klingberg from the Edwards Aquifer Authority's (EAA) Education Outreach Center (EOC) met with Braden West for a discussion on feeding practices and food items for Fountain darters kept as display organisms. Braden transferred 10 captive-born San Marcos fountain darters and some live food cultures to the EOC.

Dr. Bockrath and West swabbed and tested F1 Texas blind salamanders for Bd to identify a Bd negative individual to be showcased in the Edwards Aquifer Authority's EOC display tank.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

- EARP Staff met weekly to discuss collections, husbandry needs, and research updates
- Dr. Bockrath met with Dr. Chad Furl to discuss the status of 2023 external partner research proposals
- Desiree Moore, Dr. Bockrath, Shawn Moore, and Richelle Jackson met to discuss the status of 2022 research and timelines to completion
- Desiree Moore and Dr. Bockrath met with external partners to discuss 2023 research
- EARP staff working on the Comal Springs riffle beetle handbook met to discuss ongoing progress on the first draft

Summary of August Activities

- Peck's cave amphipod and Comal Springs salamander collection at Spring Island on August 1.
- San Marcos salamander collection below Spring Lake Dam in the San Marcos River on August 3.
- Comal fountain darter collection at Spring Island and Landa Lake on August 3.
- San Marcos fountain darter collection at Spring Lake on August 4.
- Peck's cave amphipod collection at Spring Island in the Comal River on August 10.
- Comal fountain darter collection at Spring Island in the Comal River on August 11.
- Texas wild rice collection in Section F of the San Marcos River on August11.
- Texas wild rice collection in Section B of the San Marcos River on August 12.
- Comal fountain darter collection at Spring Island in the Comal River on August 15.
- San Marcos salamander and San Marcos fountain darter collection below Spring Lake Dam in the San Marcos River on August 16.
- San Marcos fountain darter collection at Spring Lake in the San Marcos River on August 16.
- Comal fountain darter collection at Spring Island in the Comal River on August 18.
- Comal fountain darter collection at Landa Lake in the Comal River on August 24.
- Texas wild rice collection in Section B of the San Marcos River on August 25.
- Comal fountain darter collection at the "Old Channel" of the Comal River on August 25.
- Comal fountain darter collection at the "New Channel" of the Comal River on August 26.
- The first draft of the Comal Springs riffle beetle propagation handbook is complete
- Comal Springs riffle beetle Phase II and Phase III propagation trials were inventoried
- DNA was extracted from formalin preserved fountain darter tissue samples
- Texas blind and Comal Springs salamanders tagged with p-Chips were scanned weekly
- Pilot study San Marcos salamander swabs pre and post treatment were analyzed
- No significant changes were observed after the vitamin E treatment in Texas blind salamanders
- Bd test results of wild caught Texas blind salamanders were received from the San Diego Zoo.

Tables and Figures

Table 1. August's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility housed. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	87	152	0	239	71	0	24	6	302	349
Fountain darter: Comal	461	378	14	853	0	0	3	0	74	15
Comal Springs riffle beetle	NT	NT	--	--	0	0	0	0	58	48
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	0	2	10
Peck's cave amphipod	95	NT	0	95	0	0	10	4	63	257
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	2	NT	0	2	0	0	2	0	175	67
San Marcos salamander	NT	50	84	134	0	0	5	0	115	181
Comal Springs salamander	2	NT	0	2	0	0	0	0	112	96
Texas wild rice plants	10	10	0	20	0	0	0	0	195	189

Figure 1. Ben Thomas transferring fountain darters from a transport cooler to a quarantine rack at the Uvalde National Fish Hatchery

Figure 2. Braden West (left) and Desiree Moore (right) collect Comal fountain darters at the Spring Island area using a seine net.

Figure 3. Eleanor Krellenstein (left) and Braden West (right) identifying Comal fountain darters after collection using a siene net.

Figure 4. Braden West and Dr. Katie Bockrath collect Comal fountain darters at Landa Lake using a siene net.

Figure 5. Eleanor Krellenstein collecting Peck's cave amphipod.

Figure 6. Richelle Jackson reading p-Chips in Texas blind salamanders.

September 2022 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dr. Katie Bockrath and Adam Daw

With contributions from

Desirée Moore, Ben Thomas, and Braden West

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staffing

Hiring actions to bring on permanent positions at both SMARC and UNFH continue. The status of the positions are listed below.

Location	Position	Status
San Marcos	Research Biologist (GS 9/11)	Application closed and waiting on applicant list from HR
San Marcos	Biologist (GS 7/9)	Application closed and received applicant list from HR. We are reviewing applications.
San Marcos	Biological Technician (GS 5/7)	Position posted on USAJOBS.gov
San Marcos	Biological Technician (GS 5)	Waiting for HR to list positions
Uvalde	Biologist (GS 7/9)	Application closed and received applicant list from HR. We are reviewing applications.
Uvalde	Biological Technician (GS 5/7)	Position posted on USAJOBS.gov
Uvalde	Biological Technician (GS 5)	Waiting for HR to list positions

Species Collection

On September 7, Ben Thomas and Emily Nielsen collected Comal Springs fountain darters from around Spring Island. Organisms were taken to the UNFH.

On September 7, staff collected San Marcos salamanders from below Spring Lake Dam. Organisms were taken to the SMARC.

On September 15, EARP staff and SCA interns from the UNFH and SMARC in conjunction with USFWS SCUBA divers collected San Marcos salamanders from Spring Lake (Figures 1-3). Organisms were taken to the SMARC.

Husbandry

Uvalde

Thomas and Adam Daw continued training Neilsen on refugia operations. Staff spent most of their time with daily animal care and system maintenance. The Spergion well stopped operating on September 11 due to an electrical short on the main electrical lines. After the electrical lines were replaced, the Wilson well was started on the 13th. No adverse effects were observed to the refugia organisms during the water outage.

SMARC

SMARC staff moved the large oval tank out of the refugia and replaced it with two rectangular tanks brought up from the UNFH. Braden West plumbed the two tanks for use with Fountain darters. Desirée Moore tagged 16 Texas blind salamanders in the refugia stock with the p-chips before incorporation into the Refugia. West completed construction on the new display tanks. West continued to work on updating Survey123 forms used by the refugia for data collection. West and Moore assembled storage racks in the EARP building breezeway (Figure 4) to allow more efficient storage of equipment. EARP staff and SCA interns assisted with the remodeling for the genetics lab.

Animal Health

No activity to report.

Task 2 Research

Comal Springs Riffle Beetle Pupation

Israel Prewitt (BIO-WEST) inventoried Phase II (Density) and Phase III (Biofilm) flowthrough tubes. Richelle Jackson (SCA) monitored flow and water temperature of the study tubes weekly. Phases and trials are staggered so no conclusions can be drawn until the study concludes. Trial 1 of the Density series has concluded. Trials 2 and 3 are ongoing but larval numbers are declining. The biofilm trial was last to begin, and larvae are still present in these tubes. Pupation and eclosion continue to occur in the remaining density and biofilm trial tubes. All remaining trials will continue to be inventoried monthly until no larvae remain.

Comal Springs Riffle Beetle Exposure to Staphylococcus

Dr. Katie Bockrath and Desirée Moore met with Dr. Camila Carlos-Shanley to discuss the genetic results. There are significant differences in the microbial diversity of beetles held at SMARC than those at Uvalde and Comal Springs. The microbial diversity of beetles held at Uvalde and collected from Comal Springs beetles were not significantly different. Dr. Camila Carlos-Shanley has submitted a draft Final Report for review.

Captive Propagation for San Marcos Salamanders Handbook

Staff have compiled the relevant reports and publications and are writing the handbook. A first draft has been compiled.

Comal Springs Riffle Beetle Population Genetics

No updates to report.

*Comal Springs Riffle Beetle (***Heterelmis comalensis***) Refugia Manual*

The writing team met to discuss the progress of the handbook, identify missing content, and to make document revisions.

Batrachochytrium dendrobatidis *(Bd) Infection Treatment in Aquatic Salamanders*

The first full trial is complete, and the second trial is in progress (Figure 5). No mortalities or ill effects of treatment have been observed.

P-Chip Tag Effects on **Eurycea** *spp.*

Texas blind and Comal Springs salamanders were scanned weekly. Yovani Valdes (SCA) was the novice reader this month (Figure 6). No mortalities have occurred. One Comal Springs salamander's p-Chip either shifted and is now unreadable or it was ejected. All other tags are readable.

*Catalog of Historical Fountain Darter (***Etheostoma fonticola***) Tissue and DNA Viability Testing*

Approximately 350 individual fountain darter historical tissue samples were selected to complete DNA extractions to test DNA viability across preservation methods and preservation years. DNA was extracted from 103 samples and DNA quality was measured using PCR and Gel Electrophoresis visualization. DNA extractions are on track for completion in October. The

SOPs for tissue archiving, DNA extraction and DNA viability testing are drafted and under review.

Additional Accomplishments

- Permanent positions with SMARC and Uvalde were opened for application. All positions continue to progress through HR.
- Modifications to the interior of the EARP building continued. The hallway and the genetics lab are fully painted, and new baseboard was installed.
- Shelving was installed in the breezeway to allow chillers and large items to be stacked and lifted off the floor.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

- Dr. Katie Bockrath and Desirée Moore met with Dr. Shannon Brewer to discuss 2023 research proposal and budget
- Dr. Katie Bockrath and Desirée Moore met with Dr. Chris Nice to discuss 2023 research proposal and budget
- Dr. Katie Bockrath and Desirée Moore met with Ruben Tovar to discuss 2023 research proposal and budget
- Dr. Katie Bockrath and Desirée Moore met with Dr Camila Carlos-Shanley to discuss the genetic results of sequencing the larvae from the Staphylococcus exposure trials
- Desirée Moore and David Britton met with Chad Furl and Kristy Kollus to discuss 2022 research progress and 2023 research proposals
- The Comal Springs riffle beetle handbook team met twice to discuss the progress of the handbook and to make revisions.
- EARP staff met weekly to discuss ongoing research, collections, and husbandry needs.

Summary of September Activities

- Transitioning term positions to permanent positions at both SMARC and UNFH are progressing.
- Comal Springs fountain darters were collected from around Spring Island.
- San Marcos salamanders were collected from Spring Lake and below Spring Lake Dam.
- New show tanks were set up at SMARC
- Instead of VIE, P-Chips were used to tag Texas blind salamanders being incorporated into the refugia.
- New shelving was installed in the breezeway at SMARC
- Renovations for the genetics lab are complete
- Dr. Camila Carlos-Shanley submitted a draft final report on the *Staphylococcus* exposure experiment.
- BIO-WEST checked the progress of the density and biofilm CSRB propagation trials.
- The first full Bd treatment trial was completed.
- P-Chip tagged Comal Springs and Texas blind salamanders were scanned by a professional and a novice reader.
- DNA continues to be successfully extracted from preserved historical fountain darter specimens.

Tables and Figures

Table 1. September's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility housed. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NA	NT	--	--	142	128	15	13	429	463
Fountain darter: Comal	NA	102	17	119	0	0	4	0	70	15
Comal Springs riffle beetle	NT	NT	--	--	0	0	9	0	49	48
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	0	2	10
Peck's cave amphipod	NT	NT	--	--	73	0	5	4	131	251
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	0	0	1	0	174	66
San Marcos salamander	90	NT	3	93	0	0	7	5	108	176
Comal Springs salamander	NT	NT	--	--	0	0	0	1	112	95
Texas wild rice plants	NT	NT	--	--	10	20	0	0	205	209

Figure 1. SCA Interns assisting with San Marcos salamander collections at Spring Lake.

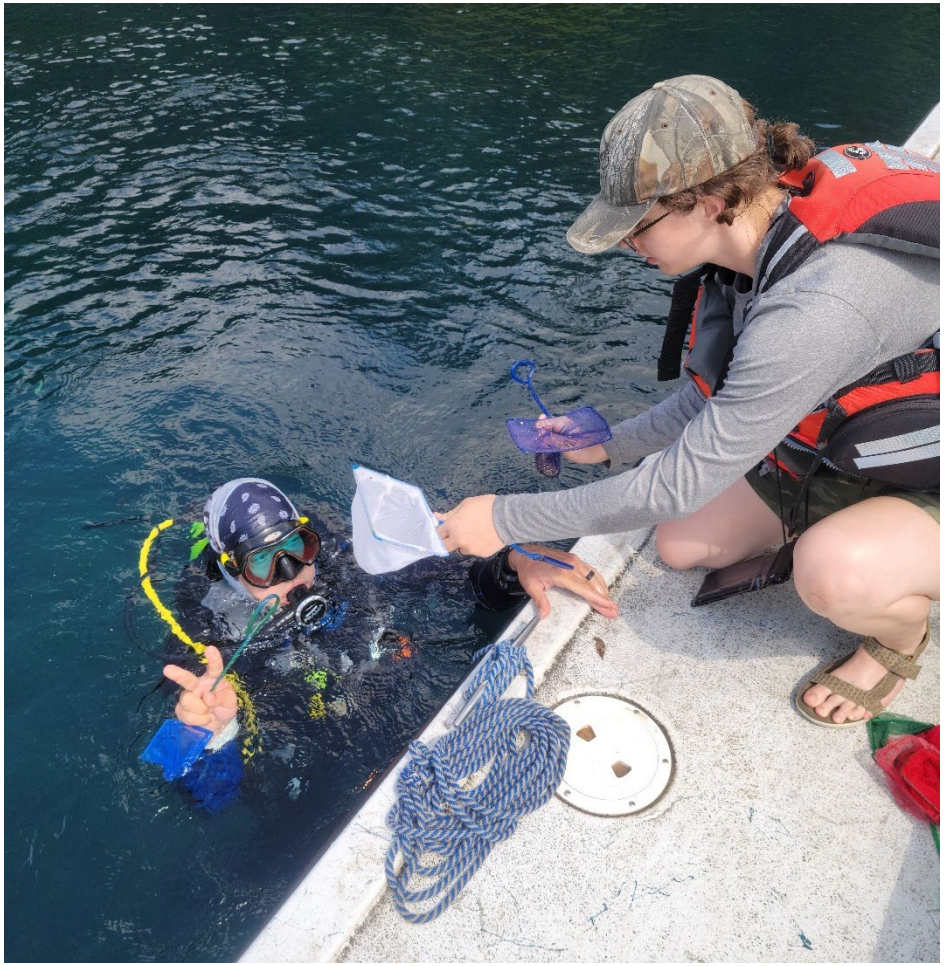


Figure 2. SCA Intern Celeste Palmquist trading nets with a USFWS diver, Channing St. Abuin, collecting San Marcos salamanders.

Figure 3. USFWS diver, Justin Crow, with a San Marcos salamander in a net, collected from Spring Lake.

Figure 4. Braden West (left) and Desirée Moore (right) standing in the breezeway at SMARC after installing new shelving.

Figure 5. Richelle Jackson (SCA) assisting with Bd treatments in San Marcos salamanders.

Figure 6. Yovani Valdes (SCA) serves as a novice reader to scan p-Chips.

October 2022 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dr. Katie Bockrath and Adam Daw

With contributions from

Desirée Moore, Ben Thomas, and Braden West

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staffing

Hiring actions to bring on permanent positions at both SMARC and UNFH continue. The status of each position is listed below.

Location	Position	Status
San Marcos	Research Biologist (GS 9/11)	Application List received from HR.
San Marcos	Biologist (GS 7/9)	Interviews complete. SMARC is checking references.
San Marcos	Biological Technician (GS 5/7)	Application period has closed. We are waiting on the applicant list from HR.
San Marcos	Biological Technician (GS 5)	Waiting for HR to post the position.
Uvalde	Biologist (GS 7/9)	Interviews complete. Uvalde made an offer.
Uvalde	Biological Technician (GS 5/7)	Application period has closed. We are waiting on the applicant list from HR.
Uvalde	Biological Technician (GS 5)	Application period has closed. We are waiting on the applicant list from HR.

Species Collection

On October 11, Braden West obtained 52 San Marcos River fountain darters from the bi-annual survey of the San Marcos River (Middle section) conducted by BIO-WEST. These fish were sent to the Southwestern Fish Health Unit at the USFWS Southwestern Native Aquatic Resources and Recovery Center (Dexter, NM) for health screening.

On October 24, West obtained 119 Comal River fountain darters from the bi-annual survey on the Comal River (Landa Lake) conducted by BIO-WEST. Sixty of these fish were sent to the Southwestern Fish Health Unit for health screening, and the rest were quarantined for incorporation into the refugia.

On October 25, West obtained 46 Comal River fountain darters from the bi-annual survey of the Comal River (Old Channel) conducted by BIO-WEST. Sixty of these fish were sent to the Southwestern Fish Health Unit for health screening, and the rest were quarantined for incorporation into the refugia.

On October 28, Randy Gibson donated 110 Pecks cave amphipods to the refugia at SMARC. These were caught during the bi-annual survey of the Comal River.

Husbandry

Uvalde

Ben Thomas and Emily Nielsen transferred fountain darters from quarantine into refugia tanks. Adam Daw and Thomas assembled epoxy lab tables for use in the UNFH and SMARC invertebrate husbandry areas. New shelving and the epoxy tabletops were installed in the Invertebrate Room. The EARP staff conducted a thorough cleaning of the refugia space.

SMARC

On October 13, six wild-caught Texas blind salamanders were p-Chip tagged and incorporated them into the refugia population. Daw, Thomas, and Braden West removed the old Invert counting structure and installed new shelving and the epoxy tabletops; this will allow more space for conducting inventories and training. Daw connected the new water quality monitor on the invertebrate system to the stations WiFi and it can now be remotely monitored. The EARP staff conducted a thorough cleaning of the refugia space. West, Juan Martinez, and Celeste Palmquist repaired a leaking well water line in the SMARC greenhouse.

Animal Health

Sixty Comal River fountain darters and 46 San Marcos River fountain darters were shipped to the USFWS Southwestern Fish Health Unit for parasite and viral screening.

Task 2 Research

Comal Springs Riffle Beetle Pupation

Phase II (Density) and Phase III (Biofilm) flowthrough tubes were inventoried. Trial 1 of Phase II is complete. Most tubes from Trials 2 and 3 of the Phase II are complete and there are 3 larvae remaining across 2 two tubes. The adults in Phase II have been breeding in the density tubes and new early instar larvae are present. Phase III tubes continue to produce adults but *Microcylloepus pusillus* larvae are also present and potentially pupating. The number of *Heterelmis comalensis* and *M. pusillus* adults continue to be tracked. Water flow and water temperature of the tubes was checked weekly. Phases and trials are staggered so no conclusions can be drawn until the study concludes. All remaining trials will continue to be inventoried monthly until no larvae remain.

Comal Springs Riffle Beetle Exposure to Staphylococcus

No updates to report.

Captive Propagation for San Marcos Salamanders Handbook

Contributing authors have written the document and it is under internal review and revision.

Comal Springs Riffle Beetle Population Genetics

Supplies for DNA extractions were ordered. F1 adults and larvae mortalities used in the Propagation study have been stored in ethanol to test DNA extraction methods.

*Comal Springs Riffle Beetle (*Heterelmis comalensis*) Refugia Manual*

Contributing authors have written the document and it is under internal review and revision.

Batrachochytrium dendrobatidis *(Bd) Infection Treatment in Aquatic Salamanders*

All treatment trials are complete and a visiting Directorate Fellow from USFWS Science Applications assisted with the last treatment (Figure 1). Reagents were ordered to complete all DNA extractions from the swabs and all Bd qPCR detection tests.

P-Chip Tag Effects on Eurycea *spp.*

Texas blind and Comal Springs salamanders were scanned weekly. Kevin Rubio (SCA) was the novice reader this month and successfully scanned the tagged salamanders (Figure 2). Two mortalities, in total, have occurred but are not isolated to a tagging treatment or species. One tagged Texas blind and one negative control Comal Springs salamander make up the total mortalities for this study. One Comal Springs salamander's p-Chip either shifted and is now unreadable or it was ejected. All other tags are readable. Thus far, there is a 100% tag retention rate and a 97% survival rate for Texas blind salamanders. There is a 98% retention rate and 97% survival rate for Comal Springs salamanders.

*Catalog of Historical Fountain Darter (*Etheostoma fonticola*) Tissue and DNA Viability Testing*

The backordered cryovials for organizing and archiving tissue samples arrived and an additional 143 samples were added to the archive. There are now 1300 tissues samples, representing samples from all collection years and preservation types, cataloged, and archived. DNA extractions from these archived tissues continues and 185 of the 334 target samples are extracted. DNA quality and quantity continue to be measured. The SOPs for tissue archiving, DNA extraction and DNA viability testing are drafted and under review.

Additional Accomplishments

- Permanent positions with SMARC and Uvalde were opened for application. All positions continue to progress through HR.
- The hallway and genetics lab in the EARP building were painted after the wall was completed. While a few items need to be purchased, the genetics lab is fully functional.
- The EARP staff showcased the Refugia as part of a larger SMARC tour given to attendees of the Habitat Conservation Plan Coalition Conference.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

- On October 6, EARP research staff met with Kristy Kollaus Smith to discuss the status of planned 2023 research projects.
- On October 26, USFWS Region 2 Regional Director Amy Lueders and Regina Santos-Aviles the Regional District Director for Congressman Tony Gonzales toured the UNFH, including the refugia (Figure 3).
- On October 26, the EARP was showcased during the National Habitat Conservation Plan Coalition Conference (Figures 4-6).
- EARP staff met weekly to discuss ongoing research, collections, and husbandry needs.

Summary of October Activities

- Comal Springs fountain darters were collected from Landa Lake and the Old Channel.
- San Marcos fountain darters were collected from the Middle San Marcos
- Pecks cave amphipods were collected by Randy Gibson and donated to the EARP
- Fountain darters in quarantine were incorporated into the refugia population at UNFH
- Texas blind salamanders in quarantine were p-Chip tagged and incorporated into the refugia population at SMARC
- Remote monitoring equipment was installed on the invert system at SMARC
- Chytrid fungus (Bd) treatment trials concluded
- CSRB Density and Biofilm propagation tubes were inventoried
- p-Chipped tagged salamanders were scanned with an expert and novice reader
- DNA is successfully being isolated for historical fountain darter tissue samples
- The San Marcos salamander and Comal Springs riffle beetle handbooks are under revision and internal review

Tables and Figures

Table 1. October's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility housed. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	52	NT	0	52	0	21	11	8	418	476
Fountain darter: Comal	165	NT	0	165	196	142	1	0	265	157
Comal Springs riffle beetle	NT	NT	--	--	0	0	13	0	36	48
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	0	2	10
Peck's cave amphipod	110	NT	0	110	0	0	0	0	131	251
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	0	0	1	0	173	66
San Marcos salamander	NT	NT	--	--	0	0	6	1	102	175
Comal Springs salamander	NT	NT	--	--	0	0	2	1	110	94
Texas wild rice plants	NT	NT	--	--	0	0	0	0	205	209

Figure 1. Visiting USFWS Science Applications Directorate Fellow assisting with Chtryid Fungus (Bd) treatment trials.

Figure 2. Kevin Rubio (SCA) scanning salamanders tagged with p-Chips and serving as a novice reader.

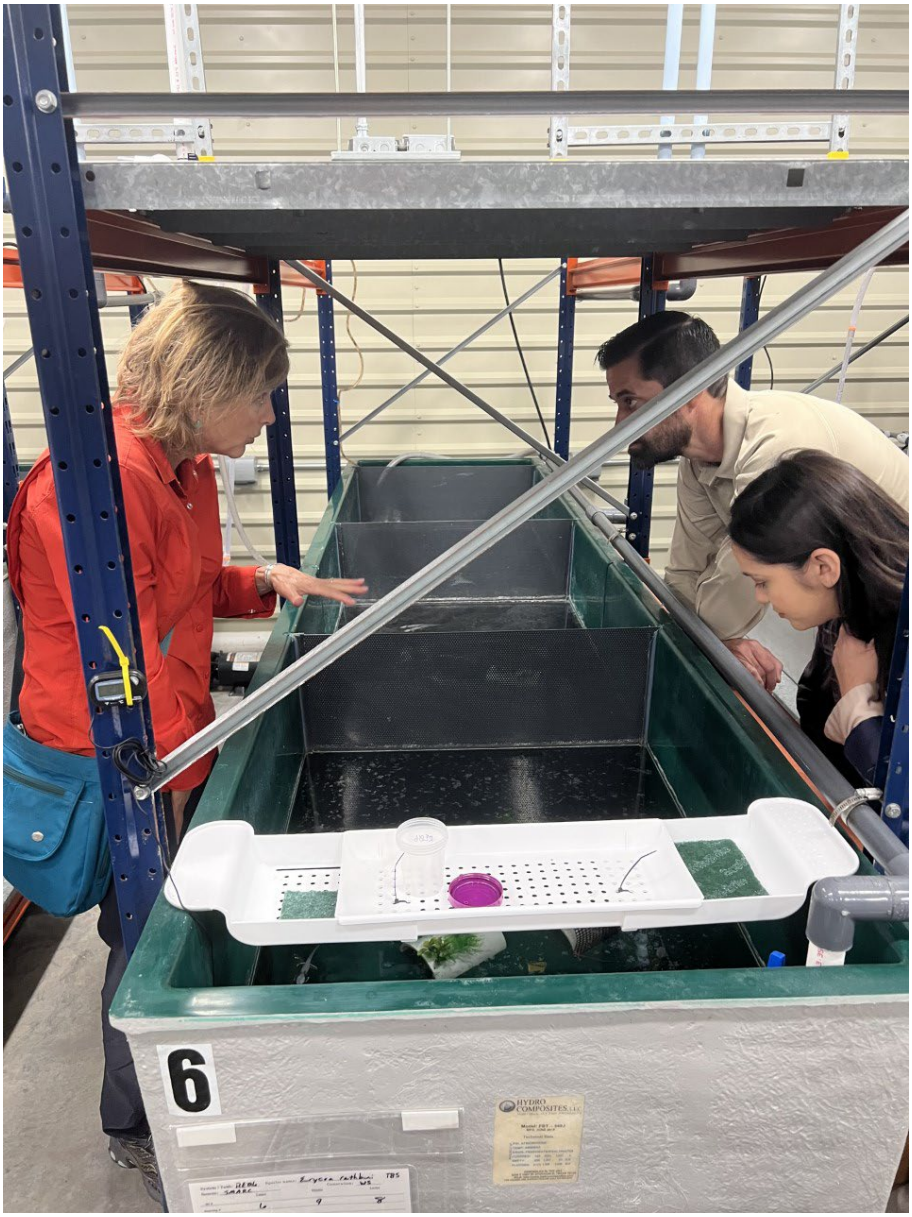


Figure 3. Adam Daw (USFWS) discussing the Texas blind salamanders with Amy Lueders (USFWS Region 2 Regional Director) and Regina Santos-Aviles (Regional District Director for Congressman Tony Gonzales)

Figure 4. Attendees of the Habitat Conservation Plan Coalition Conference tour the San Marcos Aquatic Resources Center and Edwards Aquifer Refugia Program.

Figure 5. Braden West discussing the Edwards Aquifer Refugia Program Husbandry and Collection program with the attendees of the Habitat Conservation Plan Coalition Conference.

Figure 6. Dr. Katie Bockrath discusses the research activities of the Edwards Aquifer Refugia Program with attendees of the Habitat Conservation Plan Coalition Conference.

November 2022 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dr. Katie Bockrath and Adam Daw

With contributions from

Desirée Moore, Ben Thomas, and Braden West

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staffing

Hiring actions to bring on permanent positions at both SMARC and UNFH continue. The status of each position is listed below.

Location	Position	Status
San Marcos	Research Biologist (GS 9/11)	Selection has been made.
San Marcos	Biologist (GS 7/9)	Braden West accepted the offer and is official in this new position.
San Marcos	Biological Technician (GS 5/7)	Interviews are scheduled for the week of Dec. 19
San Marcos	Biological Technician (GS 5)	Waiting for HR to post the position.
Uvalde	Biologist (GS 7/9)	Dominique Alvear accepted offer and will start Dec. 18th.
Uvalde	Biological Technician (GS 5/7)	Ben Thomas accepted offer and will start Dec. 18.
Uvalde	Biological Technician (GS 5)	Interviews finished and candidates ranked.

Species Collection

November 7, SMARC EARP staff set traps for Texas blind salamanders at Johnson well. Traps were checked three times a week for two weeks and retrieved on November 21. Four Texas blind salamanders were captured, with two retained for the refugia at the SMARC.

November 30, Ben Thomas and Emily Nielsen collected 67 fountain darters from the Comal River, near Spring Island (Figure 1). They also transported back 37 Peck's Cave amphipods collected by Braden West, Eleanor Krellenstein, and Daniela Cortez from Spring Island to the UNFH.

Husbandry

Uvalde

Thomas and Nielsen transferred Texas wild rice from rice tank 14 (R14) to rice tank 13 (R13). During the transfer, they potted F1 rice tillers from the transferred rice into new mesh fabric plots to evaluate their potential use versus the currently utilized solid plastic pots. Thomas disassembled the research rack in the refugia that was used for the San Marcos spawning experiment. Nielsen assisted Thomas with moving the empty aquaria from the rack to the mezzanine above the refugia. The plan is to use the newly open space as a live food production area. Thomas and Nielsen conducted organism inventories of refugia tanks. Thomas and Nielsen swabbed salamanders in quarantine, which will be sent to the San Diego Zoo, San Diego, CA for Bd/Bsal check. Adam Daw constructed new brine shrimp hatching containers and new equipment drying racks for the UNFH and the SMARC. Daw finished construction of experimental tubes that will be used for a Pecks Cave Amphipod (PCA) movement study (Figure 2).

SMARC

West completed construction of the new invertebrate sorting area within the SMARC refugia room. The new work area includes two benches, a sink, and expanded overhead storage. The entire area is protected by a chemical and water-resistant curtain to maintain a light-free environment while conducting inventory of light-sensitive species. Increased space in the work area will also allow staff to more efficiently train new staff on working with EARP invertebrates. West dismantled the final remaining wooden racks in the quarantine space at the SMARC. In place of the wooden racks, a new multi-tank quarantine system is being constructed. In addition to assisting with daily husbandry tasks Eleanor Krellenstein updated facility SDS sheets. Rachelle Jackson assisted with daily husbandry tasks and cleaned the AHAB unit used for salamander Bd treatment trials.

Animal Health

The health report on the wild Comal River and San Marcos River fountain darters conducted by the Southwestern Native Aquatic Resources Recovery Center's Southwestern Fish Health Unit (Dexter, NM) was completed. The fish from the Comal River tested negative for Small Mouth Bass Virus, a first in several years. Ten Comal River darters were examined for parasites, nine had *Centrocestus fromosanus* and four had monogenean parasites. The San Marcos River Fountain darters were negative for tested

viruses. Ten fish were examined for parasites, four had *Centrocestus formosanus* and one was observed with myxosporidean parasites on the gills.

Task 2 Research

Comal Springs Riffle Beetle Pupation

Phase II (Density) trails are complete. Phase III (Biofilm) flowthrough tubes were inventoried. Water flow and water temperature of the tubes was checked weekly. There are not significant differences in pupation rates among density treatments, suggesting that at least 40 larvae can be housed in a single flowthrough tube. A draft report was submitted to the EAA for review.

Comal Springs Riffle Beetle Exposure to Staphylococcus

The report was submitted for EAA review.

Captive Propagation for San Marcos Salamanders Handbook

The handbook was completed and submitted for EAA review.

Comal Springs Riffle Beetle Population Genetics

Interim report was submitted for EAA review.

*Comal Springs Riffle Beetle (*Heterelmis comalensis*) Refugia Manual*

Contributing authors continued working on the handbook. A draft was submitted to the EAA for review.

Batrachochytrium dendrobatidis *(Bd) Infection Treatment in Aquatic Salamanders*

All trials have concluded. There were no mortalities associated with the treatment. The DNA from the swabs was extracted. A draft report was submitted to the EAA.

P-Chip Tag Effects on Eurycea *spp.*

All salamanders were scanned weekly. Celest Palmquist (SCA) served as the novice scanner and successfully scanned all tagged salamanders (Figure 3). A draft report was submitted to the EAA for review.

*Catalog of Historical Fountain Darter (*Etheostoma fonticola*) Tissue and DNA Viability Testing*

The viability testing of historical fountain darter DNA was completed. Tissue with ethanol preservation and climate-controlled storage resulted in the highest viability testing (87.74%), while tissues preserved in formalin and stored in harsh conditions are completely unviable (0%). As long as samples are stored in climate-controlled conditions, DNA remains viable. A very small proportion of outside stored tissue samples are suitable for future genetics research (Table 2). A draft report has been submitted to the EAA for review.

Additional Accomplishments

- Agreements with external research partners progressed. All partnered documents were submitted to GrantSolutions.
- EARP research team met with external partners to finalize 2023 research objectives and budgets.
- Dr. Katie Bockrath and Adam Daw held interviews for the new permanent staff positions with the EARP.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

- All EARP staff contributed to the monthly report.
- 2023 research proposals were submitted to the EAA for review
- 2022 draft research reports were submitted to the EAA for review
- Revised 2023 Work Plan was submitted to the EAA for review

Task 6 Meetings and Presentations

- The EARP team attended the EAA Contractor Appreciation event. Thank you to the EAA for hosting.
- The EARP team met weekly to discuss ongoing research, collections, and refugia needs.

Summary of November Activities

- Fountain darter and Peck's cave amphipod collections at Spring Island
- Texas bind salamanders collected at Johnson's Well
- Texas wild rice was repotted at Uvalde
- A new invertebrate inventory station was constructed at San Marcos Aquatic Resources Center
- Texas blind salamanders in quarantine were swabbed for Bd testing.
- Fountain darters collected from the Comal River tested negative for Large Mouth Bass Virus.
- Research projects are wrapping up.
- Draft research reports were submitted to the EAA for review.
- 2023 research proposals were submitted to the EAA for review.
- Revised 2023 work plan was submitted to the EAA for review.

Tables and Figures

Table 1. November's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for November 2022. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	--	--	0	0	5	10	413	466
Fountain darter: Comal	NT	67	11	78	104	24	1	3	368	178
Comal Springs riffle beetle	NT	NT	--	--	0	0	NA	NA	36	48
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	NA	2	10
Peck's cave amphipod	NT	37	0	37	30	0	22	41	139	210
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	2	NT	2	4	0	0	0	0	173	66
San Marcos salamander	NT	NT	--	--	0	0	4	8	98	167
Comal Springs salamander	NT	NT	--	--	0	0	0	0	110	94
Texas wild rice plants	NT	NT	--	--	0	0	0	4	205	205

***Table 2. Proportion of successful PCR amplifications for DNA extracted from tissues in different storage conditions. Tissues were preserved in either ethanol or formalin and stored either outside* (not climate-controlled) *or inside* (climate-controlled).**

Preservation Solution and Storage Location	Percent Successful PCR Amplification
Ethanol, outside	11.19
Formalin, outside	0
Formalin, inside	37.5
Ethanol, inside	87.74

Figure 1. Emily Nielsen collecting fountain darters at Spring Island (phot crdit: Ben Thomas/USFWS)

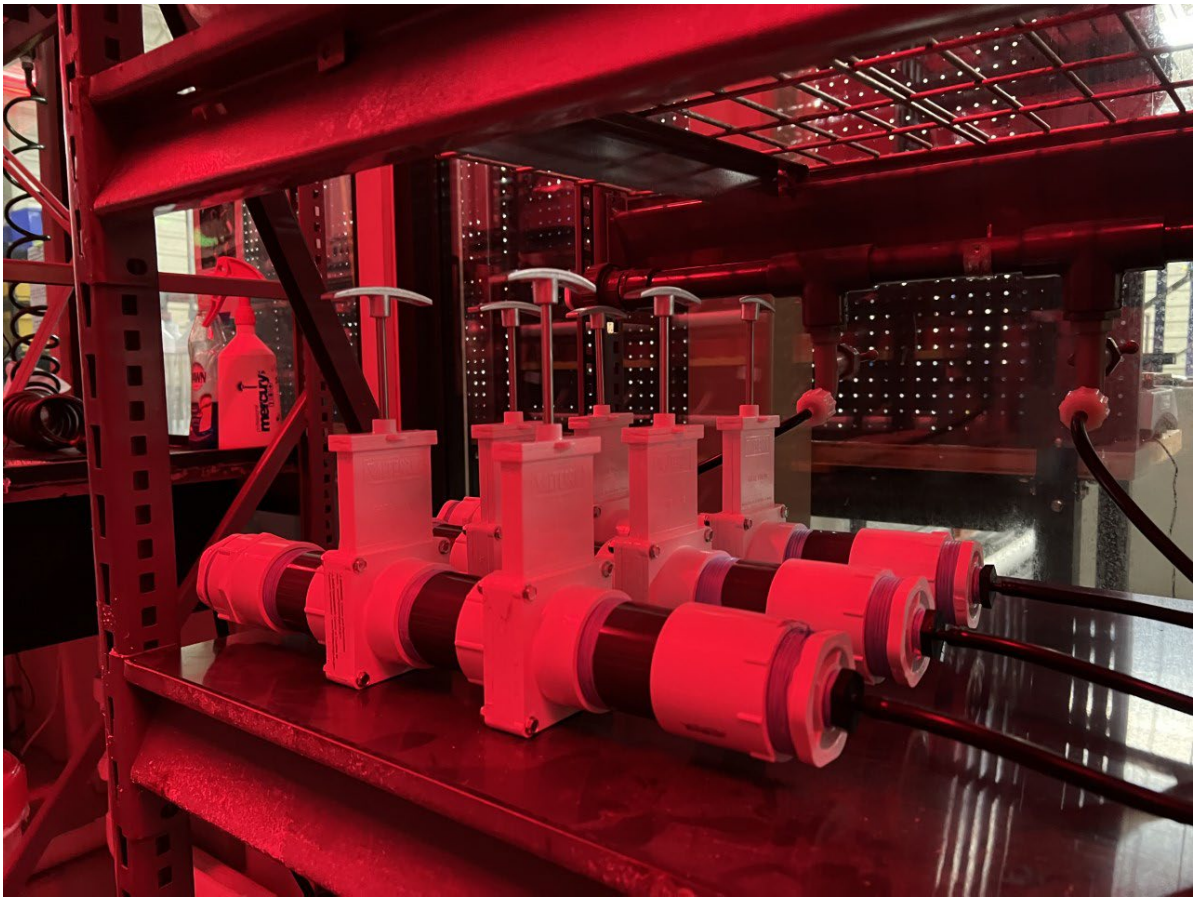


Figure 2. Experimental tubes for a Pecks Cave Amphipod (PCA) movement and offspring exclusion study (photo credit: Adam Daw/USFWS)

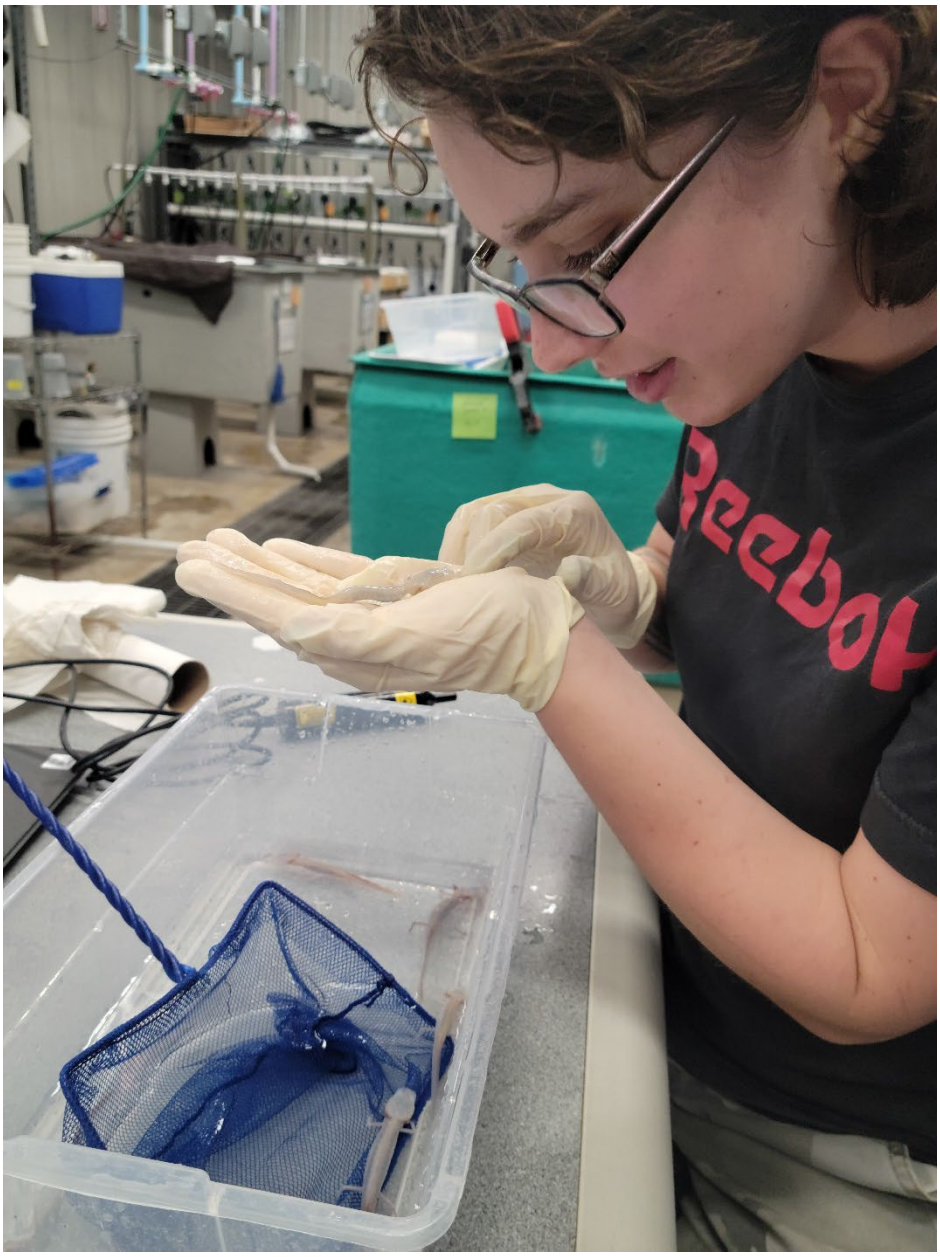


Figure 3. Celeste Palmquist scanning p-Chip tagged Texas blind salamanders (photo credit: Desiree Moore/USFWS)

December 2022 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dr. Katie Bockrath and Adam Daw

With contributions from

Desirée Moore, Ben Thomas, and Braden West

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staffing

Hiring actions continue to progress to bring on permanent positions at both SMARC and UNFH. Currently, 5 of the 7 positions have been filled. The status of each position is listed below.

Location	Position	Status
San Marcos	Research Biologist (GS 9/11)	Desirée Moore has filled this position.
San Marcos	Biologist (GS 7/9)	Braden West has filled this position.
San Marcos	Biological Technician (GS 5/7)	Shawn Moore has accepted an offer for this position.
San Marcos	Biological Technician (GS 5)	Waiting for HR to send the announcement for approval.
Uvalde	Biologist (GS 7/9)	Dominique Alvear has filled this position.
Uvalde	Biological Technician (GS 5/7)	Ben Thomas has filled this position.
Uvalde	Biological Technician (GS 5)	No selection was made. The position will be readvertised.

At the UNFH, Ben Thomas started in his new position as a Biological Technician (GS 5/7), he was previously a term GS5 with the EARP at the UNFH. Dominique Alvear started as a Biologist (GS 7/9) at the UNFH. She recently received her master's degree from Texas A&M University in Genetics/Genomics where she worked on infectious disease, and livestock breeding. During her undergraduate at the University of Texas Rio Grande Valley she worked on the phylogenetics of spring snails in Texas.

At the SMARC, Braden West started his new position as a Biologist (GS 7/9), he was previously a term GS5 with the EARP at the SMARC. Shawn Moore accepted the Biological Technician (GS 5/7) position. Shawn was an SCA intern that worked on the 2022 Fountain Darter Tissue Archive and DNA Viability study.

Species Collection

No species collections occurred in December

Husbandry

Uvalde

Adam Daw and Thomas started training Alvear on EARP husbandry SOP's. Thomas and Alvear continued inventorying organisms as part of our bi-annual inventory.

SMARC

West authored three SOP's (Standard Operating Procedure) covering animal food sources in the EARP. A newly written SOP refined the operation of captive *Daphnia* cultures used for live feed by the EARP. Braden, Eleanor Krellenstein, and Richelle Jackson also revised the protocol for *Artemia* culture used for live feed. Braden also worked with Eleanor and Richelle to refine the procedure for intake and management of blackworms used for live feed. Krellenstein and Jackson assisted West with the bi-annual refugia organism survey.

Animal Health

Skin swabs of salamanders in quarantine were sent to the San Diego Zoo for Bd/Bsal testing.

Task 2 Research

Comal Springs Riffle Beetle Pupation

The final report was submitted for EAA review

Comal Springs Riffle Beetle Exposure to Staphylococcus

The report was submitted for EAA review.

Captive Propagation for San Marcos Salamanders Handbook

The handbook was completed and submitted for EAA review.

Comal Springs Riffle Beetle Population Genetics

DNA extractions were initiated to test the extraction protocol on F1 larval and F2 adult riffle beetles. Because of the impermeable chitin exoskeleton, the beetles need to be smashed while in the DNA extraction buffer for the extraction enzymes to gain access to riffle beetle tissue (Figure 1). The interim report was submitted for EAA review.

*Comal Springs Riffle Beetle (*Heterelmis comalensis*) Refugia Manual*

The handbook was submitted to the EAA for review.

Batrachochytrium dendrobatidis *(Bd) Infection Treatment in Aquatic Salamanders*

The data from qPCR testing on the swabs collected during the treatment trials was summarized. At the current dosage, there is not observable effect, negative or positive, on the change of Bd status in San Marcos salamanders. The report was submitted to the EAA for review.

P-Chip Tag Effects on **Eurycea** *spp.*

All salamanders were scanned weekly. David Britton was the novice reader for December and successfully scanned all tagged salamanders (Figure 2). The snout to ventral length was measured for all tagged salamanders (Figure 3). The report was submitted to the EAA for review. A manuscript was drafted for peer-review publication.

*Catalog of Historical Fountain Darter (***Etheostoma fonticola***) Tissue and DNA Viability Testing*

The report was submitted to the EAA for review.

Additional Accomplishments

- Agreements with external research partners progressed. All FWS documents were submitted to GrantSolutions.
- Dr. Katie Bockrath and Adam Daw held interviews for the new permanent staff positions with the EARP.
- Desiree Moore and Braden West gathered Texas blind salamander tail clip samples to share with Dr. Chris Nice (Texas State University) and Pete Diaz (USFWS Texas Fish and Wildlife Conservation Office) for a genetic assessment study.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

- All EARP staff contributed to the monthly report.
- Revised 2023 research proposals were submitted to the EAA for review

Task 6 Meetings and Presentations

- The EARP team met weekly to discuss ongoing research, collections, and refugia needs.
- Dr. Katie Bockrath, Desirée Moore, and Adam Daw met with Dr. Chad Furl and Kristy Smith to discuss 2023 research proposals and 2022 research results.
- Shawn Moore gave a presentation to SMARC staff and SCA interns on the fountain darter tissue archive and DNA viability research.
- EARP Staff virtually attended the EAHCP Year-end Joint Stakeholders Meeting.
- EAA staff visited the UNFH for a tour of the site (Figure 4)

Summary of December Activities

- Updated research reports were submitted to the EAA for review.
- Revised 2023 research proposals were submitted to the EAA for review.
- 2022 research studies were concluded
- Updated 2022 research reports were submitted to the EAA for review
- Texas blind salamander tail clips were shared with Dr. Chris Nice for a future genetic assessment study
- EARP staff attended EAA quarterly meeting and the EAHCP Year-end meeting

Tables and Figures

Table 1. December's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for December 2022. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	--	--	0	0	11	11	402	454
Fountain darter: Comal	NT	NT	--	--	0	0	55	1	313	177
Comal Springs riffle beetle	NT	NT	--	--	0	0	NA	NA	36	48
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	NA	2	10
Peck's cave amphipod	NT	NT	--	--	0	22	NA	9	139	232
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	0	0	1	1	172	65
San Marcos salamander	NT	NT	--	--	0	0	2	3	96	167
Comal Springs salamander	NT	NT	--	--	0	0	0	1	110	93
Texas wild rice plants	NT	NT	--	--	0	0	0	0	205	205

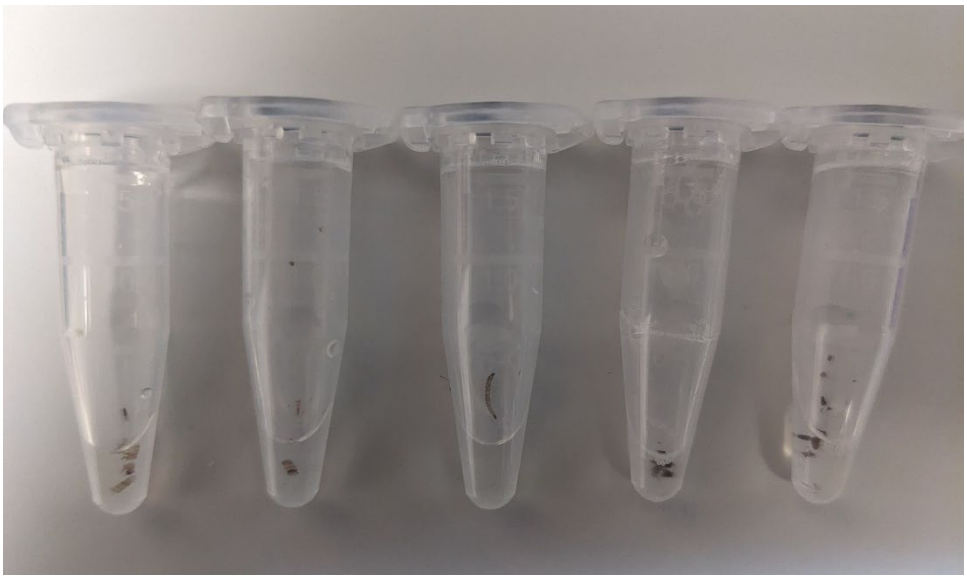


Figure 1. Comal Springs riffle beetle F1 adult and F2 larvae DNA extraction test. The center vial is a larva, which remained intact after attempting to break it up. The other vials are adult beetles who were successfully crushed. Accessing the body cavity via crushing results in more successful DNA extractions.

Figure 2. Dr. David Britton scans p-chip tagged salamanders.

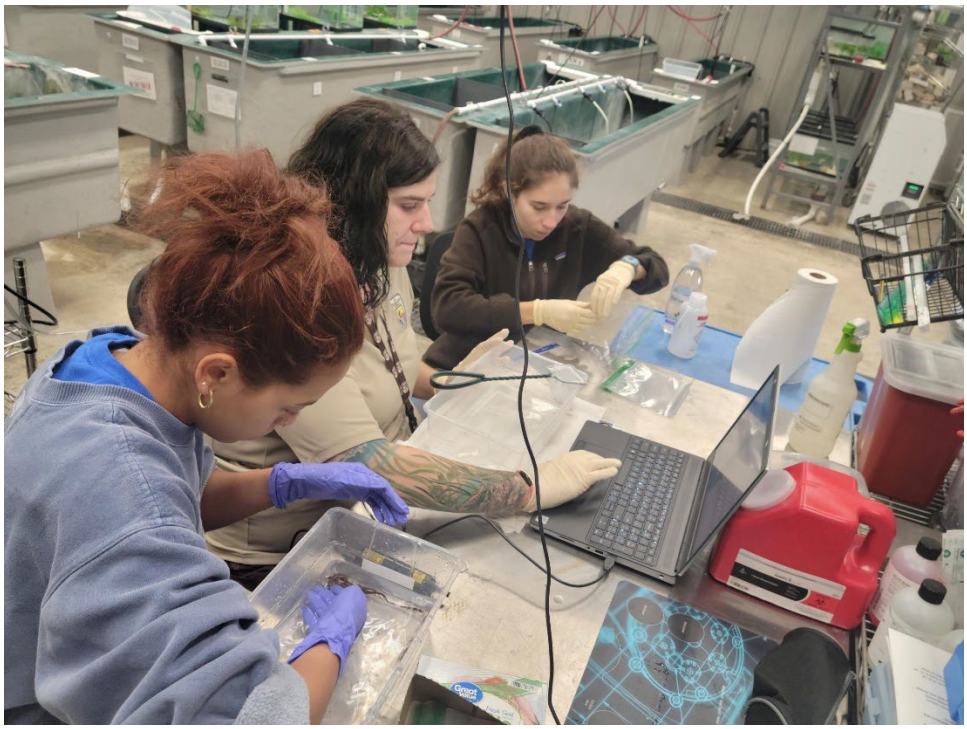


Figure 3. Richelle Jackson (right), Desirée Moore (center), and Eleanor Krellenstein complete final p-chip scans and measure snout to ventral lengths on all aquatic salamanders in the p-chip study.

Figure 4. EAA staff touring the UNFH EARP facility. Shown here is Scott Storment (left, green shirt), Damon Childs (back left), Olivia Ybarra (center, blue jacket), Dr. Chad Furl (back right), and Adam Daw (front right).

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/1049 November 11, 2023

Memorandum
To: Adam Daw, 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 23-02).

On October 26, 2022, Southwestern Fish Health Unit (SFHU) staff received 49 fountain darters (*Etheostoma fonticola*) collected 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 at latitude 29.8764° and longitude -97.9320° in Hayes County, Texas, and the river water temperature was 23°C.

Assays and examinations for the sampled fish included virology and parasitology. Viral screening of 49 fish included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected using standard cell lines. 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 (2020 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 4 of 10 fish examined. In addition to *C. formosanus*, one fish was observed with myxosporidean parasites associated with the gills. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo. No viruses were detected by cell culture.

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 23-02 for all follow up correspondence.

cc: Huseyin Kucuktas, Southwestern Fish Health Unit/ Southwestern Native ARRC
David Britton, San Marcos Aquatic Resources Center

FOD Parasite Data Sheet - Form P-03

Case History No. 23-02

Date examined: 10-26-2022 Date Collected: 10-19-22

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)	268	305	371	317	306	194	259	221	252	157
Total Length (mm)	23	26	27	26	25	23	25	22	25	20

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

Mature (left gills only)	L	2, 1, 1, 0	0	0	0	0	0	0	0	0	0
Immature (left gills only)	L	0	0	2, 8, 6, 7	2, 3, 4, 2	0	0	0	0	0	0, 1, 6, 1

Monogenea	L	0	0	0	0	0	0	0	0	0	0
Myxobolus sp.	L	0, 2, 16, 3	0	0	0	0	0	0	0	0	0
Other	L	0	0	0	0	0	0	0	0	0	0

Examiner signature

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/1050 November 30, 2022

Memorandum

To: Adam Daw, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the Comal River, Texas (Case Number 23-03).

On October 26, 2022, Southwestern Fish Health Unit (SFHU) staff received 60 fountain darters (*Etheostoma fonticola*) collected from the Comal River (GNIS ID: 1372140). These fish were collected using dip net by staff from the San Marcos ARC and shipped live to the SFHU laboratory. The location was recorded at latitude 29.7142° and longitude -98.1358° Comal County, Texas, and river water temperature at the time of collection was 23°C.

Assays and examinations for the sampled fish included virology and parasitology. One fish of the 60 fish received was dead on arrival and was therefore not included for testing. Viral screening of 59 fish included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected in the standard cell lines used. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC for the Edwards Aquifer Refugia Program. Screening for *Centrocestus formosanus* was conducted by examination of all left side gills from 10 fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2020 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 9 of 10 fish examined and monogenean parasites were observed in 4 of 10 fish examined. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo. No viruses were detected by cell culture.

If you have any questions about test methodology and 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 23-03 for all follow up correspondence.

cc: Huseyin Kucuktas, Southwestern Fish Health Unit
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 23-03

Date examined: 10-26-2022 Date Collected: 10-25-22

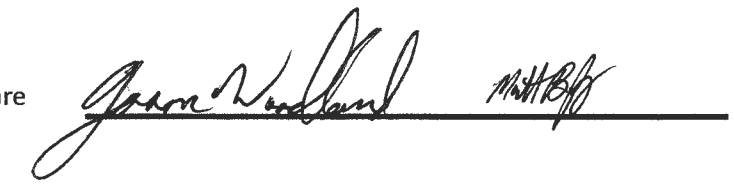
Collection site: Comal River

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	275	380	295	186	186	202	254	190	161	218
Total Length (mm)	32	35	33	30	29	30	32	28	27	30

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

Mature (left gills only)	L	1, 1, 0, 2	0	0, 1, 4, 0	0, 1, 2, 0	0	2, 0, 0, 0	6, 2, 2, 1	0	0, 0, 1, 0	0
Immature (left gills only)	L	0	0, 3, 2, 3	0, 0, 0, 1	0	0	0	0	1, 2, 0, 1	1, 2, 0, 1	0, 2, 0, 1

Monogenea	L	0, 3, 1, 1	0	0	0	0	0	1, 0, 1, 0	1, 0, 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 

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

March 22, 2022

In Reply Refer To:
FWS/R2/FR-SFHU/1016
Memorandum

To: Adam Daw

From: Trista Becker

Subject: Texas blind salamander diagnostic case 22-10

On Feb 1, 2022 three Texas blind salamanders were submitted as a diagnostic case to the Southwestern Fish Health Unit. Two animals were submitted live and one was a mortality previously saved in ethanol (head removed for genetics). The two animals were observed as lethargic and had large visible swellings/growths externally on the backs (1-2 lesions per animal, see Fig. 1a). They were humanely euthanized in buffered MS222 upon arrival and a stained smear examined from the gills and one of the lesions. Fatty deposits were observed along with external bacterial flora in the wet mounts. Some fat deposits could be visualized engulfed by macrophages on Dif Quick stained smears (Fig. 1b).

Figure 1a: Back end of one live Texas blind salamander submitted for exam showing large raised lesions on the caudal dorsum area of the tail.

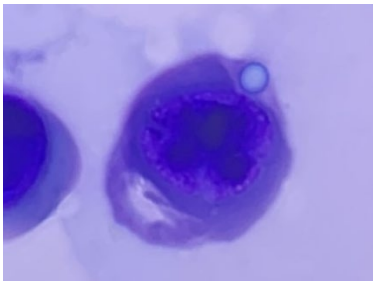


Figure 1b: 100x oil immersion view of a Dif Quick stained impression smear from one raised lesion showing a macrophage with engulfed fat deposit.

All three animals were submitted to Washington State University for histopathologic analysis. The final report determined all three animals had severe, chronic steatitis which appeared to be sterile (likely due

to a dietary cause, not pathogen-associated). Two of the salamanders also had signs of chytrid disease on the skin of the toes with probable intralesional fungal thalli of *Batrachychytrium dendrobatidis.*

A prescription for itrafungol will be provided to treat the chytrid lesions by removing the fungus from the population as a potential confounding morbidity factor. Since the steatitis was not identified as a pathogen-related issue and the chronicity signs indicate it has been ongoing, a thorough diet analysis is warranted if the animals have been in captivity for long-term. Recommended areas to examine are whether excessive amount of unsaturated fats are being fed, the potential for rancidity of fatty feeds (examine expiration dates and feed storage methods), and antioxidant levels such as vitamin E. Soaking feeds in 100-400 iu/kg body weight vitamin E supplement prior to feeding could also be tried, as long as it does not impact palatability.

Please don't hesitate to contact us for follow ups or with any new issues that may arise.

Sincerely,

Dr. Trista Becker, MS, DVM, CertAqVet

CC: David Britton

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/1017 April 06, 2022

Memorandum
To: Adam Daw, San Marcos Aquatic Resource Center

From: Huseyin Kucuktas, 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 22-35).

On March 24, 2022, Southwestern Fish Health Unit (SFHU) staff received 12 fountain darters (*Etheostoma fonticola*) collected from the San Marcos River, (Spring Lake Location; 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 at latitude 29.89414° and longitude -97.92987° in Hays County, Texas, and the river water temperature was 23°C.

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:
Neither adult, nor immature *Centrocestus formosanus* was observed in any of 10 fish examined. However, Monogenean parasites were observed on single or multiple gill arches from 8/10 fish. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo.

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 22-35 for all follow up correspondence.

cc: Trista Becker, Southwestern Fish Health Unit/ Southwestern Native ARRC
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 22-35

Date examined: 3/24/2022 Date Collected: 3/22/2022

Collection site: San Marcos River, Spring Lake Location

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	214	79	80	165	257	138	126	174	94	81
Total Length (mm)	29	21	23	29	33	26	26	29	16	22

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	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Monogenea	L	**1,2,2,1**	**2**,0,0,0	0,0,0,0	**1**,0,0,0	0,**1,2**,0	0,0,0,0	**1**,0,0,**1**	**1**,0,0,**1**	0,0,0,**1**	0,**1**,0,**1**
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

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/1017 April 06, 2022

Memorandum
To: Adam Daw, San Marcos Aquatic Resource Center

From: Huseyin Kucuktas, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the Comal River, Texas (Case Number 22-36).

On March 24, 2022, Southwestern Fish Health Unit (SFHU) staff received 13 fountain darters (*Etheostoma fonticola*) collected 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 fountain darters at latitude 29.71436° and longitude -98.13593° in Comal County, Texas, and the river water temperature was 23°C.

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:
Neither adult, nor immature *Centrocestus formosanus* was observed in any of 10 fish examined. However, Monogenean parasites were observed on single or multiple gill arches from 3/10 fish. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo.

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 22-36 for all follow up correspondence.

cc: Trista Becker, Southwestern Fish Health Unit/ Southwestern Native ARRC
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 22-36

Date examined: 3/24/2022 Date Collected: 3/22/2022

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)	162	284	95	95	90	65	92	52	61	38
Total Length (mm)	21	25	19	18	17	16	18	16	16	13

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	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Monogenea	L	0,**3**,**2**,0	0,0,0,0	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,**1**,**1**,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

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/1040 August 29, 2022

Memorandum
To: Adam Daw, 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 22-68).

On August 23, 2022, Southwestern Fish Health Unit (SFHU) staff received 10 fountain darters (*Etheostoma fonticola*) collected from the Comal River (Landa Lake location; 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 fountain darters at latitude 29.7145° and longitude -98.1357° in Comal County, Texas, and the river water temperature was 24°C.

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 (2020 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 2 of 10 fish examined. Monogenetic trematodes were also observed on the gills from 4 of 10 fish examined. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo.

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 22-68 for all follow up correspondence.

cc: Trista Becker, Southwestern Fish Health Unit/ Southwestern Native ARRC
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 22-68

Date examined: 8/23/22 Date Collected: 8-11-22

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)	136	183	239	167	246	189	255	157	245	225
Total Length (mm)	26	27	25	28	30	29	30	26	30	31

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

Examiner signature MB JAK

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/1041 August 29, 2022

Memorandum
To: Adam Daw, 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 22-69).

On August 23, 2022, Southwestern Fish Health Unit (SFHU) staff received 10 fountain darters (*Etheostoma fonticola*) collected from the San Marcos River, (Spring Lake Location; 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 at latitude 29.8941° and longitude -97.9299° in Hays County, Texas, and the river water temperature was 23°C.

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 (2020 edition) and standard SFHU protocols.

Results:
Mature *Centrocestus formosanus* was observed in 1 of 10 fish examined. Monogenetic trematodes were also observed on gills from 6 of 10 fish examined. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo.

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 22-69 for all follow up correspondence.

cc: Trista Becker, Southwestern Fish Health Unit/ Southwestern Native ARRC
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 22-69

Date examined: 8/23/22 Date Collected: 8-17-22

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)	206	192	243	235	170	116	172	182	68	52
Total Length (mm)	28	28	30	31	25	20	21	21	20	19

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

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

Monogenea	L	0, 2, 2, 0	0, 0, 1, 1	0, 2, 0, 0	0, 2, 0, 0	0	0	0	1, 1, 0, 2	0, 2, 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

Revised on 9/20/2017

San Diego Zoo Wildlife Alliance

Molecular Diagnostics Report
Amphibian Disease Laboratory
Beckman Center for Conservation Research

Set ID: 6667 Wednesday, March 30, 2022

UVALDE NATIONAL FISH HATCHERY

Amphibian ID	Common Name	Species	Chytrid PCR [1]	Bsal PCR [2]	Date Collected
21S023	Texas Blind Salamander	Eurycea rathbuni	Equivocal	Negative	11/2/2021
21S024	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	11/2/2021
21S025	San Marcos Salamander	Eurycea nana	Negative	Negative	11/2/2021
21S026	San Marcos Salamander	Eurycea nana	Negative	Negative	11/2/2021
21S027	San Marcos Salamander	Eurycea nana	Positive	Negative	11/2/2021
21U006	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	10/27/2021
21U007	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	10/27/2021
21U008	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	10/27/2021
21U009	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	10/27/2021
21U010	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	10/27/2021
21U011	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	10/27/2021
21U012	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	10/27/2021
22S028	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022
22S029	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022
22S030	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022
22S031	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022
22S032	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022

Positive chytrid skin swab indicate the presence of DNA from the amphibian chytrid fungus. Antifungal treatment with follow-up PCR is suggested before animals are intorduced into the zoo collection. Occasionally, multiple treatment cycles are required to clear animals from infection. Equivocla results may indicate the presence of small amounts of fungal DNA. Re-testing of animals and follow up witht he laboratory are recommended.

[1] Taqman PCR for Amphibian Chytrid Fungus (Batrachochytrium dendrobatides) [2] Taqman PCR for Chytrid Fungus (Batrachochytrium salamandrivorans)

UVALDE NATIONAL FISH HATCHERY

Amphibian ID	Common Name	Species	Chytrid PCR [1]	Bsal PCR [2]	Date Collected
22S033	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022
22S034	Texas Blind Salamander	Eurycea rathbuni	Positive	Negative	2/22/2022
22S035	Texas Blind Salamander	Eurycea rathbuni	Equivocal	Negative	2/22/2022
22S036	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022
22S037	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022
22U001	Comal Springs Salamander	Eurycea tridentifera	Equivocal	Negative	2/17/2022
22U002	Comal Springs Salamander	Eurycea tridentifera	Negative	Negative	2/17/2022
22U003	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	2/17/2022
HMP.T2.T19	San Marcos Salamander	Eurycea nana	Negative	Negative	11/12/2021
REA.T3.T20	San Marcos Salamander	Eurycea nana	Positive	Negative	1/23/2022

Positive chytrid skin swab indicate the presence of DNA from the amphibian chytrid fungus. Antifungal treatment with follow-up PCR is suggested before animals are intorduced into the zoo collection. Occasionally, multiple treatment cycles are required to clear animals from infection. Equivocla results may indicate the presence of small amounts of fungal DNA. Re-testing of animals and follow up witht he laboratory are recommended.

[1] Taqman PCR for Amphibian Chytrid Fungus (Batrachochytrium dendrobatides) [2] Taqman PCR for Chytrid Fungus (Batrachochytrium salamandrivorans)

Molecular Diagnostics Report
Amphibian Disease Laboratory
Beckman Center for Conservation Research

Set ID: 6978 Monday, August 29, 2022

SAN MARCOS AQUATIC RESOURCES CENTER

Amphibian ID	Common Name	Species	Chytrid PCR [1]	Bsal PCR [2]	Date Collected
1000A	Texas blind salamander	Eurycea rathbuni	Positive	Negative	3/20/2022
15A	Texas blind salamander	Eurycea rathbuni	Positive	Negative	3/20/2022
22S038	Texas blind salamander	Eurycea rathbuni	Positive	Negative	7/14/2022
22S039	Texas blind salamander	Eurycea rathbuni	Positive	Negative	7/14/2022
22S040	Texas blind salamander	Eurycea rathbuni	Positive	Negative	7/14/2022
22S041	Texas blind salamander	Eurycea rathbuni	Positive	Negative	7/14/2022
22S042	Texas blind salamander	Eurycea rathbuni	Negative	Negative	7/14/2022
22S043	Texas blind salamander	Eurycea rathbuni	Negative	Negative	7/14/2022
5A	Texas blind salamander	Eurycea rathbuni	Negative	Negative	3/20/2022
998A	Texas blind salamander	Eurycea rathbuni	Positive	Negative	3/20/2022
999A	Texas blind salamander	Eurycea rathbuni	Negative	Negative	3/20/2022

Positive chytrid skin swab indicate the presence of DNA from the amphibian chytrid fungus. Antifungal treatment with follow-up PCR is suggested before animals are intorduced into the zoo collection. Occasionally, multiple treatment cycles are required to clear animals from infection. Equivocla results may indicate the presence of small amounts of fungal DNA. Re-testing of animals and follow up witht he laboratory are recommended.

[1] Taqman PCR for Amphibian Chytrid Fungus (Batrachochytrium dendrobatides) [2] Taqman PCR for Chytrid Fungus (Batrachochytrium salamandrivorans)

San Diego Zoo Wildlife Alliance

Molecular Diagnostics Report
Amphibian Disease Laboratory
Beckman Center for Conservation Research

Set ID: 6667 Friday, March 11, 2022

UVALDE NATIONAL FISH HATCHERY

Amphibian ID	Common Name	Species	Chytrid PCR [1]	Bsal PCR [2]	Date Collected
21S023	Texas Blind Salamander	Eurycea rathbuni	Confirmation Required	Negative	11/2/2021
21S024	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	11/2/2021
21S025	San Marcos Salamander	Eurycea nana	Negative	Negative	11/2/2021
21S026	San Marcos Salamander	Eurycea nana	Confirmation Required	Negative	11/2/2021
21S027	San Marcos Salamander	Eurycea nana	Positive	Negative	11/2/2021
21U006	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	10/27/2021
21U007	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	10/27/2021
21U008	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	10/27/2021
21U009	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	10/27/2021
21U010	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	10/27/2021
21U011	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	10/27/2021
21U012	Comal Springs Salamander	Eurycea tridentifera	Confirmation Required	Negative	10/27/2021
22S028	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022
22S029	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022
22S030	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022
22S031	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022
22S032	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022

Positive chytrid skin swab indicate the presence of DNA from the amphibian chytrid fungus. Antifungal treatment with follow-up PCR is suggested before animals are intorduced into the zoo collection. Occasionally, multiple treatment cycles are required to clear animals from infection. Equivocla results may indicate the presence of small amounts of fungal DNA. Re-testing of animals and follow up witht he laboratory are recommended.

[1] Taqman PCR for Amphibian Chytrid Fungus (Batrachochytrium dendrobatides) [2] Taqman PCR for Chytrid Fungus (Batrachochytrium salamandrivorans)

UVALDE NATIONAL FISH HATCHERY

Amphibian ID	Common Name	Species	Chytrid PCR [1]	Bsal PCR [2]	Date Collected
22S033	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022
22S034	Texas Blind Salamander	Eurycea rathbuni	Positive	Negative	2/22/2022
22S035	Texas Blind Salamander	Eurycea rathbuni	Confirmation Required	Negative	2/22/2022
22S036	Texas Blind Salamander	Eurycea rathbuni	Negative	Negative	2/22/2022
22S037	Texas Blind Salamander	Eurycea rathbuni	Confirmation Required	Negative	2/22/2022
22U001	Comal Springs Salamander	Eurycea tridentifera	Confirmation Required	Negative	2/17/2022
22U002	Comal Springs Salamander	Eurycea tridentifera	Confirmation Required	Negative	2/17/2022
22U003	Comal Springs Salamander	Eurycea tridentifera	Positive	Negative	2/17/2022
HMP.T2.T19	San Marcos Salamander	Eurycea nana	Negative	Negative	11/12/2021
REA.T3.T20	San Marcos Salamander	Eurycea nana	Positive	Negative	1/23/2022

Positive chytrid skin swab indicate the presence of DNA from the amphibian chytrid fungus. Antifungal treatment with follow-up PCR is suggested before animals are intorduced into the zoo collection. Occasionally, multiple treatment cycles are required to clear animals from infection. Equivocla results may indicate the presence of small amounts of fungal DNA. Re-testing of animals and follow up witht he laboratory are recommended.

[1] Taqman PCR for Amphibian Chytrid Fungus (Batrachochytrium dendrobatides) [2] Taqman PCR for Chytrid Fungus (Batrachochytrium salamandrivorans)

San Diego Zoo Wildlife Alliance

Molecular Diagnostics Report
Amphibian Disease Laboratory
Beckman Center for Conservation Research

Set ID: 221045 01/03/23

USFWS San Marcos Aquatic Resources Center

Bd, Bsal [qPCR] multiplex Verified on: 01/03/23

Sample ID	Species		Sample	Collection Date	Test	Result
22U004	Brook salamander	skin swab	08/02/22	Bsal [qPCR]		not detected
22U004	Brook salamander	skin swab	08/02/22	Bd [qPCR]		not detected
22U005	Brook salamander	skin swab	08/02/22	Bsal [qPCR]		not detected
22U005	Brook salamander	skin swab	08/02/22	Bd [qPCR]		not detected
22U006	Brook salamander	skin swab	08/02/22	Bsal [qPCR]		not detected
22U006	Brook salamander	skin swab	08/02/22	Bd [qPCR]		positive
22U007	Brook salamander	skin swab	08/02/22	Bsal [qPCR]		not detected
22U007	Brook salamander	skin swab	08/02/22	Bd [qPCR]		confirmation required
22U008	Brook salamander	skin swab	08/02/22	Bsal [qPCR]		not detected
22U008	Brook salamander	skin swab	08/02/22	Bd [qPCR]		positive
22U009	Brook salamander	skin swab	08/02/22	Bsal [qPCR]		not detected
22U009	Brook salamander	skin swab	08/02/22	Bd [qPCR]		positive
22U010	Brook salamander	skin swab	08/02/22	Bsal [qPCR]		not detected
22U010	Brook salamander	skin swab	08/02/22	Bd [qPCR]		not detected
22U011	Brook salamander	skin swab	08/02/22	Bsal [qPCR]		not detected
22U011	Brook salamander	skin swab	08/02/22	Bd [qPCR]		not detected
22U012	Brook salamander	skin swab	08/02/22	Bsal [qPCR]		not detected
22U012	Brook salamander	skin swab	08/02/22	Bd [qPCR]		positive
22U013	Brook salamander	skin swab	08/04/22	Bsal [qPCR]		not detected
22U013	Brook salamander	skin swab	08/04/22	Bd [qPCR]		positive
22U014	Brook salamander	skin swab	08/04/22	Bsal [qPCR]		not detected
22U014	Brook salamander	skin swab	08/04/22	Bd [qPCR]		confirmation required
22U015	Brook salamander	skin swab	08/04/22	Bsal [qPCR]		not detected

22U015	Brook salamander	skin swab	08/04/22	Bd [qPCR]	positive
22U016	Brook salamander	skin swab	08/04/22	Bsal [qPCR]	not detected
22U016	Brook salamander	skin swab	08/04/22	Bd [qPCR]	positive
22U017	Brook salamander	skin swab	08/04/22	Bsal [qPCR]	not detected
22U017	Brook salamander	skin swab	08/04/22	Bd [qPCR]	confirmation required
22U018	Brook salamander	skin swab	08/04/22	Bsal [qPCR]	not detected
22U018	Brook salamander	skin swab	08/04/22	Bd [qPCR]	positive
22S044	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S044	Brook salamander	skin swab	09/16/22	Bd [qPCR]	not detected
22S045	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S045	Brook salamander	skin swab	09/16/22	Bd [qPCR]	not detected
22S046	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S046	Brook salamander	skin swab	09/16/22	Bd [qPCR]	positive
22S047	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S047	Brook salamander	skin swab	09/16/22	Bd [qPCR]	not detected
22S048	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S048	Brook salamander	skin swab	09/16/22	Bd [qPCR]	positive
22S049	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S049	Brook salamander	skin swab	09/16/22	Bd [qPCR]	positive
22S050	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S050	Brook salamander	skin swab	09/16/22	Bd [qPCR]	positive
22S051	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S051	Brook salamander	skin swab	09/16/22	Bd [qPCR]	positive
22S052	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S052	Brook salamander	skin swab	09/16/22	Bd [qPCR]	not detected
22S053	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S053	Brook salamander	skin swab	09/16/22	Bd [qPCR]	not detected
22S054	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S054	Brook salamander	skin swab	09/16/22	Bd [qPCR]	confirmation required
22S055	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S055	Brook salamander	skin swab	09/16/22	Bd [qPCR]	not detected
22S056	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S056	Brook salamander	skin swab	09/16/22	Bd [qPCR]	not detected
22S057	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S057	Brook salamander	skin swab	09/16/22	Bd [qPCR]	confirmation required
22S058	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected

22S058	Brook salamander	skin swab	09/16/22	Bd [qPCR]	not detected
22S059	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S059	Brook salamander	skin swab	09/16/22	Bd [qPCR]	not detected
22S060	Brook salamander	skin swab	09/16/22	Bsal [qPCR]	not detected
22S060	Brook salamander	skin swab	09/16/22	Bd [qPCR]	not detected
22S061	Brook salamander	skin swab	08/12/22	Bsal [qPCR]	not detected
22S061	Brook salamander	skin swab	08/12/22	Bd [qPCR]	not detected
22S062	Brook salamander	skin swab	08/03/22	Bsal [qPCR]	not detected
22S062	Brook salamander	skin swab	08/03/22	Bd [qPCR]	not detected
22S063	Brook salamander	skin swab	11/10/22	Bsal [qPCR]	not detected
22S063	Brook salamander	skin swab	11/10/22	Bd [qPCR]	not detected
22S064	Brook salamander	skin swab	11/10/22	Bsal [qPCR]	not detected
22S064	Brook salamander	skin swab	11/10/22	Bd [qPCR]	confirmation required
22S065	Brook salamander	skin swab	08/01/22	Bsal [qPCR]	not detected
22S065	Brook salamander	skin swab	08/01/22	Bd [qPCR]	positive
22S066	Brook salamander	skin swab	08/01/22	Bsal [qPCR]	
22S066	Brook salamander	skin swab	08/01/22	Bd [qPCR]	
22S067	Brook salamander	skin swab	09/07/22	Bsal [qPCR]	not detected
22S067	Brook salamander	skin swab	09/07/22	Bd [qPCR]	positive
22S068	Brook salamander	skin swab	09/07/22	Bsal [qPCR]	not detected
22S068	Brook salamander	skin swab	09/07/22	Bd [qPCR]	not detected
22S069	Brook salamander	skin swab	09/07/22	Bsal [qPCR]	not detected
22S069	Brook salamander	skin swab	09/07/22	Bd [qPCR]	positive
22S070	Brook salamander	skin swab	09/07/22	Bsal [qPCR]	not detected
22S070	Brook salamander	skin swab	09/07/22	Bd [qPCR]	positive
22S071	Brook salamander	skin swab	09/07/22	Bsal [qPCR]	not detected
22S071	Brook salamander	skin swab	09/07/22	Bd [qPCR]	positive
22S072	Brook salamander	skin swab	09/07/22	Bsal [qPCR]	not detected
22S072	Brook salamander	skin swab	09/07/22	Bd [qPCR]	not detected

22S066 Sample missing, not found in submission bag

Results are Pending for Confirmatory: Bd [qPCR]
Results are Pending for Confirmatory: Bd [qPCR]
Results are Pending for Confirmatory: Bd [qPCR]
Results are Pending for Confirmatory: Bd [qPCR]
Results are Pending for Confirmatory: Bd [qPCR]

Results are Pending for Confirmatory: Bd [qPCR]

A "confirmation required" result means a follow up up test with more technical replicates is in process. A final report will follow.
Lab contact: phone 760-291-5470 or x5471 or x5472, email AmphibianLab@sdzwa.org