2020 Refugia Work plan

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

This annual work plan and associated cost estimate have been developed per the requirements of contract number 16-822-HCP for the Implementation of the Refugia Program under the EAHCP. The tasks and subtasks that follow provide the details for the services to be performed in 2020, which provide for the maintenance of a refugia population of the Covered Species (Table 1) including the salvage, propagation, and restocking of the species, if species-specific habitat triggers occur and species are extirpated, plus research conducted on the Covered Species.

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

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

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

Long-term Objective:

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

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

2020 Assumptions:

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

accommodate realized outcomes.

- Target numbers for the standing and refugia stocks to be housed at both the UNFH and SMARC are established by the USFWS-EAA Refugia Contract (Contract # 16-822-HCP).
- Species capture rates are expected to be similar to historic values.
- Mortality rates of specimens held in captivity are expected to be similar to historic values.
- Target species collection numbers from the 2019 Work Plan are expected to be reached.
- Staff members remain employed at the two Service facilities throughout the performance period.

Target for 2020 Task 1. Refugia Operations:

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

Table 2: Species target refugia numbers and census

Species	Standing Stock	Refugia Stock	Salvage Stock	Anticipated SMARC census (Jan 2020)	Anticipated SMARC census (Dec 2020)	Anticipated UNFH census (Jan 2020)	Anticipated UNFH census (Dec 2020)
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	150	215
Texas Blind Salamander	500	500 including specimens within the standing stock	500	110	250	15	40
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	80	115	50	80
Peck's Cave Amphipod	500	500 including specimens within the standing stock	500	250	250	160	250
Comal Springs Riffle Beetle	500	500 including specimens within the standing stock	500		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	*	*	*	*

for 2020 we plan on collecting Comal Springs riffle beetles mainly to support research purposes rather than standing stock, until we can increase survivability in captivity
*catch rates and hatchery survival are uncertain given the rarity of the species

Collection: In 2020, we will collect Covered Species as required to reach and maintain target standing and refugia stock numbers as shown in Table 2. Species collections will be coordinated with other ongoing EAHCP activities (e.g. Biological Monitoring Program) so that collections for refugia do not adversely impact other efforts. Species specific collections will be carried out through a variety of passive and active collection methods. Prior to collections, Hazard Analysis Critical Control Point (see Appendix A of the 2017 EAA Work Plan) will be conducted to minimize aquatic invasive species transfer. Collection efforts will be documented and reported to EAA. Captured specimens will be divided between the SMARC and UNFH facilities in order to ensure redundancy and to expedite the obligation to establish and maintain two refugia populations at separate locations. All species will be held in respective quarantine areas until their health has been assessed. Once it is determined that specimens are free from pathogens, parasites, and invasive species they will be incorporated into the general refugia population. USFWS will share reports, including test results, produced as part of the quarantine process. Species-specific collection plans generally follow those detailed within the 2019 Work Plan; however, collection efforts vary based upon collection and knowledge gained during the previous year's collection efforts. The following sections briefly describe planned 2020 collection, maintenance, and propagation efforts for each species.

Fountain Darters:

COLLECTION—Fountain darters in 2020 will be collected from the San Marcos River primarily in coordination with the Spring and Fall Biomonitoring events to create efficiencies and reduce habitat disturbance. After fountain darters are collected via drop nets for biomonitoring, USFWS staff will retain them for refugia purposes. Specimens will be collected along a longitudinal gradient. Fish will be collected proportionally from the three sections of the San Marcos (upper = Spring Lake, Middle = Spring Lake dam to Rio Vista dam, lower = below Rio Vista dam to Capes dam). Historically, approximately 20% of the fountain darters collected annually succumb to natural mortality. If unusual mortality events occur, they will be thoroughly investigated, and summary reports will be conveyed to the EAA as part of the monthly reports. As a result, fish collections will target additional fish so that as individuals perish the remainder within the captive population should not decrease below the target number between collection events.

Due to the detection of largemouth bass virus (LMBV) in Comal fountain darters throughout the Comal River, all fountain darters from Comal will be maintained in quarantine facilities in consideration of other species located on the two stations. Higher mortality rates of incoming Comal fountain darters have increasingly caused concern as the mortality continues and no root cause has been pinpointed despite extensive testing and evaluation with the USFWS Fish Health Unit. We will conduct exposure trials of non-LMBV+ F1 fountain darters to LMBV+ darters to determine the infection rates and if the non-LMBV+ fish exhibit the same mortality rates. This will be the first step in investigation of the high mortality rates. We will also consult with veterinarians on potential treatments (not already tried) to reduce incoming mortality rates. The next steps would include determining if LMBV is vertically transferred to offspring, the feasibility of producing a population of F1 fountain darters from the remaining non-LMBV Comal fountain darters, and evaluating the mortality rate of Comal fountain darters in the wild. Until we have a better understanding of the high mortality rates of incoming Comal fountain darters we will suspend collections from the wild, unless salvage is needed.

As part of quarantine procedures, a subset of fish (N = 60 per river) will be sent to the southwest regional Fish Health Unit or equivalent facility for pathogen (bacteria, virus, and parasite) testing

prior to specimen incorporation into the general refugia population following standardized methods outlined within USFWS and AFS-FHS (2016) and AFS-FHS (2005); reports will be provided to EAA.

MAINTENANCE—Water quality (i.e., temperature, pH, dissolved oxygen, total dissolved gasses) will be monitored and recorded weekly. Fountain darters will be fed live foods reared or purchased. Ponds will be utilized to produce zooplankton and amphipods. Amphipods will be collected from other managed ponds and raceways. Black worms will be purchased when necessary along with other food resources (i.e. blood worms, brine shrimp, etc.) if the need arises. Food items are not routinely examined for pathogens. However, if they are suspect and tested for pathogens all diagnostic results will be conveyed to the EAA within monthly reports.

PROPAGATION—Standing and refugia stocks for each river will be maintained to produce F1 generation fish for research purposes. Fish will be maintained by their geographical locations. If reintroduction is warranted, subsets from each geographical location will be communally spawned. Subset groups will be culled to an equal number of progeny prior to release.

Texas wild-rice:

COLLECTION—Texas wild-rice tillers will be collected from San Marcos River reaches (Fig. 1), with a break during summer months when wild-rice does not fare well due to heat stress. In 2020 collections for SMARC will target stands that are not already part of the refugia population or require supplementation. Collections for UNFH will continue to build their refugia numbers and representative locations. The refugia populations will reflect the wild populations in both their respective proportion and genetic diversity that was historically documented within San Marcos River (Wilson et al. 2016). During tiller collection, the GPS coordinates, area coverage, and depth of the stand or individual plant will be recorded so the exact location of the clone is known. For larger stands, tillers will be collected at the beginning, middle and end of the stand, or every 20% of the stand's total length for the largest stands. Tiller collection will be done by wading and SCUBA diving. Please note that during the 2018 Texas wild-rice survey no plants were found in Section I. Sections J and K were not surveyed. Plants were found in sections E, G, and H. All sections will be re-evaluated during the 2019 Texas wild-rice survey.

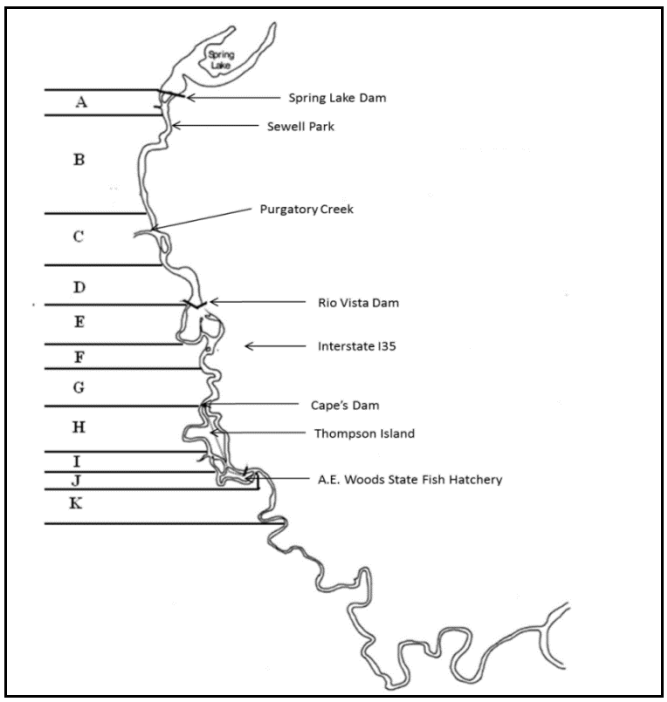


Figure 1 Letters define designated San Marcos River reaches where Texas wild-rice is collected for refugia populations.

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

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

Texas blind salamanders:

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

by Texas State University staff.

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

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

San Marcos salamanders:

COLLECTION—San Marcos salamanders will be collected up to quarterly from below Spring Lake dam and with SCUBA teams in Spring Lake (Table 3). The drift net on Diversion Springs will be checked routinely and specimens will be kept from this location as space in quarantine and need allows. Collection efforts will be coordinated with the EAHCP Biological Monitoring Program. All specimens will be transported live and maintained in the SMARC and UNFH refugia. Historically, approximately 30% of the San Marcos salamanders collected annually succumb to natural mortality. As a result, salamander collections will target additional specimens so that as individuals perish the remainder within the captive population should not decrease below the target number between collection events.

MAINTENANCE—As part of quarantine procedures, representatives of group housed salamanders in quarantine will be non-lethally cotton swabbed. These samples will be processed at SMARC 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. Duplicate swabs will be retained in case further testing is warranted. Chytrid

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

PROPAGATION—Standing and refugia stocks will be maintained to encourage reproduction. All progeny will be maintained separately by generation. If reintroduction is warranted, pair-wise and group mating will be employed to produce offspring. Stocking will occur once juveniles have reached 30 mm total length.

Comal Springs salamanders:

COLLECTION—Comal Springs salamanders will be collected up to quarterly from Comal Spring Runs 1-3 and Spring Island and surrounding areas (Table 3) by hand with dipnets using snorkelers. Close coordination with the EAHCP biological monitoring program will take place 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, Comal salamanders for refugia will be collected at a rate of no more than 10% of salamanders observed in those specific locales per daily sampling trip. A SCUBA team will be used for a portion of these collection efforts if necessary. Annual natural mortality will be recorded.

MAINTENANCE—As part of quarantine procedures, representatives of group housed salamanders in quarantine will be non-lethally cotton swabbed. These samples will be processed at SMARC 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. Duplicate swabs will be retained in case further testing is warranted. Chytrid testing will occur in batches where groups of five swabs will be pooled for analysis. Duplicate individual swabs will be retained in case further testing is warranted. All salamanders will be held in quarantine for at least 30 days and until test results have returned. Chytrid (Bd) fungus has caused mortalities in amphibian species; however, some species appear to have innate immunity. Previous tests of wild caught salamanders at SMARC (both Texas Blind and San Marcos) have almost always tested positive for Bd. Clinically, the salamanders appear normal and do not have any lesions or signs of disease. Positive testing for Bsal will be treated more cautiously as it has not yet been documented in this area (or anywhere in North America); these salamanders would remain in quarantine until further study and recommendations from FWS Fish Health. Salamander tank and system maintenance such as acid washing and system sterilization will occur annually or

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

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

Comal Springs riffle beetle:

COLLECTION—Comal Spring riffle beetle collections for standing and refugia stocks will occur four times a year from a variety of locations: Spring Runs 1, Spring Run 3, Western Shore, and areas surrounding Spring Island (Table 3). Riffle beetles will be collected with cotton lures following EAHCP standard operating procedures (Hall 2016). No specific spring orifice will be sampled two times in a row. All riffle beetle adults and larvae will be collected from the lures. Standing stock numbers will be reduced to 75 per station until propagation methods are refined and better knowledge of population numbers and meaningful standing stock numbers are derived. Standing stock number will be evaluated yearly by the Comal Springs riffle beetle Work Group. Additional collections for research purposes may be required outside of standing stock collections.

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

PROPAGATION—Propagation methods for this species are being developed.

Peck's Cave amphipod:

COLLECTION—Peck's Cave amphipod collection for standing stock will occur up to four times annually (Table 3). Adult Peck's cave amphipods will be collected with drift nets and by hand collection at variety of locations (drift nets: Spring Run 3, N = 2; Spring Island and associated Spring Lake habitats: hand collection). Special collection events will occur in January, February, and March for 2020 research purposes at UNFH, but will be counted towards standing stock numbers, the fourth collection event for UNFH will be in November. During these special collections SCUBA divers will be utilized to collect by hand at deeper locations so collections can be spread out. SMARC will collect amphipods in March, June, September, and December.

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

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

Comal Springs dryopid beetle:

COLLECTION—Comal Springs dryopid beetles will be collected primarily through the use of wooden lures and hand picking from submerged wood found in the Comal Spring system. If dryopid beetles are found on cotton lures used for Comal Spring riffle beetles they will also be retained (Table 3). We will potentially conduct two events of trapping in Panther Canyon Well during the year as access to the well and staff time allows. These will be bottle traps checked weekly for a month.

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

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

Edwards Aquifer diving beetle:

COLLECTION—Drift nets will be used to collect Edwards Aquifer diving beetle (Table 3). Drift nets will be set at a variety of locations where the species has been collected in the past (Texas State University Artesian Well N = 1; and Diversion Springs N = 1). Drift nets will be deployed and checked by USFWS staff when we are able to sample Texas State University Artesian Well (when not being used by Texas State staff).

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

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

Texas troglobitic water slater:

COLLECTION— Texas troglobitic water slater are primarily found in Artesian Well on Texas State Campus. Recent research by Dr. Will Coleman shows these are deep aquifer species that are rarely found at the surface. Dr. Coleman was unable to keep any alive for extended periods of time, as all specimens he collected came out of the spring damaged. We will continue to work with invertebrate experts in the field to determine what might be the optimum way to collect this species. Drift nets will be deployed and checked by USFWS staff when we are able to sample Texas State University Artesian Well (when not in use by Texas State staff).

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

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

Table 3. A tentative schedule for all species sampling during 2020. Collections listed here are subject to change with extenuating circumstances such as weather and coordination with external partners. EEA and partners will be notified of sampling dates as they become known or changed.

Edward's Aquifer Species Collection Plan 2020			
Date (month)	**Interval**	**Location**	**Target Species**
January	Check 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
January	Collect lures	Spring Runs, Landa Lake	CSRB, CSDB, PCA, TTWS
January	1 day sampling event, hand pick from downed wood	Landa Lake	CSDB
January	1 day sampling event, hand pick	Landa Lake	Peck's Cave amphipod, UNFH
February	Check for 2 consecutive weeks	Primer's Fissure & Johnson's Well	Texas blind salamander
February	1 day sampling event	San Marcos River	Texas wild rice
February	1 day sampling event, hand pick	Landa Lake	Peck's Cave amphipod, UNFH
March	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
March	1-2 day sampling event	Spring Lake and below dam	San Marcos salamander
March	1 day sampling event, hand pick	Landa Lake	Peck's Cave amphipod, UNFH & SMARC
March	1 day sampling event	Comal Springs	Comal Springs salamander

Edward's Aquifer Species Collection Plan 2020			
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-day sampling event	San Marcos River	Texas wild rice
April	Throughout, coincide with bio-monitoring	San Marcos River	Fountain darters
April	Set lures	Spring Runs, Landa Lake	CSRB, CSDB, PCA, TTWS
May	Check 2 consecutive weeks	Primer's Fissure & Johnson's Well	Texas blind salamander
May	1-day sampling event	San Marcos River	Texas wild rice
May	Check lures	Spring Runs, Landa Lake	CSRB, CSDB, PCA, TTWS
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, SMARC
June	1 day sampling event	Comal Springs	Comal Springs salamander
June	Set lures	Western Shore	CSRB, CSDB, PCA, TTWS

Edward's Aquifer Species Collection Plan 2020			
Date (month)	**Interval**	**Location**	**Target Species**
July	Check for 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
July	Check lures	Western Shore	CSRB, CSDB, PCA, TTWS
August	Check for 2 consecutive weeks	Primer's Fissure & Johnson's Well	Texas blind salamander
September	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
September	1-2 day sampling event	Spring Lake and below dam	San Marcos salamander
September	1 day sampling event, hand pick	Landa Lake	Peck's Cave amphipod, SMARC
September	1 day sampling event	Comal Springs	Comal Springs salamander
October	Check every T & F for 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
October	Throughout, coincide with bio-monitoring	San Marcos River	Fountain darters
October	1 day sampling event	San Marcos River	Texas wild rice
October	Set lures	Spring Runs, Landa Lake	CSRB, CSDB, PCA, TTWS
October	1 day sampling event, hand pick from downed wood	Spring Runs, Landa Lake	CSDB

Edward's Aquifer Species Collection Plan 2020			
Date (month)	**Interval**	**Location**	**Target Species**
November	Check every T & F for 2 consecutive weeks	Primer's Fissure & Johnson's Well	Texas blind salamander
November	1 day sampling event, hand pick	Landa Lake	Peck's Cave amphipod, UNFH
November	1 day sampling event	Comal Springs	Comal Springs salamander
November	Check lures	Spring Runs, Landa Lake	CSRB, CSDB, PCA, TTWS
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	1 day sampling event, hand pick	Landa Lake	Peck's Cave amphipod, SMARC

Refugium Stocks:

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

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

PROPAGATION—Propagation for stocking is not anticipated during 2020.

Salvage Stocks:

COLLECTION—If species-specific salvage triggers defined in the EAHCP are reached, the Refugia Program, in consultation with the EAA, will accommodate salvaged organisms no more than two times during the 12-year period. If triggers for multiple species are simultaneously reached, species collections during salvage operations will be prioritized

based upon the perceived species-specific effect of reduced river and spring flow and habitat degradation (i.e. EAHCP triggers). Those species that are river obligate species (i.e., fountain darter and Texas wild-rice) or that occupy spring orifice and interstitial ground water habitats (i.e., San Marcos and Comal Springs salamander, Peck's Cave amphipod, Comal Springs dryopid beetle) are presumed to be affected first as flows decrease. Those that reside solely within the aquifer (i.e., Edwards Aquifer diving beetle, Texas troglobitic water slater and Texas blind salamander) are presumed to be affected subsequently.

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

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

Construction/Renovation/Infrastructure/Facility: The SMARC Center Director will develop and maintain a list of warranty problems during the 1-year warranty period, forwarding items, as they occur, to the Contracting Officer (CO) and the USFWS Project Manager (COR).

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

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

Under the management of a lead supervisory biologist at both facilities, it is expected that six Biological Science Technicians, three at each station, will continue to assist with the collection, daily upkeep, maintenance, propagation, and research efforts for the ten species at the SMARC and UNFH. This includes maintaining experimental and culture production systems, keeping

records along with entering, filing, and collating data. The technicians will also generate basic summary statistics and graphic analyses of data and document program accomplishments through the composition of Standard Operating Procedures (SOPs), reports, and manuscripts. We will advertise for an additional animal care-taker position located at SMARC to help with day to day activities as Biological Science Technicians and the Supervisory Fish Biologist have additional collections and are involved in many of the refugia research projects.

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

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

Target for 2020 Task 2. Research:

The Research Plan for 2020 will involve a series of activities ranging from increasing survival rates of various invertebrate species, virus transfer in darter, to reproduction of Texas blind salamanders. The following section describes the basic components of each of these proposed 2020 activities. The budget cost for each project includes labor, materials, and oversight and research development costs. Therefore, the costs within this section may reflect differently than the budget table.

Project 1:

Title: Increasing survival rates of Peck's cave amphipod adults and F1 offspring

Species: *Stygobromus pecki*

Principal/Co-PI: Amelia Hunter, Makayla Blake, Dr. Lindsay Campbell

Overview: Different habitat enrichment items will be tried in holding containers for Peck's cave amphipods (PCA) to increase survival rates for wild stock adults. In addition, different food items will be added to test containers such as frozen tubifex worms or pellet foods, to see if they are a viable addition or alternative to fish flake that is currently given. Prototype holding containers for brooding females will be tested against the current brooding chambers employed for increased survival rates of F1 offspring.

Budget: $49,000

Benefit to the Refugia: Increased survival rates of PCA and continued refinement of propagation techniques.

Expected Results: The results of the study will be presented as a report to the EAA and if warranted an update to the PCA standard protocols.

Project 2:

Title: Continuation of increasing survival rates of Comal Springs dryopid beetle in captivity

Species: *Stygoparnus comalensis*

Principal/Co-PI: Dr. Ely Kosnicki, Bio-West, Inc

Overview: Different holding containers and habitat enrichment items will be evaluated for housing dryopid beetles and reducing the movement between containers of beetle eggs and larvae. Designs will also be tested in their ability to house larger numbers of beetles.

Budget: $40,000

Benefit to the Refugia: Increases survival rates of wild stock Comal Springs dryopid beetles in captivity and increased efficiency in F1 production.

Expected Results: The results of the study will be presented as a report to the EAA and if warranted an update to the Comal Springs dryopid beetle standard protocols.

Project 3:

Title: Continuation of San Marcos salamander reproduction

Species: *Eurycea nana*

Principal: Kelsey Anderson, Rachel Wirick, Dr. Lindsay Campbell

Overview: We plan to follow up on the information learned during 2019 on San Marcos salamander pathology reports by sourcing alternative foods that are not high in barium. We will investigate measures to reduce the number of free microsporidia spores in tank water. We will also consult an outside salamander reproductive specialist on potential changes to reproductive practices and use of amphibian hormones to induce mating.

Budget: $65,000

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

Expected Results: The results of the study will be presented as a report to the EAA, an update to the reintroduction strategy, and update to the Eurycea sp. Propagation Manual.

Project 4:

Title: Continuation of Comal Springs riffle beetle nutrition and survivorship research

Species: *Heterelmis comalensis*

Principal: Amelia Hunter, Linda Moon, Dr. Lindsay Campbell, Dr. Camila Carlos-Shanely

Overview: We plan to continue research into nutrition supplementation to increase survival rates. We will conduct a food preference test of artificial diets. We will use a food-grade 3D printer to construct matrices for biofilm growth as compared to cotton cloth. Research into the identification of the specific microbiome from Comal Springs riffle beetle gut content analysis will continue to compare wild microbiomes to those of captive microbiomes. Based on preliminary results, we will conduct a trial using cultured *Chromobaterium sp.* to determine if there are negative effects on larvae due to its presence in culture systems.

Budget: $100,000

Benefit to the Refugia: Increased survival rates of Comal Springs riffle beetles.

Expected Results: Interim reports to USFWS and EAA on the successes and failures of various techniques tried and knowledge gained.

Project 5:

Title: Continuation of increasing pupation success in the Comal Springs riffle beetle in a captive setting

Species: *Stygoparnus comalensis*

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

Overview: The purpose of this project is to identify conditions that are optimum for incurring successful pupation and eclosion for Comal Springs riffle beetles. Increased replicates of the most effective designs of holding larvae in flow-through tubes will be implemented. These designs will also be tested on larvae collected from the wild.

Budget: $75,000

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

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

Project 6:

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

Species: *Stygoparnus comalensis*

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

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

Budget: $75,000

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

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

Project 7:

Title: Continuation of evaluating three different long-term tagging methods in aquatic salamander species

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

Principal: Dr. Lindsay Campbell, Linda Moon

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

Budget: $30,000

Objectives and Methods: We will finish out a full year of evaluating the three different tag methods: Visible Implant Elastomer tags, Visual Implant Alphanumeric tags, and small Passive Integrated Transponders. Tags and injection sites will be monitored overtime for health, retention, and clarity/readability.

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

Target for 2020 Task 3. Species Propagation and Husbandry:

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

Target for 2020 Task 4. Species Reintroduction:

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

Reintroduction Plan for 2020: None

Any anticipated triggers being prepared for: Given current weather predictions, spring flows, and the Edwards Aquafer water level none are anticipated during the 2020 performance period.

Target for 2020 Task 5. Reporting:

Species specific Propagation plans (SOPs): Refine throughout year as needed.

Species specific Genetic Management plans: None during 2020.

Species specific Reintroduction plans: Refine as needed.

2020 EAHCP Annual Program reporting: USFWS will provide a year-end report of 2020 activities to the EAA no later than 1/31/2021.

Program reporting as required by ITP and TPWD: TPWD Scientific Research Permit Report will be conveyed to the EAA July 31, 2020.

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.

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 the Center director and EAHCP CSO).

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

Monthly electronic reports to EAHCP CSO: A monthly report of all activities will be provided to the EAHCP CSO. USFWS anticipates providing the report by the 10th of each month for the previous month's activities.

Target for 2020 Task 6. Meetings and Presentations:

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

Monitoring:

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

Amended U.S. Fish and Wildlife Service 2020				
	Task		**Task Budget Amount**	**Total Task Budget Amount**
1	**Refugia Operations**			**$735,466.09**
	SMARC Refugia & Quarantine Bldgs.			
	Equipment & Building Maintenance		$12,000.00	
	Utilities		$50,000.00	
	UNFH Refugia & Quarantine Bldgs.			
	Equipment & Building Maintenance		$20,000.00	
	Utilities		$51,788.23	
	Construction		$40,000.00	
	SMARC Species Husbandry and Collection		$133,146.07	
	Fish Biologist (GS-12, 165 hrs)	$9,153.97		
	Fish Biologist (GS-07, 952 hrs)	$30,678.70		
	Fish Biologist (GS-07, 952 hrs)	$30,911.26		
	Fish Biologist (GS-07, 952 hrs)	$31,463.59		
	BioTechnician (GS-05, 1049hrs)	$21,462.54		
	Biologist-Detail (GS-09)	$9,476.00		
	Weekend Walk Through		$5,000.00	
	Other Overtime		$1,500.00	
	UNFH Species Husbandry and Collection		$196,954.22	
	Fish Biologist (GS-11, 1125 hrs)	$54,587.71		
	Fish Biologist (GS-07, 1856 hrs)	$56,880.94		
	Fish Biologist (GS-07, 1170 hrs)	$39,426.24		
	Fish Biologist (GS-07, 1456 hrs)	$46,059.32		
	Weekend Walk Through		$5,000.00	
	Other Overtime		$4,500.00	
	Water Quality System Maintenance & Additions		$15,000.00	
	Divers		$2,500.00	
	Fish Health		$8,000.00	
	Amphibian Health Training Workshop		$3,000.00	
	SMARC Reimbursibles		$40,214.98	
	UNFH Reimbursibles		$40,000.00	
	Subtotal		*$628,603.50*	
	Admin Cost Subtotal		*$106,862.59*	
2	**Research**			**$667,366.55**

	BIO-WEST Life History of Dryopids		$46,872.85	
	BIO-WEST CSRB Pupation continued		$96,259.90	
	Texas State CSRB Pupation continued		$121,330.80	
	Increasing Survival Rates of PCA		$31,899.60	
	Fish Biologist (GS-07, 710 hrs)	$22,663.20		
	Fish Biologist (GS-07, 220 hrs)	$6,736.40		
	Materials	$2,500.00		
	San Marcos salamander reproduction		$62,184.71	
	Fish Biologist (GS-07, 705 hrs)	$21,587.10		
	Fish Biologist (GS-07, 420 hrs)	$12,658.80		
	WADDL diagnostics	$19,938.81		
	Materials	$8,000.00		
	Long term salamander tagging cont		$22,158.15	
	Fish Biologist (GS-07, 705 hrs)	$22,158.15		
	CSRB Nutrition Research continued		$84,737.23	
	Fish Biologist (GS-07, 925 hrs)	$28,391.20		
	Fish Biologist (GS-07, 220 hrs)	$6,914.60		
	Microbiome Analysis	$41,431.43		
	Materials	$8,000.00		
	Oversight and Research Development		$104,955.52	
	FWS Administrator (55 hrs)	$5,318.61		
	Fish Biologist (GS-12, 1466 hrs)	$73,374.42		
	Fish Biologist (GS-11, 550 hrs)	$26,262.49		
	Subtotal		*$570,398.76*	
	Admin costs for Task 2		*$96,967.79*	
3	**Species Propagation and Husbandry**			**$0.00**
	Subtotal			
4	**Species Reintroduction**			**$0.00**
	Subtotal			
5	**Reporting**			**$95,175.02**
	SMARC Staff		$45,258.58	
	FWS Administrator (40 hrs)	$3,206.33		
	Staff (GS-11, 168 hrs)	$7,490.58		
	Fish Biologist (GS-12, 393 hrs)	$19,568.74		
	Fish Biologist (GS-07, 194 hrs)	$4,943.05		
	Fish Biologist (GS-07, 194 hrs)	$4,980.49		

	Fish Biologist (GS-07, 194 hrs)	$5,069.41		
	UNFH Staff		$36,087.60	
	Staff (GS-07, 95 hrs)	$2,953.76		
	Fish Biologist (GS-11, 400 hrs)	$18,513.30		
	Fish Biologist (GS-07, 156 hrs)	$4,680.07		
	Fish Biologist (GS-07, 156 hrs)	$5,109.07		
	Fish Biologist (GS-07, 156 hrs)	$4,831.39		
	Subtotal		*$81,346.18*	
	Admin costs for Task 5		*$13,828.85*	
6	**Meetings and Presentations**			**$15,641.00**
	SMARC staff		$8,883.32	
	FWS Administrator (30 hrs)	$2,280.00		
	Fish Biologist (GS-12, 56 hrs)	$2,953.49		
	Fish Biologist (GS-07, 30 hrs)	$1,206.11		
	Fish Biologist (GS-07, 30 hrs)	$1,213.31		
	Fish Biologist (GS-07, 30 hrs)	$1,230.41		
	UNFH Staff		$4,485.05	
	Fish Biologist (GS-11, 36 hrs)	$1,719.00		
	Fish Biologist (GS-07, 30 hrs)	$904.25		
	Fish Biologist (GS-07, 30 hrs)	$957.60		
	Fish Biologist (GS-07, 30 hrs)	$904.20		
	Subtotal		*$13,368.37*	
	Admin costs for Task 6		*$2,272.62*	
		TOTAL	**$1,513,648.66**	

Total Projected (2020) Budget Summarized by Task:

Task 1: $735,466.09 (including $122,904.09 in rollover funds from 2019)
Task 2: $667,366.55 (including $223,190.55 in rollover funds from 2019)
Task 3: $0
Task 4: $0
Task 5: $95,175.02 (including $15,872.02 in rollover funds from 2019)
Task 6: $15,641

Projected (2020) Subcontractor Expenses Summarized by Task:

Task 1: Southwest Regional Fish Health Unit, Dexter, NM $8,000 (Health Diagnostics); Amphibian Health Training Workshop by Amphibian Ark $3,000

Task 2: BIO-WEST, Inc, $143,132.75; Texas State University, Dr. Weston Nowlin, $121,330.80; Washington Animal Disease Diagnostic Laboratory (WADDL) $19,938.81; Dr. Ruth Marcec-Greaves, $2,500; Dr. Camila Carlos-Shanley's Laboratory $31,431.43
Task 3: $0
Task 4: $0
Task 5: $0
Task 6: $0

2020 available budget:
$1,151,682

Estimated 2020 budget:
$1,513,648.66

Timeline of 2020 Milestones

(List major deliverables)

January	Continue with species collection
	Subcontract research awards executed
	2020 Specific Research Study Plans finalized
July	Submit and renew TPWD permit
September to December	Draft Research Reports
	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.

Cantu, V., J. N. Fries, and T. A. Ryan. 2009. An apparatus for separating live amphipods from debris. North American Journal of Aquaculture 71:6-9.

Crawford, D. M., and D. C. Tarter (1979) Observations on the life history of the freshwater amphipod, *Crangonyx forbesi* (Hubricht and Mackin), in a spring-fed cistern in West Virginia. American Midland Naturalist 2: 320-325.

Culver DC, T Pipan (2009) The biology of caves and other subterranean habitats. Oxford University Press, New York.

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.

Ulrich, G. W (1986) The larvae and pupae of *Helichus suturalis* Leconte and *Helichus productus* Leconte (Coleoptera: Dryopidae). The Coleopterist Bulletin 40: 325-334.

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.

Increasing survival rate of Peck's cave amphipods (*Stygobromus pecki*)

2020 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by David Britton, PhD

Contributions from Makayla Blake, Dr. Lindsay Campbell, Kelsey Anderson, and Jennifer Whitt

San Marcos Aquatic Resources Center

U.S. Fish and Wildlife Service

Table of Contents

Background

The Peck's cave amphipod (*Stygobromus pecki*; PCA) is a federally endangered (USFWS 1997) amphipod (family Crangonyctidae) endemic to the eucrenal habitats of two Edwards Aquifer springs, the Comal and Huaco, in Comal County, Texas. The Edwards Aquifer Authority (EAA) established a contract with the U.S. Fish and Wildlife Service (USFWS) for refugia in accordance with the Edwards Aquifer Habitat Conservation Plan (EAHCP) for ten covered species at two locations in Texas, the San Marcos Aquatic Resources Center (SMARC) in San Marcos and the Uvalde National Fish Hatchery (UNFH) in Uvalde. These refugia serves as genetic backup population that can be reintroduced if a catastrophic event destroys the wild population or habitat.

Historically, captive PCA have suffered from low survival rates in captivity. Increasing survival rates of captive PCA is fundamental to building a fully functional refugium for the species. Targeted research with captive standing stock is needed to successfully maintain healthy individuals and for the overall success of the refugia. Survival of PCA, tracked as overall survival of the UNFH standing stock, has been consistently low (29% in 2017, 24% in 2018). Survival rates at the SMARC have been better with 72% in 2017 and 58% in 2018. PCA collected in the wild are of unknown age, thus, a portion of the mortality could be due to natural senescence. PCA are known to cannibalize smaller individuals, which contribute to the morality rates (R. Gibson, pers. obs.; Nowlin et al. 2016). Additional causes for high mortality may include inconsistent water flow and temperature regulation, inadequate habitat, and poor nutrition.

Kosnicki and Julius (2019) hypothesized that increased heterogeneity of inhabitable space and increased amount of habitat material may increase survival rates of PCA. Based on this hypothesis and personal communication with one of the studies author's (E. Kosnicki, Bio-West, January 2019), modifications where made to the UNFH PCA culture boxes for pilot trials during 2019. Low-density Matala® filtration media was added as habitat media to PCA culture boxes collected in March 2019 and was added to all of the PCA culture boxes at UNFH during the May 2019 inventory. The media mimics the interstitial spaces between gravel in a riverbed and provides increased surface area, therefore increasing the heterogeneity of the habitat. The filter media, as opposed to

gravel, can be quickly assessed for organisms which decreases excessive handling, improving the efficiency of inventories.

Survival rates of PCA at UNFH were approximately 35% for the months of January-March 2019. After adding filter media as habitat, the overall survival rates increased: 57.7% in May, 79.9% in July, 88.3% in September, and 91.9% in November. Other additional improvements to the culture boxes include using a new type of box that is made of black opaque material and housing the invertebrate organisms in a climate controlled environment at UNFH. The black totes block light and, because they are deeper, allow for multiple layers of the filtration media whereas the former boxes (clear plastic, wrapped in black cloth) could only accommodate one layer. The added layers of material effectively increases usable habitat space for PCA as they do not regularly inhabit empty water column space. The completion of the new EAA refugia and quarantine at UNFH allowed the invertebrate organisms to be moved to a more controlled external environment. The invertebrate room is a section of the EAA refugia added during the construction in 2018-2019. A 660 gallon insulated sump was added in the refugia invertebrate room that preconditions well water for more optimal temperature (21°C ±1°C) and total dissolved gas values (≤100%). The PCA are housed separately by size class (small, medium, large), potentially decreasing cannibalism. It appears these changes have contributed to increase survival of PCA at UNFH, thus we propose scaling up these pilot trials to a full testable design.

Only a limited number of studies have investigated diet or nutrition of *S. pecki* (Nair 2019) and other Crangonyctidae (Dickson 1979) in the wild, and no published studies have investigated captive diet. PCA are likely predators consuming other invertebrate species (Nair 2019). We offer red krill based flaked feed to PCA in captivity in addition to leaves and biofilm. Ostracods are also present in abundance in the UNFH water supply and may possibly add to the diet of the PCA. However, these food items might not fully meet nutritional requirements for the species. We investigated acceptance of additional food items beyond what has been offered at our two facilities.

Finally, our refugia must have the ability to produce offspring for restocking purposes. While PCA females become gravid in captivity, we have had only limited success with raising captive bred individuals, as adults often cannibalize the offspring. We

have tried housing amphipods in brooding chambers consisting of a flow-through tube containing a dividing mesh to separate offspring from adults. However, we have seen limited success. In this study, we tested three prototype brooding chambers with gravid females found during inventory at the SMARC. At UNFH, brooding females were left in the experimental set up to test if captive bred amphipods survive and can be retrieved in our recently re-designed containers (as described above).

Objectives

The goal of this research is to improve survival rates of adult and captive bred PCA through an evaluation of habitat and feeding requirements. This research has three parts:

1) We compared adult PCA survival rates across three treatment groups, each utilizing a different combination of low and/or medium-density Matala® filtration material as habitat in redesigned culture boxes.

- Low-density (black) Matala® filtration media
- Medium-density (green) Matala® filtration media
- A combination of 50% low-density and 50% medium-density Matala® filtration media

2) We tested potential food sources for the captive PCA as follows.

- We monitored the behavior of small groups of PCA in chambers over a 24-hour period after a novel food type was introduced, to test whether the amphipods were attracted.
- We compared different foods for PCA consumption.
- We introduced the food types to a larger holding container to evaluation their use on a larger scale.

3) We developed a brooding chamber to increase survival of the captive bred PCA.

- Two prototype designs were developed by Dr. Lindsay Campbell with input from Makayla Blake and Amelia Hunter.
- As brooding females were found during inventory at the SMARC, they were placed in the prototype chambers and monitored.

- Brooding females at UNFH not part of the holding container experiment were placed in brooding chambers.
- Brooding females were replaced in the experimental set up at UNFH to determine if the Matala® material provided enough refuge for captive bred PCA to survive and be retrieved by technicians.

Methods

Habitat Media Evaluation

Pilot Study

Prior to 2019, transparent polypropylene, 15-34 quart storage totes were used to construct all PCA culture boxes. These were covered in black cloth or painted black (on the outside of the boxes) to block light. A pilot study was conducted at UNFH in 2019 to assess the efficacy increasing habitat heterogeneity and quantity in the all PCA culture systems by adding low-density Matala® filter media as substrate. As new amphipods were collected, they were placed in new culture boxes made from opaque black, 20 quart storage totes constructed of linear low-density polyethylene (LLDPE) filled with low-density Matala® filter media at a depth of 10 cm as habitat substrate. The low-density media was also added to all of the existing PCA culture boxes. However, the existing transparent polypropylene culture boxes had a shallower depth of only 5 cm. As the year progressed, during inventories, the older, transparent culture boxes were replaced with black opaque boxes that could hold multiple layers of the habitat media.

Current Study

To build upon the success of the pilot study, black opaque boxes and Matala® filtration media were used to build new culture boxes for the PCA. A three-by-three experimental design was implemented with three treatments varying in substrate density at UNFH. Twelve identical culture boxes were constructed of 5-gallon black storage totes with ½ inch bulkhead fittings for inflow and ¾ inch bulkhead fittings for the outflow. The ½ inch, inflow bulkhead fitting was placed 1 ¼ inch, on center, below the top edge of the box, directly in the middle of one of the short ends. On the opposite short end, a 3/4 inch, outflow bulkhead fitting was placed 2 inches, on center, from the top edge of the box. PVC fittings (½ inch) and pipe were used to create a two pronged flow bar at the bottom of the box to mimic the hydrology of a spring upwelling. Positioned on the sides, top and bottom

of the pipe, 50, 1/16th inch holes were drilled along the flow bar to facilitate flow throughout the culture box. A ¾ inch horizontal standpipe was inserted into the ¾ inch bulkhead, which was covered by a 220 µm mesh screen sock to prevent escape while facilitating adequate out flow (Figure 1).

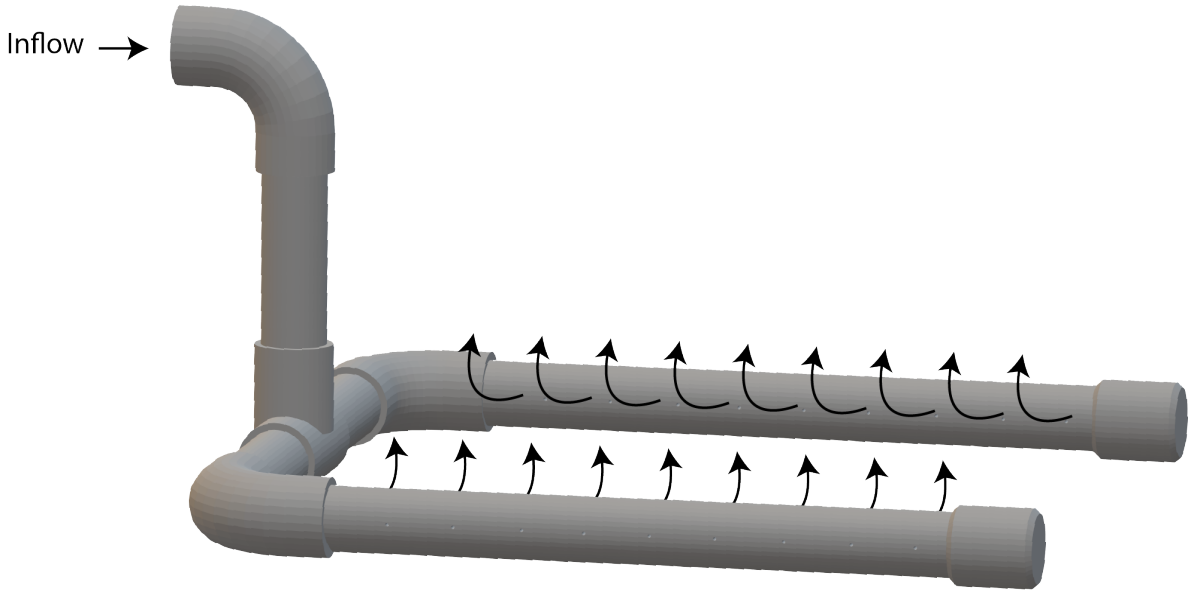


Figure 1. Two pronged flow bar constructed of ½ inch PVC

A single layer of nylon mesh was placed over the flow bars with a layer of leaves (oak, sycamore, and pecan) on the mesh. The mesh layer helped to facilitate flow below the leaves to prevent anoxic zones from forming (Figure 2).

Figure 2 Bottom layer of test PCA container showing flow bars (which PVC along the bottom), mesh, and layer of leaves.

About 10-15 grams of dry leaves were added to each treatment box. The PCA are likely predators and probably do not eat the leaves or biofilm, however, the leaves provided additional habitat for the PCA and a potential food source for ostracods, on which the PCA may feed. Ostracods are frequently found in the incoming well water at UNFH and likely provide a novel food source for the PCA. Feed is also currently supplemented with a paste of red fish flake. All of the treatments will have three layers of Matala® filter media with a single layer of leaves in-between each layer, but will not contain leaves on the top layer. The black, low-density filter media contained a surface area of 62 sq. ft. per cubic foot and 92% open space. The green medium-density filter media contained a surface area of 96 sq. ft. per cubic foot with an open space of 93%. The first treatment was assigned the black, low-density Matala® media for all three layers. The second treatment

was assigned the green, medium-density Matala® media for all three layers. The first two treatments were single media (Figure 2). The third treatment was an even mix of media of both densities (Figure 3). Each treatment was replicated in triplicate for nine total treatment boxes.

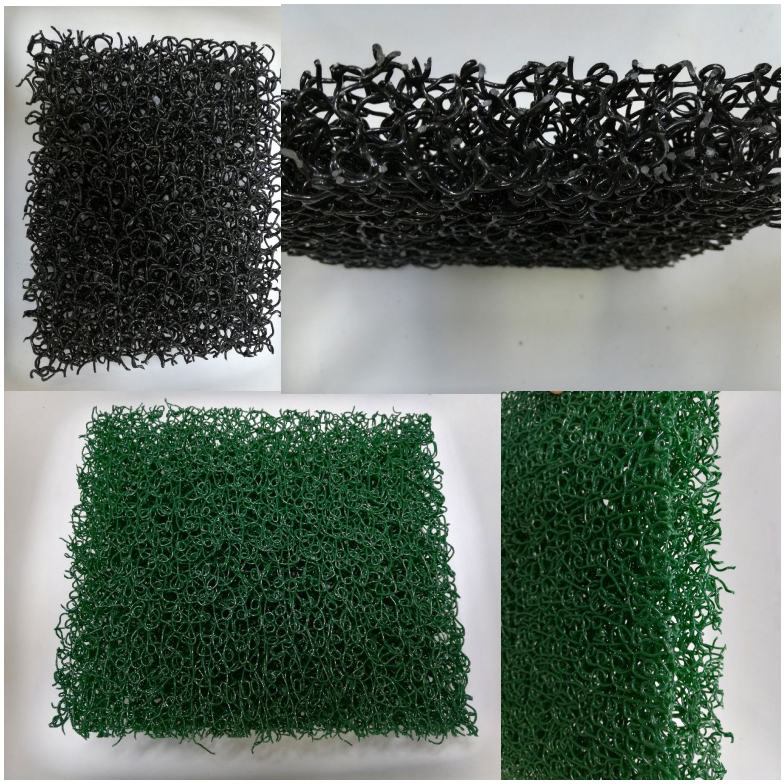


Figure 3 Top: Black low-density filter material. Bottom: Green medium density filter material.

The dimensions of the filter media placed in the boxes were standardized across all treatments. An additional culture box was constructed for each treatment type, in addition to the three needed as replicates, and were conditioned and running on the same rack for the purpose of backup and to hold any captive bred offspring.

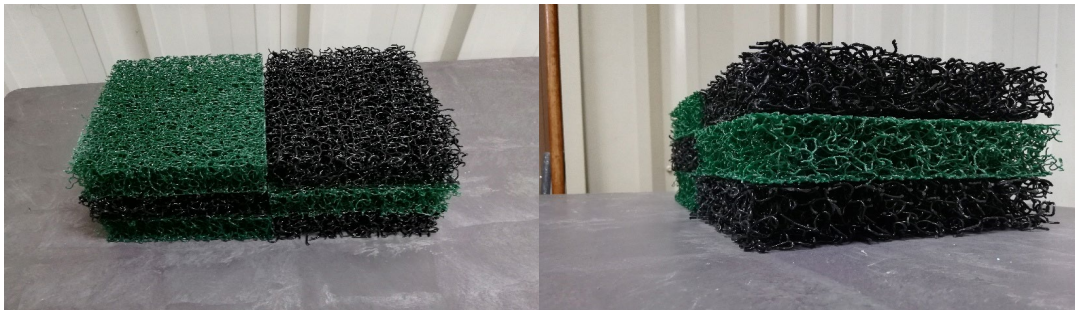


Figure 4 Mixed material density design.

Three collection events were separated by months. We collected at least 300 PCA at each collection event from Spring Island in the Comal River, New Braunfels, Texas. At least 100 surplus individuals were kept at each collection event to account for any potential losses during transport. If unneeded for this purpose, the excess organisms were added to refugia standing stocks. The first collection occurred in January 2020. The second collection occurred in February 2020 and, the third collection occurred in June 2020 (delayed by the Covid-19 pandemic). Events were temporally separated to allow for PCA population recovery within the collection area.

After the PCA were collected around Spring Island in the Comal River, the individuals were transported back to UNFH in jars containing low-density filter media and river water. The capped jars were placed in a cooler filled with river water to maintain temperature during transport. After collection, 30 wild caught PCA of random size and sex were introduced into each treatment box. The PCA from each collection event were equally and randomly distributed within one of each of the three treatment types for a total of 30 individuals in each box.

After the thirty-day quarantine, the treatment boxes were inventoried to determine survival rate for the first month, andall nine of the treatment boxes, plus the three back-up/captive-bred boxes were held on one dedicated rack in the invertebrate room in refugia at UNFH for the duration of the research..

The flow to each box was standardized to at least 0.5 GPM and flows were monitored and recorded daily for each of the culture boxes and sumps. This system used partial recirculation to ensure a constant temperature (21°C ±1°C) and flow rates (≥0.5 GPM). A submersible pump in the rack's sump tank provided water to each of the treatment boxes via a manifold. The outflow water from all boxes were diverted into the dedicated sump tank, supplemented by a fresh conditioned well water input from the larger conditioning tank. Fresh conditioned water was supplied at a rate to ensure a turn-over, of the whole system every 6-8 hours. Conditioning of fresh well water was accomplished in a large, 660 gallon sump tank where well water is degassed and chilled. This conditioned water was pumped from the large conditioning sump tank to a smaller sump dedicated for the PCA.

All treatment boxes were maintained using established culture practices. The Red flaked fish food was added to each box every Monday and Thursday. Four grams of finely ground dry flake was placed in a 100 ml cup and mixed thoroughly with 80 ml of conditioned well water. The food slurry (8 ml) was then injected into the filter media at four locations throughout the box including at different depths of the box, using a disposable pipet. The outflow screens were monitored daily and cleaned or replaced as needed. Water quality parameters within the rack's sump tank was monitored weekly using a combination of probes, titrations, and colorimeter tests. Parameters tested were: Temperature (21 °C ±1 °C), Dissolved Oxygen (>2 mg/L), Ammonia (<0.1 ppm), Nitrite (<0.1 ppm), Sulfides (< 0.1 ppm), pH (<8.5), Carbon Dioxide (<40 mg/L) and Total Gas Pressure (90-100%).

After the PCA were moved to the invertebrate room, inventories were conducted every 40-45 days to monitor survival within each treatment box. All boxes within a collection group were inventoried within a 24 hour period. Inventories were conducted on one treatment box at a time. The flow to the box was turned off and the box allowed to drain to below the standpipe by gently propping up the inflow side of the box. The filter media was carefully removed and inspected for PCA in a large white tub. Once each piece of media was checked and was clear of PCA, it was stored in a bucket of conditioned well water during the inventory. The leaves were removed and carefully placed in a 1000 ml cup filled with conditioned well water, to be sorted later. The nylon mesh was also removed, thoroughly checked in a white tub and placed in the bucket. All PCA found were placed in a holding cup containing low-density filter media and conditioned well water where temperature was monitored regularly. PCA were retained in the bottom of the box while the habitat media was removed, and were collected using a baster and placed in the holding cup, to be tallied on a data sheet. Gravid females were noted.

Once all visible PCA were removed from the box, the flow bar was carefully removed, disassembled and checked for PCA. The remaining water in the box was poured through a 75 µm sieve and placed in a white tub filled with enough conditioned well water to cover the mesh. The sieve and leaves were inspected for PCA. The box, habitat media, and PVC pieces were scrubbed and rinsed before reassembly. A similar wet weight of conditioned leaves, including some of the leaves from the box along with newly

conditioned leaves were added to each of the treatment boxes. The box was reassembled and returned to the rack to refill. Once full, the flow was temporarily shut off while the PCA were returned to the box from the holding cup. Flow was restored after 5 minutes, allowing the PCA time to disperse and settle.

Survival was assessed by comparing Survival (Alive or Dead) across three “Media Types” (Low, Medium, or Mixed Density). The experiment was replicated three times (“Collection Event” with collections in January, February, and June 2020). Although we inventoried each box once after 45 days or quarantine, and regularly every 30 days thereafter, we used only the final survival counts in our analysis. First, we tested the independence of Media Type and Collection Event with a two-way contingency table and a chi square test of independence, with the null hypothesis that survival counts at each Media Type were independent of Collection Event. Second, we tested the independence of Survival and Media Type, with a two-way contingency table and a chi square test of independence, with the null hypothesis that Survival was independent of Media Type. Significance was determined with α threshold at 0.05.

Evaluation of Alternative Food Items

We placed four PCA in small containers with potential food items for 24 hours andmonitored the container by video to evaluate predation behavior. Novel organisms were used for each food type, including a cladoceran (*Daphnia* sp.), an isopod (*Lirceolus* sp.), and an amphipod (*Hyalella* sp.). We also tested an animal-based, extruded pellet produced by Amelia Hunter in conjunction with Bozeman Fish Technology Center. We tested each food type three separate times.

PCAs were retained in their original refugium boxes prior to the experiment. One box was selected without bias for each replicate trial. Food was not removed from individual boxes, but PCAs were not given additional supplemental food for 10 days prior to the experiment. On the day of the experiment, four PCA from each box were selected without bias and moved into testing chambers. Testing chambers were housed in a dark room with constantly flowing well water (at 0.10 gpm) and a space heater was added to maintain temperature in the room and increase the water temperature to the normal temperature the PCA are held at (22.5 °C). The bottoms of the testing chambers were painted black to ease visualization of amphipods. To aid in reducing stress, a 5 cm by 10

cm piece of hard plastic, black mesh was placed in the center of each chamber and held in place with a small pebble. One PCA was placed in each chamber and allowed one hour to acclimate. After one hour, PCAs were given either a) 0.25 g of animal-based extruded "log" (cut into two pieces), b) two live *Lirceolus* sp. isopods, c) two live daphnia (*Daphnia pulex*) cladocerans, or d) two live *Hyalella* sp. amphipods. Animals and their food items remained in testing chambers for five days. On the fifth day, PCAs were removed and evaluated for general condition (activity level, color, gut content) before being returned to their original container. Testing chambers that had contained live food items were searched for carcasses and remaining live individuals. The extruded log could not be measured as it had disintegrated within 5 days and grown mold even in high flow through set ups. Cameras recorded 24-7 infrared footage of each replicate for future analysis. Testing chambers were emptied and dried between replicates and no animals were used repeatedly.

Pilot Test of Brooding Chambers

In the past several years a flow-through tube design with fine mesh separating chambers has been used to separate captive bred PCA before they were preyed upon by adults in the large containers. Limited to no success was found with this design. Thus, we have designed two new prototype brooding chambers and tested their efficacy. We used small, fish breeder chambers modified in two ways: 1) with flow from the top (Prototype A) and 2) with flow from the bottom (Prototype B). In both prototypes up to four brooding females were placed. Each was monitored for offspring and fully inventoried every 45 days.

Prototype-A had a side-attached smaller chamber for offspring to flow into (Figure 5). The container was positioned in a larger water bath. Side slits that allow for water movement was covered with fine mesh to prevent escape. Water input from the top helped encourage smaller offspring down into the second refuge chamber. Gravid females were placed in the top portion of the container above the green divider. Blue high-density Matala® material (124 sq. ft. per cubic ft and 94% open space) was placed in the depression above the opening to the lower chamber. Black, course low-density Matala® material and barrel shaped bio media were placed in the upper chamber.

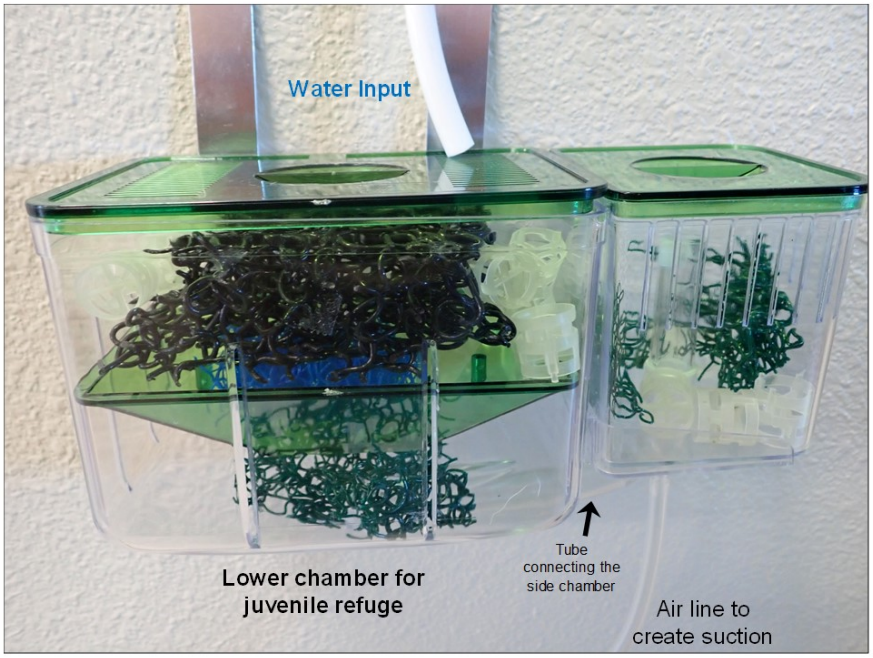


Figure 5 Prototype A brooding chamber.

We hypothesized that the large females will not be able to traverse the denser material into the bottom's refuge area for the offspring. Biologists have noticed that PCA display the behavior of burying into substrate when threatened (i.e. during collection events), so we planned to capitalize on this descending behavior for the offspring to escape any predation by adults. In the bottom chamber, small pieces of Matala® material and small glass beads were utilized as habitat for offspring. In Prototype A, we tested if suction created, by lowering the pressure in the side chamber, moved offspring out of main container to the side chamber where they would be more easily collected. Containers were shielded from light. Staff fed adults following the Standard Operating Procedures as for regular (non-experimental) containers. Small amounts of flake paste was injected into the bottom container and the side container to prevent the need of the smaller PCA to venture into the top chamber to find food.

Figure 6 Prototype B brooding chamber.

In Prototype B, (Figure 6) we did not use the side container. We routed water flow into the chamber as a whole from the bottom, following the hypothesis that downward escapement behavior of PCA is a response to upward spring flow. Habitat items and placement were similar to Prototype A. Food was injected into both the top and bottom chamber. The lower chamber was checked daily for offspring. If a large group of offspring was seen in lower container, we removed and conducted inventory on a 45 day schedule. Offspring were transferred to grow-out containers and non-gravid females were returned to their original containers.

On April 30, 2020 we moved six female PCA to brooding chambers: Four were added to the Prototype A brooding box and two were added to the Prototype B brooding box. After inventory on June 4, 2020 three gravid females from the Prototype A box were added in with one gravid female in the (single chamber) Prototype B brooding box. The non- gravid PCA were removed.

We added one additional gravid female on July 9, 2020 to the Prototype B brooding box.

On September 8, 2020 we started again with two gravid females to the Prototype A (double chamber) and two to the Prototype B (single chamber) brooding box.

Results

Habitat Media Evaluation

Overall, 73% of the collected PCA (n = 270) survived in captivity for the entirety of the experiment. The breakdown of these survivors, by habitat Media Density and Collection Event, is depicted in Table 1.

Table 1. Observed (and expected) frequencies of Peck's cave amphipods surviving after 105 days in refugia boxes by Media Density and Collection Event

		Media Density		
		Low	Medium	Mixed
Collection Event	January	17 (18.5)	26 (23.8)	22 (22.8)
	February	26 (21.6)	26 (27.8)	24 (26.6)
	June	13 (15.9)	20 (20.5)	23 (19.6)

Each box began with 30 amphipods.

The chi square test of independence (Media Density by Collection Event) was not significant ($\chi^2 = 2.75$, df = 4, p = 0.060). There was insufficient evidence to reject the null hypothesis that Collection Event was independent of Media Density.

We next examined Survival by Media Type (with Collection Event collapsed; Table 2).

Table 2. Observed (and expected) frequencies of Peck's cave amphipod Survival after 105 days at three different Media Densities

		Survival	
		Alive	Dead
Media Density	Low	56 (65.7)	26 (24.3)
	Medium	72 (65.7)	26 (24.3)
	Mixed	69 (65.7)	20 (24.3)

Each Media Type began with 90 amphipods.

The chi square test of independence (Survival by Media Type) was significant (χ^2 = 8.15, df = 2, p = 0.017). There was sufficient evidence to reject the null hypothesis that Survival was independent of Media Density.

Peck's cave amphipods held with medium-density Matala® filtration media had the highest survival in captivity (80%). Survival of amphipods held in mixed-density media (half low-density media and half medium-density media) was intermediate (77%), and survival of amphipods held in low-density was lowest (62%; Figure 7).

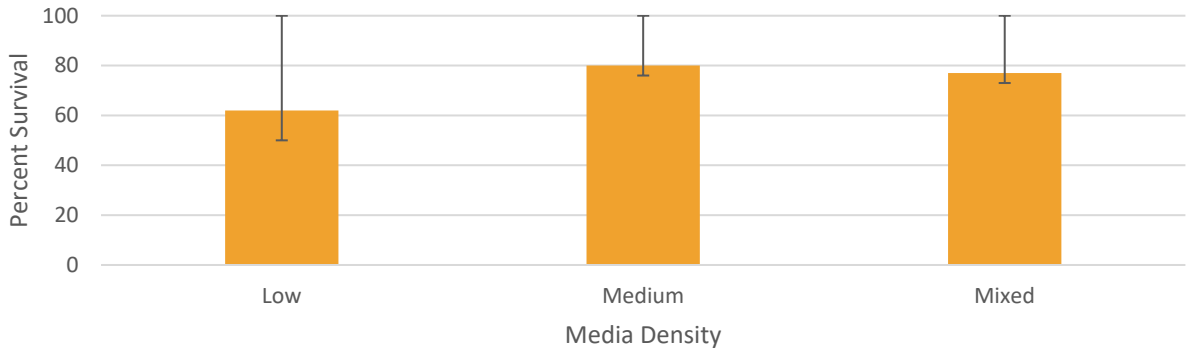


Figure 7. Percent Survival of Peck's cave amphipods by habitat media density, after 105 days in captivity. Error bars show the range across replicates.

Evaluation of Alternative Food Items

PCAs consumed one individual of *Daphnia* sp, *Lirceolus* sp., and *Hyalella* sp. in one of three replicate trials when these were offered as food. The results of the experiment are summarized in Table 3.

Table 3. Items accepted when offered as prey to Peck's cave amphipods

		Replicate		
		1	2	3
Prey Item	*Daphnia*	1 of 2	0 of 2	0 of 2
	Lirceolus	0 of 2	1 of 2	0 of 2
	Hyalella	0 of 2	1 of 2	0 of 2

No PCA expired during any of the replicate trials of the experiment. While we have observed that *Hyalella* sp. and *Lirceolus* sp. can survive long-term in boxes on the same food items as PCAs, *Daphnia* sp. expired within 5 days without supplemental food (yeast, spirulina powder or other).

Pilot Test of Brooding Chambers

We found that the availability of brooding female amphipods was limited. We tested the prototype brooding chambers with the few brooding females available, but did not find any juveniles.

PCA put into brooding chambers on April 30, 2020 were inventoried on June 4, 2020. In Prototype A (the double brooding box), four gravid females were originally

present, but we found no neonates. We found one female, no longer gravid, in the bottom section of the larger chamber. We found three still-gravid females in the larger outer box. In Prototype B (single brooding box), two adult PCA were found in the bottom section. One was gravid and one was not. Only two gravid females were originally put in this box.

After the inventory June 4, 2020, the three gravid females from the Prototype A box were added in with one gravid female in the (single chamber) Prototype B brooding box, which was better for preventing escape. The non- gravid PCA were removed.

On July 20, 2020, we found all four PCAs from the previous inventory plus an additional gravid female that we added on July 9, 2020. Three were found in the bottom open area of the box. Two were found in the single brooding chamber in the lower portion below the divide that was designed to keep adults out. None of the previously gravid females remained gravid.

On September 8, 2020, four gravid females were placed in the experimental brooding boxes-two in the single box design (Prototype A) and two in the double box design (Prototype B). Both contained in one larger black container. Each design had its own water input line. When inventoried on November 10, 2020, three of four females were found, none were still gravid. None were found outside the brooding boxes in the larger container. In the single brooding box (Prototype A), one PCA was found in bottom section no longer gravid; the other was not found. We found no juveniles.

In the double brooding box (Prototype B) we found one female in the side box, no longer gravid. Nothing was found in the main box.

Discussion

Habitat Media Evaluation

We found that Peck's cave amphipods held with medium-density Matala® filtration media had the highest survival in captivity, averaging 80% over 105 days in captivity. This is an improvement on historical survival rates at the SMARC, which averaged 72% in 2017 and 58% in 2018.

Although Kosnicki and Julius (2019) hypothesized that increased habitat heterogeneity should improve PCA survival, our experiments showed that the (heterogenous) mixed media (low density plus medium density filter media) had intermediate survival success when compared to the homogenous, low density media (lowest survival success), and the homogenous, medium density filtration material, which had the highest survival success. Medium-density filtration media similar to that of (Matala®) is recommended for future refugia for PCA.

Evaluation of Alternative Food Items

Our small-scale experiment provided evidence that PCA will consume *Daphnia* sp., *Lirceolus* sp., and *Hyalella* sp. However, *Daphnia* sp. is problematic as a food source for PCA because of its own nutritional requirements. We were unable to keep *Daphnia* sp. alive for more than 5 days after they were introduced into PCA holding chambers. A full-scale experiment comparing PCA survival under diets of *Lirceolus* sp. and *Hyalella* sp. versus a control of standard PCA is warranted as a follow up to this study. Since studies by Nair (2019) indicate PCA are carnivorous, we suggest adding in *Lirceolus* sp. and *Hyalella* sp. (once a month on different weeks) as a supplement to the normal flake slurry.

Pilot Test of Brooding Chambers

We had insufficient gravid female PCA to fully test the two prototype brooding chambers. The limited testing that we were able to perform resulted in no surviving PCA offspring. PCA will eat their young if given the opportunity. Our results suggest that offspring were consumed by adults. We were able to learn that the adult exclusion barrier was unsuccessful and needs to be improved. A full redesign of this experiment is warranted.

References

Dickson, G.W., 1979. The importance of cave mud sediments in food preference, growth and mortality of the troglobitic amphipod Crustacean *Crangonyx antennatus* packard (Crangonyctidae). *Crustaceana*, 36(2), pp.129-140.

Kosnicki, E. and Julius, E. 2019. Life-History Aspects of *Stygobromus pecki*. Technical Report to the Edwards Aquifer Authorty.

Nowlin, W.H., Schwartz, B.F, Worsham, M. and Gibson, R. 2016. Refugia Research: Development of Husbandry and Captive Propagation Techniques for Invertebrates Covered under the Edwards Aquifer Habitat Conservation Plan. Report to the Edwards Aquifer Authority.

Nair, P., 2019. Ecophysiology and Food Web Dynamics of Spring Ecotone Communities in the Edwards Aquifer, USA. Dissertation. Texas State University, San Macros, Texas.

Continuation of Captive Reproduction Techniques in San Marcos Salamanders (*Eurycea nana*)

2020 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Lindsay Campbell, PhD and Kelsey Anderson

San Marcos Aquatic Resources Center

U.S. Fish and Wildlife Service

Table of Contents

Background

San Marcos salamanders (*Eurycea nana*) are a federally threatened aquatic species endemic to San Marcos, TX and to the Edwards Aquifer. While this species has been held at the San Marcos Aquatic Resources Center (SMARC) for years as part of a captive assurance colony, we have yet to predictively produce offspring on demand. San Marcos salamanders successfully breed in captivity at SMARC, but without predictability. In case a future reintroduction event becomes necessary, we need the ability to reproduce many offspring at once, thus the impetus for these investigations. We continue to investigate potential causes for observed reproductive dysfunction behavior in captivity. In 2019, we sacrificed female individuals from wild and captive populations for toxicology and histopathology assessments to reveal potential reproduction inhibitors, such as vitamin deficiencies, heavy metals, toxins, and/or disease. Initial findings showed increased levels of barium in captive individuals. We identified high levels of barium in blackworms (*Lumbriculus* sp.), a common food source for captive amphibians nationwide. We eliminated blackworms from our captive salamanders' diet. We planned follow-up tests to assess any changes in barium levels.

Dr. Ruth Marcec-Greaves, an amphibian reproductive specialist, consulted with the Refugia program on San Marcos salamander reproduction. She recommended testing the efficacy of luteinizing hormone releasing hormone (LHRH) to stimulate reproduction. LHRH is a natural hormone present in all known vertebrates. It is released from the brain to trigger an increase in downstream sex hormones and acts as a pheromone in some species. Additionally, LHRH is safe to handle and can be reconstituted and stored frozen for up to three years.

Objectives

The objectives for 2020 were to investigate questions raised from past years' research on reproduction and to continue to evaluate different techniques to induce reproduction in San Marcos salamanders. The following are activities we pursued to meet our objectives.

1. We asked Dr. Ruth Marcec-Greave to evaluate our salamander husbandry practices and provide advice.

2. We tested the use of Luteinizing Hormone Releasing Hormone (LHRH) in a pilot study, to be scaled-up to full experiment if successful.
3. We offered different food sources to reduce barium levels in salamanders.
4. We followed up with our regional veterinarian and other animal health professionals on a broad spectrum of health tests.

Methods

LHRH Pilot Study

At the end of 2019, we contacted Dr. Ruth Marcec-Greaves, a veterinarian and salamander reproduction expert currently working at the Detroit Zoo. Dr. Greaves has specialized in assisting reproduction in several captive amphibians and created the protocol for priming and administering propagation hormone regimes for many amphibians, including the Houston toad (*Anaxyrus houstonensis*). Based on data collected during last few years of reproduction trials, we decided collectively (Dr. Marcec-Greaves, Dr. Campbell, and Kelsey Anderson) to test the effectiveness of luteinizing hormone releasing hormone (LHRH). LHRH is also known as GnRH, or Gonadotropin releasing hormone. This hormone is present in all known vertebrates and in many invertebrates. In vertebrates, it is released from the brain to trigger an increase in downstream sex hormones (see review in Okubo & Nagahama 2008). It also acts as a pheromone in some species (Moore 1983,1987). It works on multiple hormones (Luteinizing hormone and Follicle stimulating hormone, testosterone and estrogen, and others around multiple systems; Marques et al., 2018, see review in Okubo & Nagahama 2008). Dr. Marcec-Greaves travelled to the SMARC in February 2020 to assist with the pilot trial of LHRH and to evaluate our salamanders.

San Marcos salamanders are small and have an obligate aquatic lifestyle so, we decided to apply the LHRH topically rather than inject it or immerse our salamanders into a bath containing LHRH. We selected the standard topical dosage of LHRH used for amphibians (25 µg/g) for the pilot study. Hormone was topically applied on the head where pheromones are best absorbed, transmitted, and smelled. This method, called the "nose-drop" method (Marcec-Greaves *pers. comm.*), is cost-effective and allows researchers to know exactly how much hormone was deposited on the animal's vomeronasal receptor area (Figure 1).

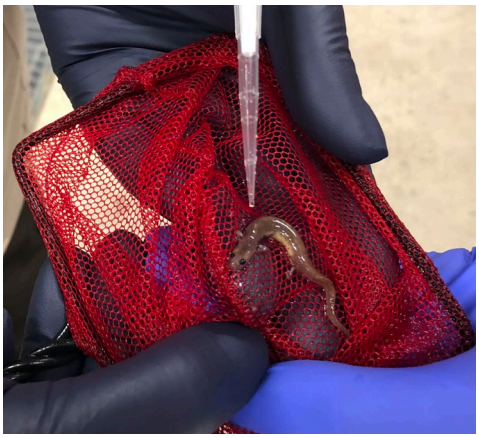


Figure 1 Application of LHRH to San Marcos salamander via the nose-drop method.

In preparation for Dr. Marcec-Greaves' visit and application of LHRH, a subset of San Marcos salamanders were separated by sex into flow-through aquaria in January 2020. The remaining male and female salamanders (11 females, 15 males) from our heritage San Marcos salamander population, and 26 individuals (13 males, 13 females) from the younger Standing Stock population, were separated by sex (and population type) and placed in aquaria completely isolated each other and other salamanders. Biologists combined the males and females into the same system (one for each population), but without access to each other a week before the application of LHRH, on February 18, 2020.

Reproduction experiments took place in two rectangular tanks (102 in length x 18 in width x 6 in water depth) with groups separated by screen dividers. Each system had a sump, a pump, a heater-chiller unit, and a UV-sterilizer. Water was on partial re-circulation, with fresh, chilled well-water input to the sump. Due to health concerns, including previously high mortality rates and disease prevalence in the heritage population, the two populations were separated into different systems. No equipment or water was shared between populations at any time during experimentation.

The day before application, LHRH powder was reconstituted using distilled water at a concentration of 2 mg LHRH:1 mL water or 2 µg LHRH: 1µL water. We pipetted small aliquots (200 µL) of the solution into individual vials, labelled, and stored hormone that was not needed for pilot study application in a -20 °C freezer.

On February 25, 2020, salamanders from the two sex-segregated systems were removed and placed temporarily into coolers by sex and group. The tanks were cleaned and filled with new habitat items and video monitoring cameras were hung above each section. For LHRH application, a biologist held one individual in a damp net while a second person applied 12.5 µL of LHRH solution (25 µg) over the nose and face using a micropipette. Each animal was held in the net after application for 30 seconds before being placed into a dish with clean well water. Pipette tips were changed between individuals. Hormone administration was demonstrated and overseen by Dr. Marcec-Greaves (Figure 2).

Figure 2 Dr. Lindsay Glass Campbell (Left) holds a salamander as Kelsey Anderson (Right) applies LHRH. Dr. Ruth Marcec-Greaves (Center) oversees the technique.

Salamanders were treated in groups of six, before being added to their designated tank section. Females were loaded into a section first, then the males. In short, all males were dosed with LHRH, but only half of the females were dosed. The treatment groups from the salamanders that had been sex separated were: Heritage females (five) and males (five) dosed, heritage females (five) not dosed and males (five) dosed, Standing stock females (six) and males (six) dosed, and Standing Stock females (six) not dosed and males (six) dosed (Figure 1). Biologists then collected 12 gravid females and 12 males from mixed-sex systems to compare with the salamanders that had previously been separated by sex. Of these, six females received hormone and six did not, all males received hormone (Figure 3). These were added to the Standing Stock system in their own sections.

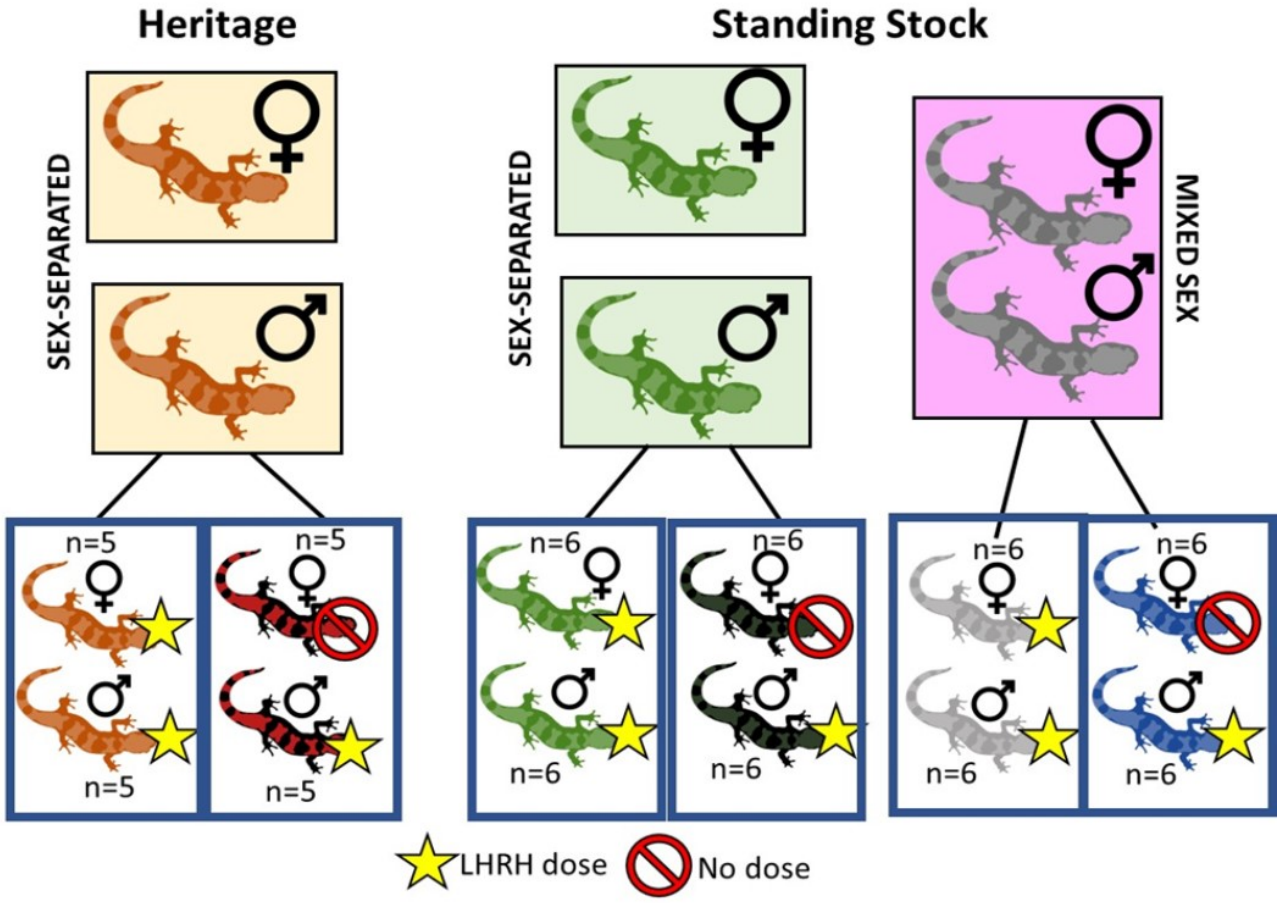


Figure 3 Diagram illustrating how each population was divided into treatments. A subset of salamanders from the Heritage and Standing Stock populations were separated by sex prior to the LHRH treatment. We also tested salamanders from the Standing Stock population that had been in mix-sex tanks prior to LHRH treatment.

The lights were dimed in the room after salamanders were placed and low activity was conducted around the tanks for the remainder of the week. During the first 24 hours, fresh well-water input was turned off to the systems to maximize the circulation of pheromones. Low levels of fresh well water continued for the next three days, before returning to normal input levels. The experiment ran for three months, after which animals were moved back into either Standing Stock systems or the heritage system. Staff removed egg clutches within 72 hours of oviposition to a separate glass aquaria.

LHRH Upscaled Experiment

After no deleterious effects from LHRH were observed in the pilot experiment, we attempted a large-scale experiment using all mature captive wild stock San Marcos salamanders. In preparation for this trial, all Standing Stock San Marcos salamanders were sex-segregated into four systems (two male, two female), with 50 to 65 animals within each system on May 29, 2020.

Biologists prepared recirculation set-ups for each of the four tanks, including pumps, sumps, and UV-sterilizers. Though in place, systems were not converted to individual recirculation set ups until the day salamanders were put in their treatments groups and LHRH was applied.

Dr. Marcec-Greaves recommended a higher dosage of LHRH (50 µg/g) due to low enthusiasm for courtship observed on the video recordings and lower than expected reproductive output during the pilot experiment. For this full-scale study, we applied LHRH only to males, as they initiate courtship in this species by producing pheromones in their mental glands and rubbing them on the females to induce receptivity. In our digital video of these salamanders in the 2019 and 2020 pilot experiments, males did not appear to be as interested in initiating and pursuing courtship as expected, many times not participating in courtship behavior at all. We also considered the possibility that the addition of hormones might increase female ovarian ruptures, though no negative results were seen in the pilot experiment. We prepared the LHRH solution following the same procedure as the pilot study, but at the higher recommended concentration, on the day of application.

On August 27th, biologists removed the salamanders from the systems and temporarily placed the sexes in separate large coolers of fresh water. The four systems were cleaned and habitat items were replaced. Biologists switched the systems from flow-through to recirculation. The treatment groups were randomly assigned to each of the four tanks: two tanks where males were dosed with LHRH and two control tanks where males were not dosed (no females received LHRH).

Groups of 25 females were selected without bias and distributed into the four tanks. All females selected exhibited some level of gravidity; those that were non-gravid or appeared in poor condition were not used for this trial (n=3). We allowed females at least 30 minutes to explore the tank before adding males.

Males were netted in groups of five and held in a large beaker of water. One at a time, each male received LHRH dose on the nose/head. To increase absorption of the hormone, each male was placed into a dish covered in wet paper towels for three minutes. The salamander skin remained moist and they did not exhibit any signs of distress. After three minutes, LHRH-dosed males were transferred to a cooler with others from their treatment group (25 per group). In total, 50 males received LHRH and 50 males did not receive LHRH.

Biologists dimmed the lights after salamanders were placed and conducted minimal activity around the tanks for the remainder of the week. During the 48 hours following males and female combination, the systems were set to full recirculation (with no incoming fresh water) to retain pheromones. Then, for another 48 hours, fresh water input was resumed, but kept at a low level. Finally, normal input flow of fresh well-water resumed, taking the system back to partial re-circulation augmented with fresh water flow-through. Cameras recorded interactions for the first 72 hours after combination. As eggs were observed, staff removed eggs within 72 hours of oviposition to a separate glass aquaria.

Salamanders are to remain in these systems for 6 months after LHRH application. As of November 2020, this study was still in progress; historical records from SMARC indicate more clutches were produced between November and February for this species.

Salamander Health

After one year without blackworms as a food source, we sent an additional three female individuals from our heritage population of 3+ year old captives and three females from the Standing Stock population (that were not in the scaled-up LHRH experiment) for heavy metal testing. The results were compared to that from 2019 to assess whether blackworms were associated barium levels. We also received a histology report from salamanders sent in 2019.

Habitat Alteration

At UNFH we set up two research systems for pilot testing habitat modifications to increase San Marcos salamander reproduction. Each system is capable of holding 20, 5.5-gallon aquaria per shelf. Staff purchased equipment for water conditioning: including coarse filtration through 100 and 50 micron pleated filters, UV sterilization of 40 ms/cm/sec, a sedimentation collection box and biofilter. Staff prepared 25 5.5-gallon aquaria by cutting holes and installing new bulkhead fittings, filtration, sterilization, delivery, supply plumbing, and 25 additional 5.5-gallon aquaria. All the habitat items needed for the study have been obtained and constructed. However, staffing limitations has led to postponement of the habitat alteration study until 2021.

Results and Discussion

LHRH Studies

We observed no deleterious impacts from LHRH in either the pilot experiment or the scaled-up experiment. However, we have not observed any increase in clutches laid after the application of LHRH.

Dr. Marcec-Greaves reviewed video of the pilot experiment with biologists and confirmed that courtship behavior was occurring, but at lower than expected levels. This was consistent with what we observed in 2019, and lead to the decision to increase the dose of LHRH in the scaled-up experiment. Three clutches were produced during the pilot experiment from Standing Stock groups that had been sex separated. The sex separated, male-only LHRH dosed group produced two of the three clutches. Neither the Standing Stock groups that had not been separated nor the heritage salamanders that had been separated produced egg clutches.

We decided to use large groups of salamanders for the scaled-up trial, as in practice this would be the most efficient way to produce offspring rather than dividing them into smaller groups. We agreed with Dr. Marcec-Greaves that the larger rectangular tank without dividers held more room for the salamanders to court and separate themselves from one another. Habitat enrichment of rocks and artificial vegetation also allowed for salamanders to distance themselves from one another in the tank as needed.

After three months, salamanders produced three clutches, two of these clutches came from groups where males received LHRH and the other from a control group. As of November 2020, salamanders still remain in their experimental reproduction groups and more clutches may be produced. The study will be re-evaluated six-months post LHRH application (in February 2021). Potentially, application of higher doses of LHRH to the females could be explored, but it is unclear if higher dosages of LHRH would produce increased oviposition of clutches.

Many salamander species in the wild naturally separate by sexes (Marcec-Greaves *pers. comm.*). Sexes later come closer together where they sense each other, and then finally interact for mating. We discussed an alternative husbandry plan with Dr. Marcec-Greaves where we would sex separate the San Marcos salamanders for a while, sharing water but not access to each other, and then combine them throughout the rest of the year. We would try to mimic this in refugia by cycling through these periods throughout the year. For example, salamanders would be separated by sex in different systems for three months. Then for two weeks, water would be exchanged between systems with males and females (or in same tanks but separated by dividers). Then we would combine the sexes in systems for three months. Finally, we would restart the cycle by separating the salamanders again.

We speculate that when the salamanders have constant access to potential mates, there might not be as much of a drive to mate as that resource is always available. Separation for a period of time and then combination could create an instinctive behavior to act on a resource (access to mates) that has not previously been available. The partial separation phase may also be important to prime hormone production in males and females for courtship. When animals are combined in mixed groups, recirculation of water is recommended by Dr. Marcec-Greaves heighten the matting response due to retaining pheromones being produced.

Salamander Health

There was no decrease in barium levels in captive salamanders tested in 2020 versus those tested in 2019 (Figure 4). We thought that after removing food high in barium, salamander bodies would naturally clear some of the accumulated barium, but our results do not support this hypothesis. Selenium levels of captive individuals were lower than wild individuals (Figure 5), while copper levels are also slightly elevated in captives compared to wild individuals (Figure 6). Zinc levels generally increased with time in captivity (Figure 7). We still do not know what impacts these different levels may have, if any, on the salamanders. A full table with individual results from the metal panel can be found at the end of the document (Table 1)

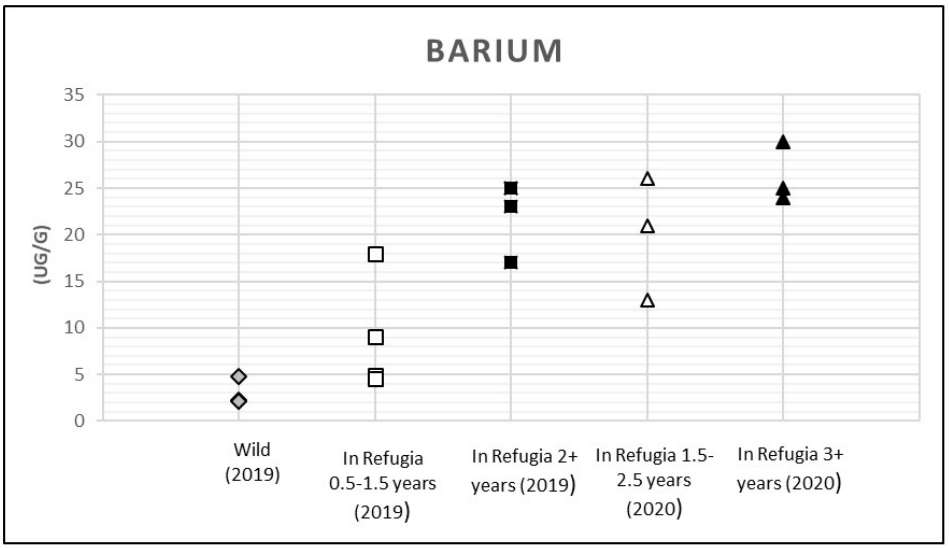


Figure 4 Barium levels in wild salamanders, captive salamanders tested in 2019, and captive salamanders tested in 2020.

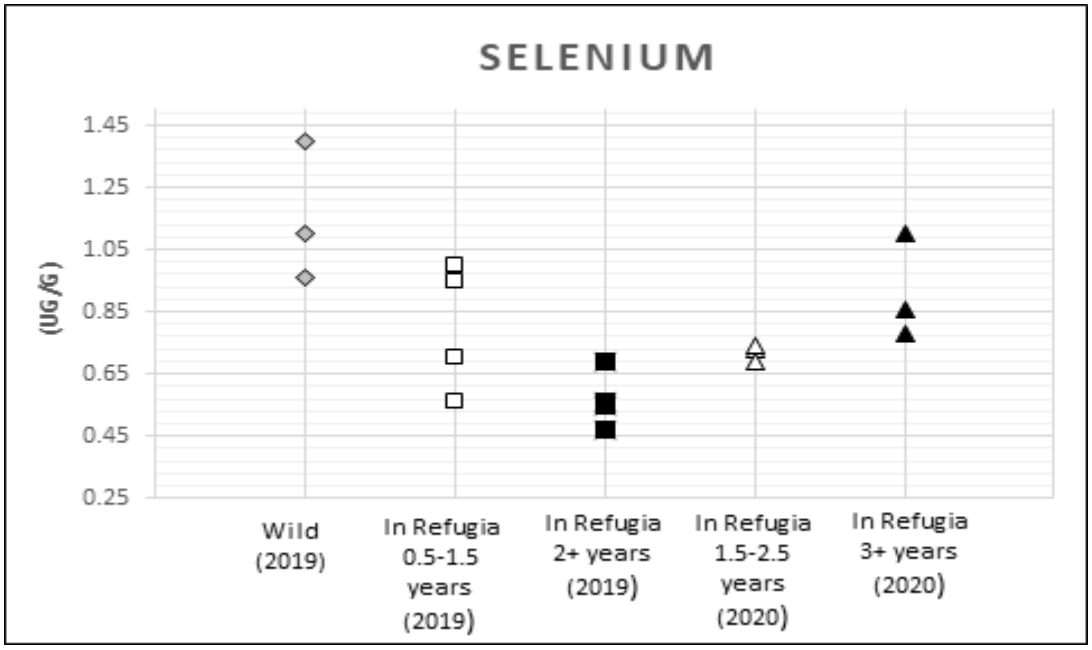


Figure 5 Selenium levels in wild salamanders, captive salamanders tested in 2019, and captive salamanders tested in 2020.

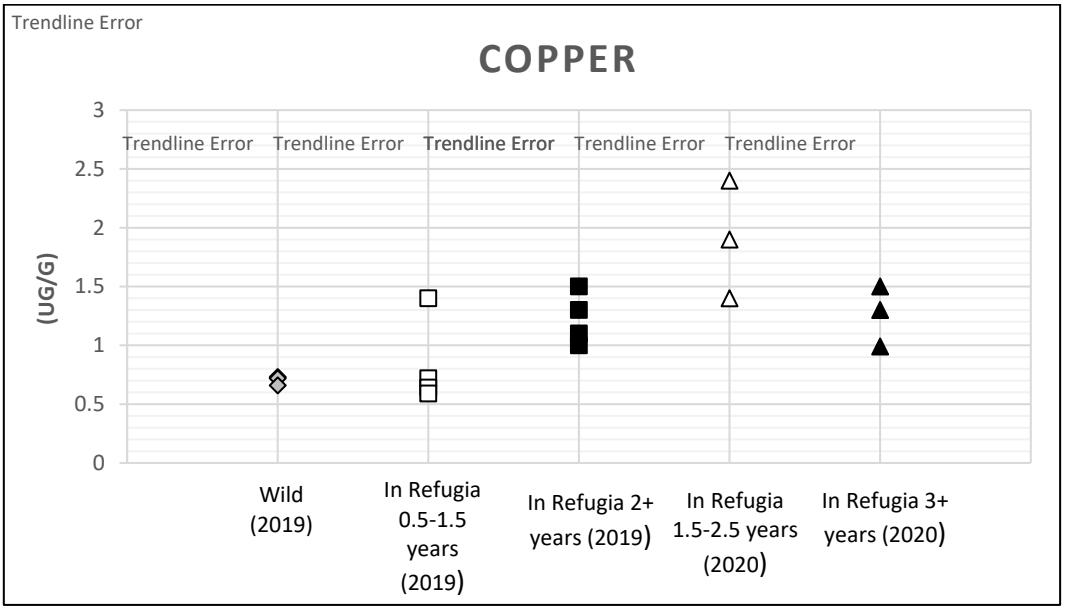


Figure 6 Copper levels in wild salamanders, captive salamanders tested in 2019, and captive salamanders tested in 2020.

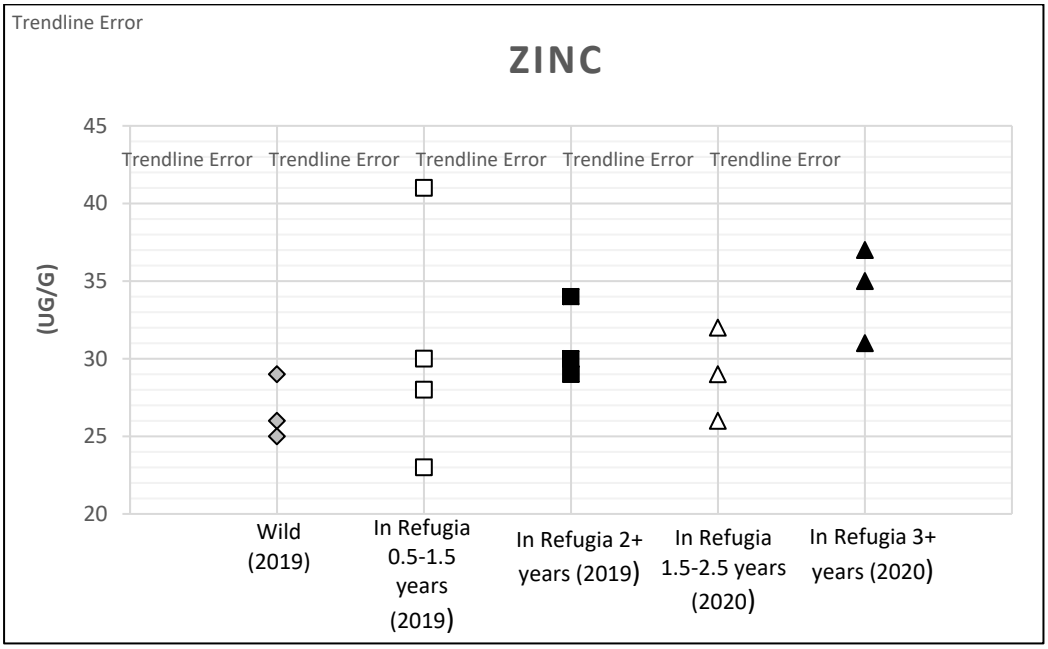


Figure 7 Zinc levels in wild salamanders, captive salamanders tested in 2019, and captive salamanders tested in 2020.

Dr. Allen Pessier sent us a final histology report of the salamanders we sent to him in 2019, noting ovarian microsporidiosis (Figure 8 and report PDF attached). One of the salamanders labelled “recent captive” had ovarian microporidiosis, which he believed likely started in the wild. Mycobacteriosis was observed in both short- and long-term captive salamanders. Dr. Pessier said:

> *The pattern of mycobacterial lesions in these salamanders is interesting. Many develop lesions in the coelomic cavity and lesions tend to be more severe in the caudal body. Possibilities would include ascending infections from the environment via the cloaca/reproductive tract or via introduction of environmental bacteria via skin punctures…*

He also found that (Figure 9):

> *Toe tip chytridiomycosis was observed in both shorter term captives and freshly wild caught salamanders. Infection has previously been observed in diagnostic submissions. Chytridiomycosis in the San Marcos Salamander appears to be mostly an incidental finding although I have suspected in some cases that chytrid-associated hyperkeratosis may have pre-disposed to secondary fungal/Oomycete/bacterial infections of the feet. Because this species only appears to have keratinized skin on the feet chytrid infections remain limited to this location.*

We plan to complete the habitat manipulation portion of this study in 2021. At that time a full synthesis of all the various findings from 2018 to 2021 on San Marcos salamander reproduction and health will be assembled.

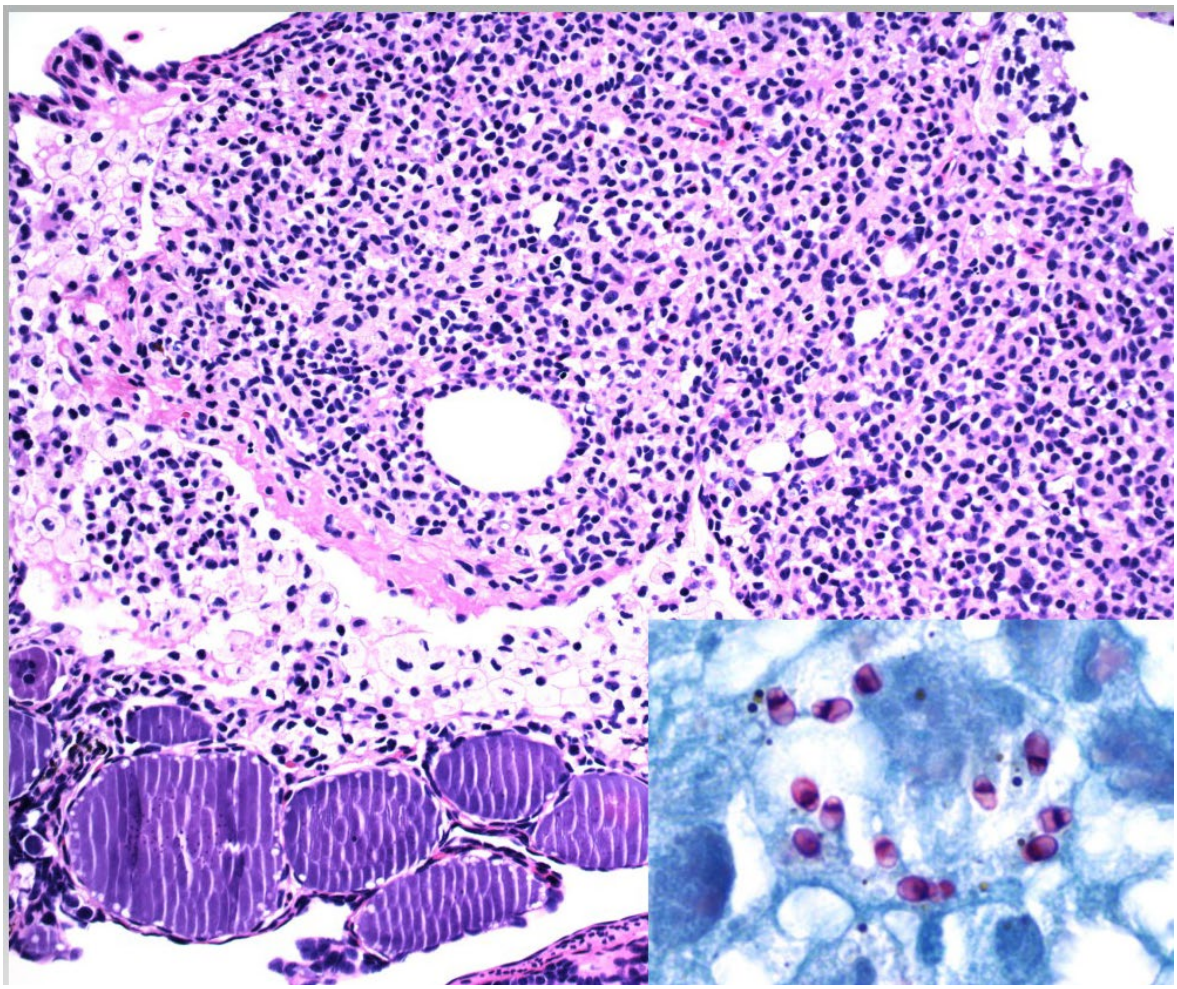


Figure 8 Section of San Marcos salamander ovary with histiocytic inflammation (top 2/3 of the photo). Normal ovarian cells are seen at the bottom of the photo as large purple cell. The inset shows microsporidial spores within the area of inflammation stained with Fite's acid fast.

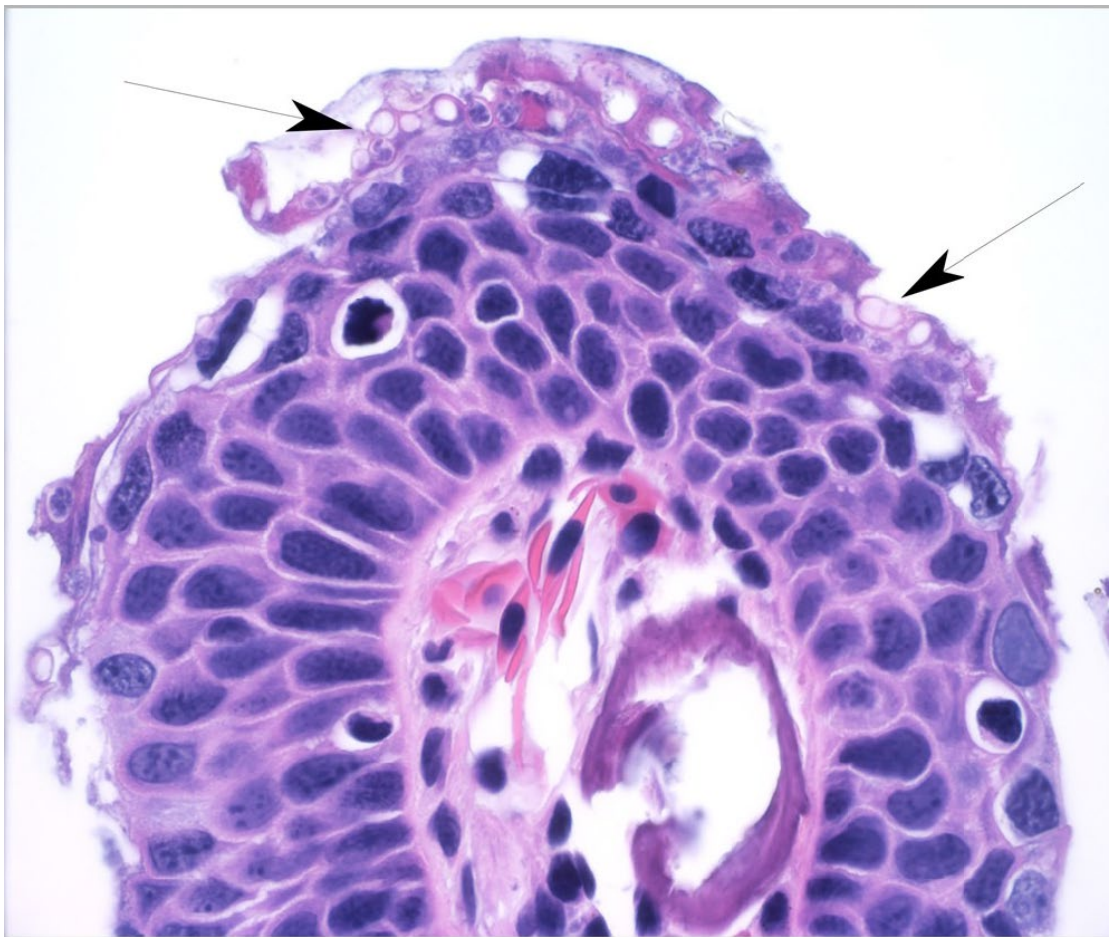


Figure 9 Thalli of the chytrid fungus *Batrachochytrium dendrobatidis* (arrows) within a focus of hyperkeratosis on the toe.

Table 1 Trace mineral results from whole body analysis of representative salamanders from three groups: wild, heritage and younger captives. Heritage and younger captives were re-tested in 2020.

Mineral	Wild (2019)			Heritage Refugia (2019)				Younger Refugia (2019)				Heritage Refugia (2020)			Younger Refugia (2020)		
	W1	W4	W5	C4	C5	C6	CX	C1	C2	C3	C15	HA	HB	HC	NHA	NHB	NHC
	Results (ug/g)			Results (ug/g)				Results (ug/g)				Results (ug/g)			Results (ug/g)		
Calcium	8600	9800	8300	11000	10000	8800	8800	8700	11000	4600	10000	N/A	N/A	N/A	N/A	N/A	N/A
Phosphorus	7700	8000	7200	7300	7600	7200	7000	6900	7700	9200	7100	N/A	N/A	N/A	N/A	N/A	N/A
Chromium	0.06	0.052	0.073	0.057	0.054	0.052	0.052	0.051	0.055	0.046	0.21	0.2	0.051	0.045	0.06	0.044	0.05
Manganese	4.5	8.9	6.3	7.3	3.1	6.6	3.3	3.5	8	7.4	3.2	6.7	7.7	5.8	2.8	6.3	3.5
Iron	16	25	17	25	27	26	26	40	32	31	27	38	33	23	19	19	26
Cobalt	<0.03	0.038	<0.03	<0.03	0.027	0.022	0.022	0.044	0.051	0.046	<0.04	0.022	0.024	<0.03	<0.03	<0.04	<0.03
Copper	0.73	0.72	0.66	1.5	1.3	1.1	1	0.72	0.64	0.59	1.4	1.3	1.5	0.99	2.4	1.9	1.4
Zinc	25	29	26	34	30	29	29	30	23	41	28	35	31	37	29	26	32
Arsenic	0.031	0.035	0.032	<0.03	<0.02	0.024	0.02	0.035	0.041	<0.03	<0.04	0.037	0.054	0.073	0.057	0.056	0.056
Selenium	0.96	1.4	1.1	0.55	0.56	0.69	0.47	0.7	1	0.95	0.56	1.1	0.86	0.78	0.73	0.69	0.74
Molybdenum	<0.03	<0.03	<0.03	<0.33	<0.02	<0.02	<0.02	<0.03	<0.03	<0.03	<0.04	0.026	0.024	<0.03	<0.03	<0.04	<0.03
Cadmium	<0.03	<0.03	<0.03	<0.33	<0.02	<0.02	<0.02	<0.03	<0.03	<0.03	<0.04	<0.02	<0.02	<0.03	<0.03	<0.04	<0.03
Barium	2.2	4.8	2.1	23	25	17	25	18	4.9	4.6	9.1	24	25	30	21	13	26
Lead	<0.03	0.2	0.035	0.05	0.045	0.037	0.036	<0.03	0.036	0.068	<0.04	0.051	0.066	0.066	0.03	<0.04	0.035

FINAL REPORT

INCREASING PUPATION SUCCESS IN THE COMAL SPRINGS RIFFLE BEETLE IN A CAPTIVE SETTING

Prepared for:

San Marcos Aquatic Resources Center
U.S. Fish and Wildlife Service
500 East McCarty Lane
San Marcos, TX 78666

Prepared by:
Ely Kosnicki, PhD – Senior Invertebrate Ecologist
BIO-WEST, Inc.
1405 United Drive, Suite 111
San Marcos, TX 78666

TABLE OF CONTENTS

Executive summary

Maintaining a captive-propagating population of the Comal Springs riffle beetle *Heterelmis comalensis* is an important goal of the Edwards Aquifer Habitat Conservation Plan (EAHCP). The main objective of this study was to examine factors that may enhance captive pupation success. A second objective was to track the fecundity of first generation captive reared (F1) females and to use this information to estimate how many adults would be required to maintain a captive population.

Trials of flow-through tubes utilizing 2-inch PVC pipe and fittings were used to test whether or not late-instar larvae given quality-flow habitats and access to more air would improve pupation success and eclosion to adult. Different resource materials were also tested. Post-trial treatments were implemented to examine other factors that may influence pupation; trials included small flow-through tubes, starvation, terrestrial, and modified flow-through container habitats. Twenty adult females reared from these experiments, and four additional F1 females from other studies, were paired with males and tracked for a number of viable larvae produced over time. Two additional trials, one with small and one with medium-sized larvae, were also implemented in order to get a better idea of larval survivorship.

Late-instar larvae that were given more access to air were more likely to successfully pupate (*F-value* = 17.852.431, *p-value* = 0.0029), with most individuals eclosing to adult. Late-instar larvae given conditioned cotton, wood, and leaves, were not found to pupae at a higher rate compared to late-instar larvae that were only provided leaves. Post-trials were mainly observational but resulted in 16 out of 75 larvae pupating. From the 24 females tracked until death, 703 larvae were produced with an average of 29.3 ± 37.1 larvae per female. Female size was not found to be related to number of larvae produced but number of days over all inspections was (*F-value* = 47.870, $p < 0.001$, $R^2 = 0.685$). Five out of 22 larvae pupated and eclosed to adult from the medium-sized trial after 183 days and 15 out of 63 larvae pupated (14 eclosing to adult) from the small-sized larvae trial after 211 days. A total of 74 adults eclosed during this study and the sex ratio of F1 adults produced was not different from a 50:50 ratio ($X^2 = 1.515$, *p-value* = 0.218).

From this study, it can be estimated that a colony consisting of 10 females surviving 60 days with unlimited access to mates would produce ca. 185 larvae. Conservatively using a 12 % survival rate (half that of the observations from the small-size trial), 22 larvae would be expected to become adults. With a 50/50 sex ratio demonstrated in this study, 11 would be F2 females. If F2 females have the same fecundity and survivorship as F1 females, a perpetual captive colony could be expected.

Introduction

The Comal Springs riffle beetle (*Heterelmis comalensis*) Bosse, Tuff, and Brown (1988) is a troglobitic beetle from the family Elmidae (Coleoptera) known from springs at Comal Springs and San Marcos Springs, in Comal County, TX and Hays County, TX, respectively (Gibson et al. 2008). Critical habitat designated for this species includes 15.56 ha of surface area at Comal Springs and San Marcos Springs, collectively (USFWS 1997 and 2013). All known locations of this species are found within the Edwards Aquifer, the primary water source for the city of San Antonio and agricultural production within Central Texas (Edwards Aquifer Authority 2019. https://www.edwardsaquifer.org/science-and-maps/about-the-edwards-aquifer, accessed 13 June 2019).

Heterelmis comalensis, like other species of the Edwards Aquifer face many threats to their ecosystem, including but not limited to over pumping of the aquifer, pollution, and adverse effects due to invasive species (Bowles and Arsuffi 1993). Having a functional refuge is a requirement of the US Fish and Wildlife Service (USFWS) (1996) and maintaining a captive-propagating population is a key goal of the Edwards Aquifer Habitat Conservation Plan (EAHCP). Although wild collections have been kept at the USFWS San Marcos Aquatic Resources Center (SMARC) since 1996, maintaining a self-propagating population has had varied success (Fries 2003, Huston and Gibson 2015). Numerous larvae can be produced within captivity; however, getting first captive generation (F1) larvae to become adults has been more challenging. This study was designed to investigate factors that could increase pupation success of captively reared larvae.

Extensive field collections by Bowles et al. (2003) showed that pupae of *Heterelmis comalensis* were rarely sampled but were found in January, April, July, and October, indicating that they are non-seasonal as is similar for emergence patterns of other elmid species (Shepard 2002). Huston and Gibson (2015) provide the only known observations of captive pupae of *H. comalensis*. Pupae did not make pupal chambers, but were found under rocks, leaves or within folds of conditioned cotton substrates (Gibson et al. 2008). Pupae are exarate, have hydrophobic hairs, and will float to the water surface if disturbed from underwater air pockets. Most importantly, pupae were observed to have an air bubble associated with them and the pupae died when the air bubble was removed. These observations along with morphological inspection revealing that they have no other modifications for respiring underwater, indicate that *H. comalensis* pupae respire through the use of a compressible gas gill.

Evidently, flow is of great importance to the survival of adults and larvae and there has been noted success in utilizing flow-through tubes (BIO-WEST 2017 and Gibson personal communication 2018). Field studies have shown that *H. comalensis* is restricted to active springs (Cooke et al. 2015) and laboratory studies have shown that adult beetles tend to move in the direction of flow (Cooke et al. 2015), but may move towards flow (BIO-WEST 2002), presumably, when conditions approach stagnation (see Cooke et al. 2015). Also, a long-held belief has been that a conditioned cotton substrate has been one of the more important resources for rearing this species in captivity and may be important for pupation success (Huston and Gibson 2015; BIO-WEST 2017). The objectives of this study were to examine these parameters with regard to improving pupation success.

Although numerous larvae are produced in captivity, little is known about female fecundity. Recently, male and female adults have been able to be reliably separated, using internal structures that can be seen with careful lighting techniques (Kosnicki 2019). With this, newly reared females can be paired with males so that numbers of captively reared individuals can be determined. By establishing an estimate of female fecundity coupled with the success of newly hatched larvae to eclose to adults, a simplified life table could be developed in order to estimate the minimum number of individuals need for maintaining self-propagating captively-reared colony. The data collected from this study suggests that 11 F1 females given access to a mate is enough to maintain such a colony under conditions similar to this study.

Methods

Pilot testing and late-instar study

Heterelmis comalensis is a federally listed endangered species. Because of the limitation on available test subjects for research purpose, a series of pilot studies were implemented with varying parameters with the intent of using the most successful for a study design. Because pupation success was the

ultimate goal of this study, late-instar larvae were obtained from the USFWS at the SMARC. Late-instar larvae were identified as having a head capsule width of 0.406 ± 0.015 mm and were considered in the final or penultimate instar (see BIO-WEST 2017). Late-instar larvae were chosen because they would be relatively close to pupation, requiring less time to assess results, and would provide a proof of concept to be implemented for earlier instars. To assess pupation success with given parameters, a series of trials with late-instar larvae were implemented.

For each trial, test subjects were placed into flow-through tubes constructed from 19 cm (7.5 in) long 2 in PVC pipe and fittings (**Fig. 1**). The housings consisted of female slip/threaded couplings fitted over each end of the pipe and welded. Caps were made for each end by fitting a 2 in slip x ½ in threaded bushing into a 2 in male threaded female slip coupling with a 255 µm plastic mesh placed in between and welded to form a seal. A ½ in threaded spigot was screwed into the bushing with plumber's tape to create a seal. The threaded couplings of the caps were then screwed into each end of the housing to create the flow-through tube. A hose leading from a flow-bar valve supplied with fresh well water directly from the Edwards Aquifer was attached to one of the flow-through tube spigots. Each tube had a full capacity of 650 -700 mL but 470-520 mL minus the screened off cap space.

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

Each trial utilized two flow-through tubes, one placed in an upright position with the water source flowing from the bottom, thus filling most of the tube save for air bubbles formed by atmospheric gases coming out of pressure. The second tube of each trial was placed horizontally on its side, allowing for a small gap of air to be maintained. The difference between a tube placed horizontally or upright was ca. 50 – 100 mL of air space.

Pilot trials utilized various materials packed in different configurations and different flow regimes in order to identify conditions that would be appropriate to consistently test for pupation rates of tubes with more air pockets (horizontal position) compared to tubes with less air (upright position). Packing materials consisted of combinations of conditioned leaf, wood, and cotton cloth, plastic mesh, and limestone. Conditioned leaves were almost exclusively *Platanus occidentalis* L., with some of the earlier pilot trials including leaves of *Ehretia anacua* (Terán and Berland), and/or *Juglans* sp. Dried leaves were placed in flow-through containers at the SMARC and submerged with Edwards Aquifer well water. After biofilms developed on the leaves for two to four months, the leaves were considered conditioned as a resource for *H. comalensis* subjects. Conditioned wood consisted of 1 cm diameter poplar dowels cut into lengths of 15 cm and were submerged in flow-through containers of Edwards Aquifer well water for at least three months. Conditioned cotton cloth consisted of 10 x 10 cm square cuts of 40/60 % polyester/cotton blend bed sheets. Cotton cloths were submerged in flow-through containers containing Edwards Aquifer well water and were considered usable after allowing biofilms to

accumulate for one to three months. One-millimeter plastic mesh was either used to line the circumference of the inside of the tube housing on which other materials could be placed and slide into the tube, or the mesh was rolled into a 1 cm diameter tube of 15 cm length and held together with hot glue. These plastic mesh tubes were placed in the center of the flow-through tube housing to help maintain flow through the center of the tube. Limestone rocks of large gravel to small pebble sizes were submerged in Edwards Aquifer water before use within a tube. Flow was measured by filling a beaker to ca. 500 mL and timed using a stop watch. This measure was performed three times and the average was used to determine the discharge of each tube at the start and completion of each trial.

Flow-through tubes of the same trial contained the same packing material and had similar flow regimes at the start of the trial, only differing by being placed in an upright or horizontal position as described above. After each tube was packed with resource material, 20 late-instar larvae of the same F1 cohort were added to each tube, respectively. The tube was then capped and a hose with flowing Edwards Aquifer water was fit to one end. Discharge was measured about once per month and after ca. 90 days the entire contents of the tube were inspected for larvae, pupae, and adults; additional biological information such as exuvia or early instar larvae was also recorded. Pilot trials were considered to have a successful packing configuration and flow regime if one of the tubes reached a benchmark of four or more of its subjects (≥ 20 %) transforming into a pupa, with the assumption that larvae may not pupate unless certain conditions are met (see Brown 1973). Once either tube of a pilot trial was found to have four or more subjects transform into a pupa or adult, that trail was replicated two more times with the same packing configuration and flow regime. The goal was to have at least three trials of two separate packing configurations, one with cotton and one without, so that two-way ANOVA could be used to test between packing configuration (with vs. without cotton cloth) and tube position (upright vs. horizontal).

Post trials and fecundity study

Larvae that had not transformed to pupae or adult at the end of their initial trial were relaunched within the same container with the same conditions in the case where eight or more (≥ 40 %) living larvae remained. Larvae remaining at the end of other trials were moved to another environment in order to assess pupation success under other conditions: small flow-through tube, flow-through contain, terrestrial, or starvation. Small flow-through tubes consisted of 8 cm long 1 in PVC pipe, caped with 1 in couplings and bushings with a 255 µm mesh, similar to the flow-through tube described above. Flow-through containers consisted of a small sandwich box with a hole cut in the lid towards one end for a hose to deliver fresh water. A hole was cut on the wall of the opposite end and screened with a 500 µm mesh, sealed with hot glue. The screen served as a drain and maintained a water level depth of ca. 1 cm. Modified flow-through containers had conditioned leaf and/or cotton cloth placed on the bottom with two emergent limestone rocks placed on top of the bottom material at opposing corners, and emergent conditioned wooden dowels. Starvation habitats utilized the same flow-through container but only had emergent rocks. The terrestrial habitat was similar to the starvation habitat, except that the screen was cut on the bottom of the container so that only a film of water was provided for larvae. Pupae found alive were moved to pupation chambers. Pupation chambers were the same as the modified flow-through boxes described above for the larvae and pupae were either placed on the surface of a wooden dowel or rock, and allowed to eclose for up to one month.

Adults developed at any point during this experiment (after the initial trial, relaunch, post treatment experimentation, or pupation chamber) were measured lengthwise from the posterior tip of the scutellum to the posterior mediad of the elytra. Adult females were matched with a male from this experiment, a separate colony, or wild caught individuals and placed into a mating chamber consisting

of a 1 in flow-through tube described above. Each mating chamber contained a conditioned wood dowel and conditioned leaf. Mating chambers were inspected ca. once per month to see if the adults were still alive and to count and remove recently hatched larvae. The contents of the tube, including water, resources, and adults, were replaced back into the tube so that additional larvae could be produced and counted at a later time. When the female was alive and the male found dead during an inspection, another male (when available) was used to replace him and fecundity observations were continued for that female. Females were tracked for total fecundity until dead. The total number of larvae produced was regressed over female size and length of time over all inspections to determine if those relationships existed. In this way F1 female fecundity was determined in terms of second captive generation (F2) living-larvae produced.

Early-instar investigations

After the late-instar trials were conducted, additional trials were initiated with 22 medium-sized (presumably third – fifth instars; HCW = 0.25 ± 0.07 mm) and 63 small-sized (first – second instars; HCW = 0.16 ± 0.1 mm) F1 larvae to help gain a better understanding of larval survivorship and overall pupation success. A second small-sized flow-through tube was prepared with 53 individuals; however, this trial still had > 50 % of its larvae alive in December 2020 and is not discussed further in this report. These trials only consisted of one flow-through tube with packing from one of the successful late-instar trials. Survivorship of these trials with fecundity estimates per female was established, which allowed a preliminary estimate of the number of females needed to maintain a captive self-propagating colony to be calculated.

The first late-instar trials began on 15 January, 2019 and new trials were initiated ca. once a month up to 27 August, 2019 at which point the supply of late-instar larvae were not in sufficient supply. The final late-instar larvae trial was initiated on 27 February, 2020 and was concluded on 4 June, 2020. The medium and small sized larvae were initiated on 15 April, 2020 and concluded on 15 October, 2020, and 12 November, 2020, respectively. Water quality of the well water was checked at least once per month over the course of the study, including pH, DO mg/L, Conductivity, and temperature with a YSI ProDSS (Yellow Springs, Ohio, USA). In addition, three Thermochron iButtons, manufactured by Dallas Semiconductor (a subsidiary of Maxim Integrated Products, Sunnyvale, California, USA) temperature loggers sealed in Gorilla Tape® and placed within a flow-through tube were averaged and used to calculate degree days per trial unit. Base R (R statistical software version 3.4.1; R Core Team 2017) was used to perform 2-way ANOVA and calculate degree days, while regression analysis was performed in Microsoft Excel 2016 (Redmond, Washington, USA). Photographs were taken with a NIS-Elements imaging source package, including acquisition and analysis software, and a HD color camera (Nikon Corporation, Tokyo, Japan). The camera was mounted on a Nikon SMZ 18 stereoscope, and measurements were later taken with the cellSens standard imaging software version 1.18 (Olympus Corporation, Tokyo, Japan).

Results

Pilot testing and late-instar study

Water quality of the well water used for all flow-through containers and tubes was measured from 18 March, 2019, to 12 November, 2020. Dissolved oxygen ranged from 4.43 – 7.28 mg/L (average = 5.16 mg/L), specific conductance ranged from 0.5491 – 0.6620 µS (average = 0.6283 µS), and pH ranged from 6.52 – 7.72 (average = 7.22). The average daily temperature recorded from the data loggers from 15

January, 2019, to 12 November, 2020, was 21.7 °C ranging from 19.6 – 23.9 °C, with higher temperatures recorded during the summer months of 2019 (**Fig. 2**). Nine trials were conducted, but only six were used for experimental purposes with three trials considered as pilot trials that were largely unsuccessful (< 4 pupations within either tube) but useful to help determine flow regime limits and packing configurations. Low-flows of < 10 mL/sec tended to result in stagnant conditions with mostly dead larvae and few pupation events. Flows of > 30 mL/sec tended to result in larvae pushed against the outflow screen with no pupation events. The targeted flow was then set to ca. 15 mL/sec with a range from 10 – 22 mL/sec. It was also noted that packing too much material was detrimental, assuming that flow was limited throughout most of the housing.

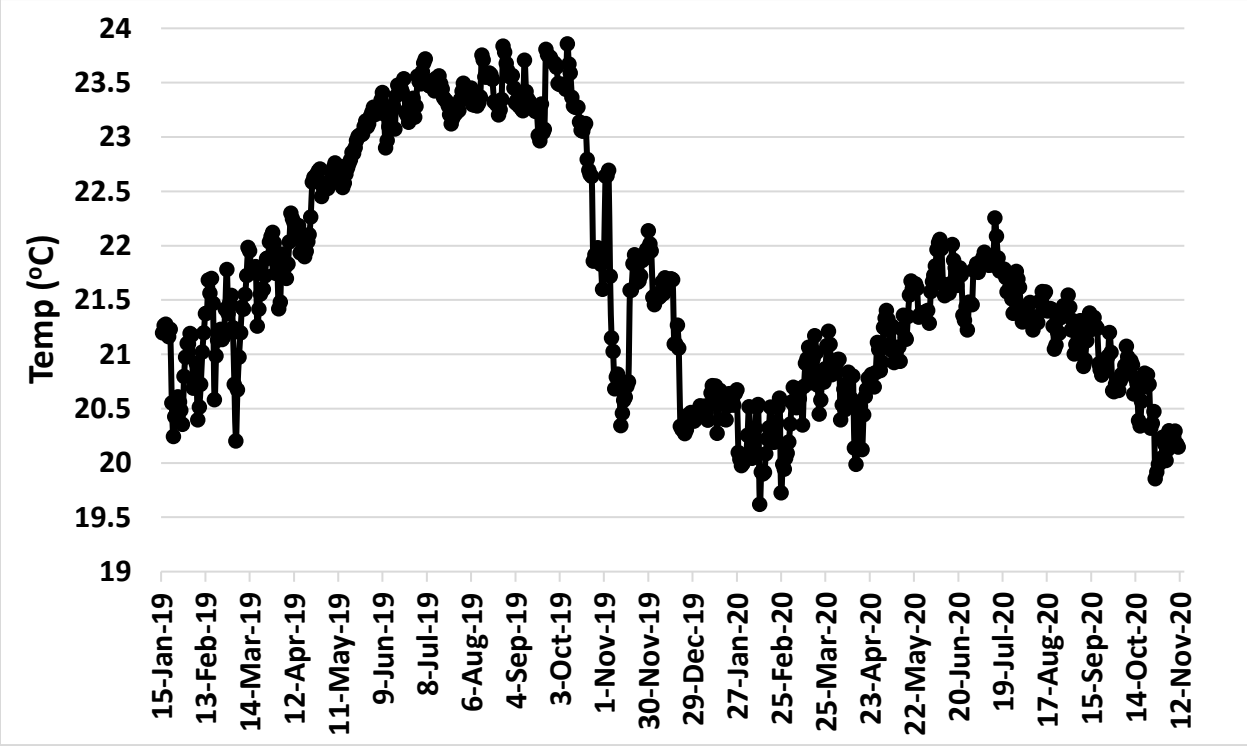


Figure 2. Mean daily water temperatures (°C) recoded by submerged data loggers throughout the study.

The second late-instar trial reached the benchmark of ≥ 20 % of the larvae pupating for the horizontal position and was replicated two more times (**Table 1**). These trials included conditioned leaf, wood, and cotton with a plastic-mesh tube in the middle. The fourth trial also reached the study benchmark; however, without the use of cotton cloth and was also replicated two more times. These trials included plastic mesh lining the inside of the housing with conditioned leaves and rocks placed in the middle. Most larvae had either transformed or died during the late-instar trials with ca. three living larvae remaining on average. However, the last two trials had 40 % or more of the original larvae still alive at the end of the trial for tubes in both positions and therefore were allowed to continue for ca. a month before moving to post-trial testing.

Table 1. All trials were run within 2-inch PVC flow-through tubes. Late-instar larvae were used for "Pilot" studies or the statistically testable trials 1 - 6. Cohorts were identified by various specific locations holding containers and refuge origination. Cohorts marked with (*) were run for an additional month after the initial check. Q_start and Q_end refer to mL/sec measured at start and end of the trial. Start-HCW was the average head capsule width of larvae starting the trial for both tubes combined. Unk adult = adults with unidentified gender due to *post mortem* degradation. Live larvae at end does not include early instar larvae that were the result of female reproduction before the end date. Proportion transformed refers to either pupae or adult, dead or alive and is out of 20 larvae for all late-instar trials, but 22 and 53 for the medium and small-size trials, respectively. Non-highlighted rows are pilot trials, green and yellow-highlighted rows represent trials utilizing the same packing materials of that color code; pink-highlighted rows represent the medium and small-sized larvae trials.

Trial	Cohort	Position	Packing material	Start date	End date	Days	Q_start	Q_end	Degree days (oC)	Average temp (oC)	Init-HCW (mm)	Male	Female	Unk adult	Live larvae at end	Pupae	Proportion transformed
Pilot1	SMARC1	Upright	rock, leaf, wood; vertical	15-Jan-19	8-Apr-19	83	15.6	14.8	1745.2	21.3	0.405	0	0	0	0	0	0.00
Pilot1	SMARC1	Horizontal	rock, leaf, wood; vertical	15-Jan-19	8-Apr-19	83	10.8	8.6	1745.2	21.3	0.405	0	1	0	2	0	0.05
1	SMARC1	Upright	mid roll; leaf, cotton, wood outside	19-Feb-19	17-May-19	87	13.9	12.4	1885.5	21.9	0.400	0	0	0	7	0	0.00
1	SMARC1	Horizontal	mid roll; leaf, cotton, wood outside	19-Feb-19	20-May-19	90	15.2	15.6	1953.7	22.0	0.400	3	3	0	0	0	0.30
Pilot2	SMARC1	Upright	mid roll; leaf outside	15-Mar-19	18-Jun-19	95	35.4	33.4	2110.2	22.5	0.394	0	0	0	5	0	0.00
Pilot2	SMARC1	Horizontal	mid roll; leaf outside	15-Mar-19	20-Jun-19	97	40.3	38.9	2156.8	22.5	0.394	0	0	0	6	0	0.00
2	SMARC2	Upright	mesh outside; leaf rock mid	12-Apr-19	18-Jul-19	97	20.4	17.3	2205.0	23.0	0.403	0	0	0	2	1	0.05
2	SMARC2	Horizontal	mesh outside; leaf rock mid	12-Apr-19	19-Jul-19	98	14.5	12.3	2228.5	23.0	0.403	5	5	0	0	2	0.60
3	SMARC2	Upright	mid roll; leaf, cotton, wood outside	21-May-19	6-Aug-19	77	21.1	18.5	1771.1	23.3	0.412	0	0	0	7	0	0.00
3	SMARC2	Horizontal	mid roll; leaf, cotton, wood outside	21-May-19	8-Aug-19	79	13.3	11.0	1817.8	23.3	0.412	2	2	1	5	1	0.30
Pilot3	SMARC3	Upright	Wood outside; mesh roll up inside	24-Jun-19	16-Sep-19	84	18.1	15.8	1943.4	23.4	0.375	1	2	0	4	0	0.15
Pilot3	SMARC3	Horizontal	Wood outside; mesh roll up inside	24-Jun-19	18-Sep-19	86	17.8	5.3	1989.9	23.4	0.375	0	1	0	0	1	0.10
4	SMARC3	Upright	mesh outside; leaf rock mid	24-Jul-19	23-Oct-19	91	18.4	14.9	2105.4	23.4	0.411	0	0	0	2	0	0.00
4	SMARC3	Horizontal	mesh outside; leaf rock mid	24-Jul-19	25-Oct-19	93	10.3	8.9	2150.7	23.4	0.411	2	3	0	0	2	0.35
5	Uvalde-SMARC*	Upright	mesh outside; leaf rock mid	27-Aug-19	3-Jan-20	129	11.9	11.0	3034.7	22.3	0.413	1	2	1	6	0	0.20
5	Uvalde-SMARC*	Horizontal	mesh outside; leaf rock mid	27-Aug-19	2-Jan-20	128	10.6	11.6	3014.2	22.3	0.413	5	1	0	5	0	0.30
6	Uvalde*	Upright	mid roll; leaf, cotton, wood outside	27-Feb-20	13-Jul-20	137	14.9	13.4	2874.5	21.1	0.436	0	0	0	11	1	0.05
6	Uvalde*	Horizontal	mid roll; leaf, cotton, wood outside	27-Feb-20	9-Jul-20	133	12.9	11.6	2787.2	21.1	0.436	0	0	1	13	1	0.10
Medium	UNK	Horizontal	mid roll; leaf, cotton, wood outside	15-Apr-20	15-Oct-20	183	11.4	13.3	3881.4	21.3	0.256	3	2	0	1	0	0.23
Small	UNK	Horizontal	mid roll; leaf, cotton, wood outside	15-Apr-20	12-Nov-20	211	12.3	13.4	4450.38	21.2	0.16	5	7	2	7	1	0.24

Results of the late-instar flow-through tube trials indicated that tube position was important with regard to pupation success at α = 0.05 (*F-value* = 17.852.431, *p-value* = 0.0029), while packing material with or without cotton had no effect (*F-value* = 3.689, *p-value* = 0.0910), and there was no interaction between factors (*F-value* = 0.803, *p-value* = 0.3963). From the six trials used in this analysis, of the 120 larvae given extra access to air 32.5 ± 14.6 % pupated (39) with most of these eclosing to adults (33). Of the 120 larvae reared in the upright position with less access to air, 5.0 ± 7.1 % pupated (six with four eclosing to adult) (**Table 1**).

Post trials and fecundity study

Because there was a limitation with regard to the number of individuals that could be used for experimentation, larvae remaining from earlier late-instar trials were placed into containers exposing them to different conditions. Eight out of 30 larvae placed within small flow-through tubes pupated. There were four out of 19 larvae that pupated within the modified flow-through container. Two out of six larvae pupated from the starvation treatment and two out of 20 pupated from the terrestrial treatment. Larvae in the terrestrial treatment were always found in the most submerged place in the container presumably seeking water. All pupae found during the first check from the late-instar flow-through trials (eight) were dead. Among the six pupae found during post-trial checks, three later eclosed to adult. Because post trials were checked about once a month, it is estimated that pupae take ca. 3 weeks to eclose to adult.

Twenty adult females from the late-instar flow-through tube trials, plus four additional F1 females from other experiments were tracked for living larvae produced over time (**Table 2**). From the 24 females tracked until death, 703 larvae were produced with an average of 29.3 ± 37.1 larvae per female. Female size was not found to be related to number of larvae produced (*F-value* = 1.064, $p = 0.314$, $R^2 = 0.046$); however, number of days over all inspections was found to be related to number of larvae produced (*F-value* = 47.870, $p < 0.001$, $R^2 = 0.685$) (**Fig 3**).

Table 2. The number of larvae produced for each of the 24 F1 females tracked for number of larvae produced over time.

Female	Larvae produced	Days	No. checks	Female length (mm)
Trial.8.1	121	174	4	1.36
Trial.7.1	115	274	6	1.34
Trial.16.1	110	294	6	1.41
Refuge3	70	113	4	1.27
Trial.16.2	60	274	3	1.32
Trial.4.2	42	49	2	1.4
Trial.4.1	32	46	2	1.29
Trial.15.1	26	195	4	1.26
Refuge2	23	71	3	1.27
Trial.8.4	16	56	2	1.22
Trial.18.1	14	77	1	1.33
ASE2.1	13	107	3	1.34
Trial.10.2	13	36	1	1.44
Refuge1	12	50	2	1.26

Trial.8.2	10	31	1	1.33
Trial.11.1	8	30	1	1.32
Trial.15.2	7	34	1	1.4
Trial.4.3	6	50	2	1.4
Trial.14.2	3	25	1	1.26
Trial.14.1	2	25	1	1.25
Trial.3.1	0	41	2	1.19
Trial.10.1	0	36	1	1.38
Trial.8.3	0	31	1	1.36
Trial.12.1	0	30	1	1.33

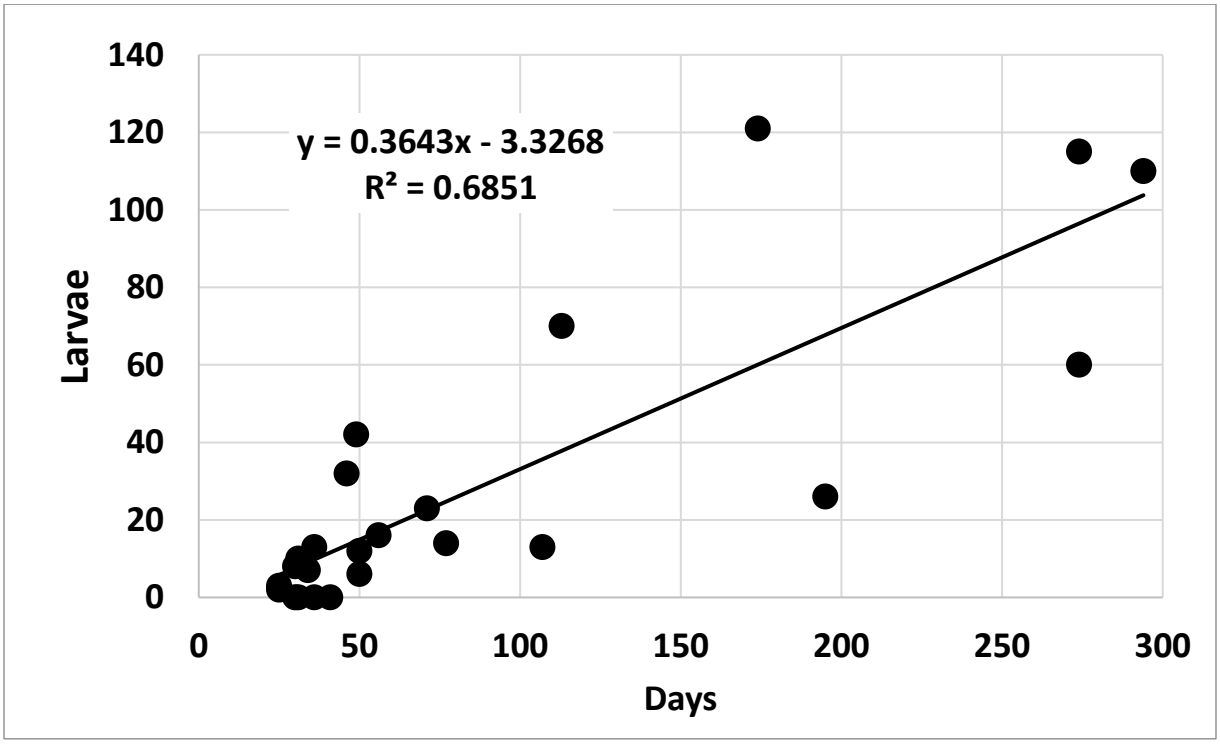


Figure 3. Relationship between number of larvae produced based on female longevity.

Early-instar investigations

Both medium-sized and small-sized larval trials included conditioned leaf, wood, and cotton with a plastic-mesh tube in the middle, placed in the horizontal position. Although late-instar larvae pupated at similar success with only conditioned leaf as a resource, this configuration was chosen because additional resources may be more important for earlier instar larvae. Five out of 22 larvae pupated and eclosed to adult from the medium-sized trial after 183 days and 15 out of 63 larvae pupated (14 eclosing to adult) from the small-sized larvae trial after 211 days, equating to 23% and 24 % pupation rates, respectively (**Table 1**).

Sex ratios

A total of 74 adults eclosed from all the trials and post-trial treatments of this study, resulting in 38 females, 28 males, and eight unidentified adults (due to degradation *post mortem*). The ratio of females to males produced during the late-instar trials (including pilot studies) was 20:19 with three individuals of unidentifiable gender. Ratios produced during post-trial, medium-sized, and small-sized were 9:1, 2:3, and 7:3, with individuals of unidentified sex at 3, 0, 2, respectively. Chi-square analysis of all reared adults, except for individuals of unidentified gender, indicated no departure from a 50:50 ratio (X^2 = 1.515, *p-value* = 0.218).

Discussion

Pilot testing and late-instar study

Flow appeared to be an influential factor with regard to pupation. As indicated during the pilot trials, very few pupation events occurred with low-flow regimes and larvae mortality was high. The pilot trial with the highest flow regimes resulted in most remaining larvae pushed against the outflow screen and no pupation events were recorded. Trials conducted with discharges between 10 – 22 mL/sec produced benchmark results for flow-through tubes in the horizontal position.

Late-instar larvae were more successful in pupating and eclosing to adult compared to their counterparts from the same cohort with less access to air. This is logical considering that larvae do not make a cocoon, pupae breath air, and pupae float. Houston and Gibson (2015) observed that larvae would seek a tightly packed space before pupation and surmised that an air bubble was created during the pupation process. Observations from this study agree with their assessments as late-instar larvae in the flow-through tube trials were frequently found within tightly packed areas, such as the threads of the caps. However, within a cave-like habitat there are probably numerous air pockets and it is likely that pupae will naturally float to these air pockets in case they are disturbed from their original place of pupation (kind of like a plan B). Flow-through tubes on their own without added air supply may be too unstable for most pupae to maintain their bubbles for the estimated 3-week period of time before eclosion to adult.

The last two late-instar trials were run for an additional month due to having 40 % or more living larvae in both treatments after the first check. It is noted that because late-instar F1 larvae were in low supply at this point in the experiment, larvae from a colony kept at the Uvalde TX USFWS refuge were transported to the SMARC to support this and other studies. The penultimate trial utilized both SMARC refuge originating larvae and Uvalde refuge originating larvae while the final trial consisted of only Uvalde originating larvae. It is possible that the larvae from the Uvalde cohort experienced more stress from being transported and handled more compared to cohorts used in earlier trials and this may have hindered their pupation success (see *Early-instar investigations* below).

It is also clear that more work is necessary with regard to gaining a better understanding of the nutrient requirements for *H. comalensis*. It has been observed that some captively reared adults appear to be less fit than others, dying within one month after enclosing and not reproducing. It is also of interest to support development of a better design for holding this species long-term. The tube design is awkward for caretakers to handle as food items need to be replenished and general habitat cleaned. Maintenance requires loosening the tube caps and emptying the contents which can cause stress and damage

individuals. Lastly, a better understanding of the wild population genetics is important to ascertain in order to maintain a similar allelic ratio in captivity.

Post trials and fecundity study

Thirteen of the 75 larvae were placed in post-trial treatments. Although these data are observational, it is likely that some larvae simply needed more time to pupate. However, there was a high incidence of pupation for those individuals that were starved. Starving larvae has been shown to decrease titers of JH and has proven to be an effective means of initiating pupation (Sparks et al. 1983). Larvae of the pleasing fungus beetle *Dacne picta* were shown to pupate after ca. 7 days of starvation, regardless to the amount of time they were allowed to feed beforehand (Sato and Suzuki 2001). Munyiri et al. (2003) showed that larvae of the longhorn beetle *Psacothea hilaris* starved during their last larval instar pupated faster than those that were fed *ad libitum*, but with reduced fitness. Among other examples, three genera of riffle beetles have been reared from freshly caught larvae by placing them in a container with no food (see Huston and Gibson 2015). Considering the natural behavior of insects to stop feeding in preparation for pupation, it is logical that pupation can be encouraged by removing food sources from "well fed" larvae; however, more research is needed to identify if this is a factor that influences *H. comalensis*.

Results from the fecundity study clearly indicate that females are iteroparous. Even more, observations showed that females do not produce eggs in the absence of a male. In several instances, a female produced eggs after being paired with a male, did not produce larvae after her mate died, but began producing larvae after another male was added to her mating chamber. Results from this study likely underestimate captive F1 female fecundity; it should be noted that there was a learning curve with regard to properly packing mating chambers and that some adult pairs died within the first month, possibly due to poor flow conditions. Furthermore, routine checks likely increased stress and chance of damage through handling. Therefore, some females would have probably lived longer and produced more larvae than results suggest if placed within more suitable habitats and if handled less often. However, this was not the case for most of the female subjects and it is likely that a number of these died early due to poor conditioning, possibly due to inefficient nutrition or excessive stress experienced during larvae development.

Even though female fecundity numbers are probably underestimated, the given information can be used to estimate how many females with access to mates in desirable conditions are needed to maintain a captive colony. A colony consisting of 10 females surviving 60 days with unlimited access to mates would produce ca. 185 larvae. Conservatively, using a 12 % survival rate (half that of the observations from the small-size trial), 22 larvae would be expected to become adults. With a 50/50 sex ratio demonstrated in this study, 11 would be F2 females. If F2 females have the same fecundity and survivorship as F1 females, a perpetual captive colony could theoretically be maintained.

Early-instar investigations

Results from this study suggest larvae take ca. seven months to eclose to adult after hatching, which is similar to previous investigations (BIO-WEST 2017). Pupation rates suggested by the medium and small-size trials indicate > 20 % of larvae will become adults which is in-line with studies of other elmid species (Elliot 2008). It was noted that the small-size trial was more successful compared to the medium-sized trial and this was not expected as it was anticipated that more mature larvae would have a greater chance of pupation success compared to earlier instars. However, it is possible that the higher success of

the small-size trial had more successful pupation and eclosion rates compared to the medium-sized trial because they were only handled once after hatching before being placed into the flow-through tube. The individuals of the medium-sized trial were held in a container that was frequently visited by another research group and thus may have been more stressed in comparison.

Conclusion

Favorable flow conditions and reasonable access to air pockets are evidentially important for pupation and eclosion. F1 females have the potential to produce > 100 larvae over time; however, more work is needed to gain a better understanding in maintaining their longevity. The results from this study indicate that there is great potential to maintain a captive self-propagating colony of *H. comalensis*. Investigations on nutrition and alternative flow-through aquaria are suggested for future studies.

Literature Cited

BIO-WEST. 2002. Comal Springs riffle beetle laboratory study: evaluation under variable flow conditions. Final Report. Prepared for the Edwards Aquifer Authority. 30 pp.

BIO-WEST. 2017. Comal Springs riffle beetle (Heterelmis comalensis): life history and captive propagation techniques. Final Report. Prepared for the Edwards Aquifer Authority. 36 pp.

Bowles, E.B., 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.

Bowles, E.B., and T.L. Arsuffi. 1993. Karst aquatic ecosystems of the Edwards Plateau region of central Texas, USA: a consideration of their importance, threats to their existence, and efforts for their conservation. Aquat. Conserv. Mar. Freshw. Ecosyst. 3: 317–329.

Bosse, L.S., D.W. Tuff, and H.P. Brown. 1988. A new species of Heterelmis from Texas (Coleoptera: Elmidae). Southwest Nat. 33: 199–203.

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

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.

Elliot, J. M. 2008. The ecology of riffle beetles (Coleoptera: Elmidae). Freshwater Reviews 1:189-203.

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. Southwest Nat. 53: 74–84.

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

Huston, D.H. and Gibson, J.R. 2015. Underwater pupation in the Comal Springs riffle beetle *Heterelmis comalensis* (Coleoptera: Elmidae). The Coleopterists Bulletin 69: 521-524.Coleopterist Bulletin.

Kosnicki, E. 2019. Determining sexual dimorphism of living aquatic beetles, *Stygoparnus comalensis* (Coleoptera: Dryopidae) and *Heterelmis comalensis* (Coleoptera: Elmidae), using internal abdominal structures. Journal of Insect Science 19:(9) 1 – 5 doi: 10.1093/jisesa/iez075

Munyiri, F. N., W. Asano, Y. Shintani, and Y Ishikawa. 2003. Threshold weight for starvation-triggered metamorphosis in the yellow-spotted longicorn beetle, *Psacothea* hilaris (Coleoptera: Cerambycidae). Applied Entomology and Zoology 38:509-515.

Sato, R., and A. Suzuki. 2001. Effect of starvation and feeding of larvae during 4th stadia on pupation and adult size in *Dacne picta* (Coleoptera: Erotylidae). Applied Entomology and Zoology 36:189-197.

Shepard, W.D. 2002. Elmidae. In: R. H. Arnett, M. C. Thomas, P. E. Skelley, and J. H. Frank (eds.), American Beetles Vol 2. (pp. 117-126). Boca Raton, FL: CRC Press LLC.

Sparks, T. C., Hammock B. D., and Riddiford L.M. 1983. The haemolymph juvenile hormone esterase of *Manduca sexta* (L.)-inhibition and regulation. Insect Biochemistry 13:529-541.

United States Fish and Wildlife Service (USFWS). 1997. Endangered and threatened wildlife and plants; final rule to list three aquatic invertebrates in Comal and Hays counties, TX, as endangered. Fed. Reg. 62: 66295–66304.

United States Fish and Wildlife Service (USFWS). 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. Fed. Reg. 78: 63100–63127.

TEXAS STATE UNIVERSITY

The rising STAR *of Texas*

July 30, 2020

Dr. David Britton
«U.S. Fish and Wildlife Service»
« San Marcos, Texas»

This letter is intended to provide a brief report of the current progress of the project "Functional genomics of bacteria associated with wild and captive-reared Comal Springs riffle beetle". Up to now, 300 bacteria were isolated from adult CSRB, water, and wood biofilms collected in the wild and in captivity. Until now, 142 isolates were successfully identified using partial 16S rRNA gene sequencing. In total, we have identified 30 genera belonging to 4 phyla. Of these 23 were found only in wild beetles, 41 only in captive beetles and 8 were found in both groups. The diversity of culturable bacteria was higher in the wild water samples than those from the refugium environment, but the wild beetles displayed lower diversity in their microbiome than their captive counterparts at the genus level. We sent 87 bacteria isolates for genomic sequencing to the Joint Genome Institute (JGI). We have received the completed genomes of 58 isolates and are expecting the remaining 29 after they continue processing samples. We are currently working on phylogenetic and functional analyses of these genomes.

I am available to answer any questions or concerns.

Sincerely,

Camila Carlos-Shanley, PhD
Assistant Professor, Dept. of Biology
Email: carlos-shanley@txstate.edu
Phone: 512-245-5283

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

2020 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Lindsay Campbell, PhD and Linda Moon

San Marcos Aquatic Resources Center

U.S. Fish and Wildlife Service

Table of Contents

Background

The ability to distinguish individuals and collective groups is essential for many ecological studies. Long-term tagging allows for effective species management by monitoring biological data over a period of years and possibly through an individual's lifespan. The methods for long-term tagging of salamanders have been growing rapidly with new technologies being developed in the last decade. Nevertheless, few studies have been made with these novel methods on fully aquatic, paedomorphic or neotenic salamanders. Salamander species pose unique challenges in long-term marking methods due to their sensitive, permeable skin and, with few exceptions (such as the large North American Hellbender), their small body size. Historically, clipping of appendages has been the most common way to mark amphibians, including salamanders. Studies have shown that toe clipping can be detrimental to salamanders, and their ability to regenerate limbs leaves this method insufficient for long-term observations (Heatwole 1961; Davis and Ovaska 2001; Kinkead et al 2006). Long-term tagging allows for data such as collection date, age or estimated age, growth rate, sex, reproduction events, offspring produced, and health to be easily identified and tracked for each individual. Tagging individual salamanders in captive reassurance populations permits researchers to distinguish organisms unsuitable for reproduction, due to repeated lack of reproduction or over-aggressive behavior during breeding. Tagging also permits researchers to distinguish salamanders suited for reproduction, including individuals found to have genetics that should be preserved in the population. Beyond captive reassurance population operations, long-term tags in individuals reintroduced to the wild would facilitate mark-recapture evaluations.

When choosing a method for long-term tagging of a salamander, Osbourn *et al.* (2011) recommended considering factors such as the impact on health, degree of invasiveness, mark longevity, and number of unique marks necessary. We focused on tagging methods that would benefit the captive reassurance populations of salamander species held at our facility, the San Marcos Aquatic Resources Center (SMARC). A variety of marking methods have been evaluated in amphibians, including coded-wire tags (Sinsch 1997), radio tags (Richards *et al.* 1994), skin pattern or pigment recognition (Grant and Nanjappa 2006; Gamble *et al.* 2008), and tattooing (Donnelly *et al.* 1994; Schlaepfer 1998).

Imaging software has been successful in tracking individuals via pigmentation and body characteristics and has advanced over the years. However, such software has not been tested for following individuals over many years of growth with aquatic, paedomorphic or neotenic salamander species. Software imaging methods are useful for mark recapture and other field studies but lack in quick tank-side identification required for captive reassurance populations where being able to move animals quickly can be warranted. Utilizing photographic identification would require caretakers to take a photo of the animal, place the individual in a single holding area, upload the photo, wait for the program to analyze, and then move the animal. By having an animal with an individual marker on its body, there is no need for the lengthy process of photographs and software. In the case of young salamanders, their pigmentation, coloring, and pattern characteristics can change quickly over time with growth or the effects of different light sources. It is unknown whether photo identification can track a juvenile salamander through adulthood accurately. For animals collected from the wild to be held in captive reassurance colonies, being able to accurately identify origin sites is important, especially for genetic management purposes.

For this study, we selected three tagging techniques used in monitoring other salamander species: visible implant elastomer (VIE), visible implant alphanumeric tags (VIA), and passive integrated transponders (PIT) (Davis and Ovaska 2001; Bailey 2004; Heemeyer *et al.* 2007; Osbourn *et al.* 2011; Appleby 2015; Whiteman 2016; Mitchell *et al.* 2017; Lunghi and Veith 2017). Of these, only VIE tagging has been evaluated in the San Marcos salamander Phillips and Fries 2009), none have been evaluated for use in the other San Marcos Aquatic Resources Center (SMARC) captive reassurance population salamanders and comparison studies among tagging techniques for all SMARC aquatic salamander species have not been performed. These tag types were selected for their effectiveness with other salamander species, ability to be quickly and easily identified tank-side, low rates of negative effects on organisms, and perceived ease of learning and implanting the tags. Analysis of these three different tagging methods with Texas blind (*Eurycea rathbuni*), San Marcos (*Eurycea nana*), and Comal Springs (*Eurycea spp.* or *Eurycea pterophila* as recently classified by DeVitt *et. al.* 2019) salamanders will provide valuable information for successful long-term marking of individual animals.

Visible Implant Elastomer (VIE) has been tested at the SMARC with San Marcos salamanders as single marks for purposes of population studies. VIEs showed no ill effects on growth or mortality rates to the salamanders (Phillips and Fries 2009). Pilot tagging with individual color codes has been undertaken on Texas blind salamanders at SMARC from 2017 through 2018. VIE marks to indicate salamander sex and year captured on San Marcos salamanders have also been tested on a portion of the captive reassurance population. VIE is a useful tagging method for salamanders due to several benefits. Subcutaneous VIE tags can be placed with small needles, and therefore can be used on small individuals. Multiple color combinations can be used with VIE tags to create unique identifying marks and VIEs have projected longevity as a mark. Common drawbacks using this method include tag migration or breakage, misidentification of tag color, elastomer product loss due to hardening, and the need for multiple marks to create unique tags (Davis and Ovaska 2001; Marold 2001; Heemeyer *et al.* 2004). Due to these shortcomings, comparisons with other tagging methods is warranted. Our objective was to select the best tagging method(s) for salamanders held at SMARC and other salamander holding facilities.

Visible implant alphanumeric (VIA) tags were originally developed for fishes and are the newest of the three tagging types to be tested in this study. The tags are made up of a small rectangular fluorescent piece of plastic containing an alphanumeric code on one side, which consists of one letter and two numbers. Studies have had conflicting results on the efficacy of VIA tags in salamanders (Osbourn *et al.* 2001; Lunghi and Veith 2017). Injector malfunction can cause the tag to be inserted upside-down without an easy way to reposition the tag (Lunghi and Veith 2017). If the skin pigmentation of the salamander is dark, or if the tag is inserted too deep, the tag may not be easily read or could be misread (Osbourn *et al.* 2011). However, when placed properly under the skin, VIA has been found advantageous due to its small size (1.2 mm X 2.7 mm) and has been successfully used on salamanders weighing ~1.2 grams (Osbourn *et al.* 2011) with no loss in tag product and has 10,000 unique tags available.

Passive integrated transponder (PIT) tags transmit a unique code when a Radio Frequency Identification (RFID) reader sends a radio frequency to the transponder. This method has been extensively used in many organisms but has been limited in the use of small species in the past, due to tags' relatively large size of 10 to 14 mm in length (Gibbons and Andrews 2004). A newer, smaller 8.4 mm PIT tag has been successfully implanted in salamanders 1.5 to 3.9 grams in weight (Mitchell *et al*. 2017; Ousterhout *et al.* 2014). The average adult San Marcos salamander held in captive reassurance population weighs 0.67 grams with an average total length of 63 mm. The 8.4 mm tag may make it possible to implant PIT tags in salamanders in the size range of San Marcos and Comal Springs salamanders, but no published studies have tested it on salamanders of this size class. This method might be useful for larger adult Texas blind salamanders but may not the best option for smaller species.

Objectives

Our goal was to evaluate the utility of three different, long-term tagging methods in Texas blind, San Marcos, and Comal Springs salamanders. Evaluation consisted of two fronts: first, compare tag retention and readability over a period of twelve months; this includes the evaluation of novel versus experienced taggers and readers. Second, evaluate the use of tag type for the use in each species and whether they are useful for individual tagging or group tagging purposes.

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

Methods

Initial Tag evaluation

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

We tested implanting PIT tags subcutaneously into the upper tail musculature rather than the body cavity of salamanders to prevent internal organ damage and to reduce the risk of the injection site compromising the body cavity. We first placed six PIT tags in the tails of F1 Texas blind salamanders that were 5.6 – 7.1 cm snout-vent length (SVL). After one month of evaluation, the salamanders had no infection at insertion site and tolerated the PIT tags (movement and swimming ability were not compromised). We then implanted PIT tags in five F1 Texas blind salamanders that were 4.3 – 5.5 cm SVL. Over 45 percent of the Texas blind salamanders shed their tags. After this evaluation period, we decided that injecting PIT tags into smaller salamander individuals for all three species would be imprudent.

Tag Insertion

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

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

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

We quickly recognized that horizontal tags on the smaller bodied salamanders would not be feasible for San Marcos and Comal Springs salamanders with VIE. The average SVL of the Texas blinds with horizontal VIE tags was 43.3 mm (8.8 SD), while the average SVL of the VIE groups of San Marcos salamanders was 27.9 mm (3.3 SD) and Comal Springs salamanders 32.9 (2.9 SD). We made the decision to tag the San Marcos and Comal Springs salamanders with vertical lines and add a second group of smaller Texas blind salamanders (26.4 mm SVL, 2.6 SD) also tagged vertically. Figure 1 shows the corresponding positional locations for VIE tags and the comparison between vertical and horizontal tag composition.

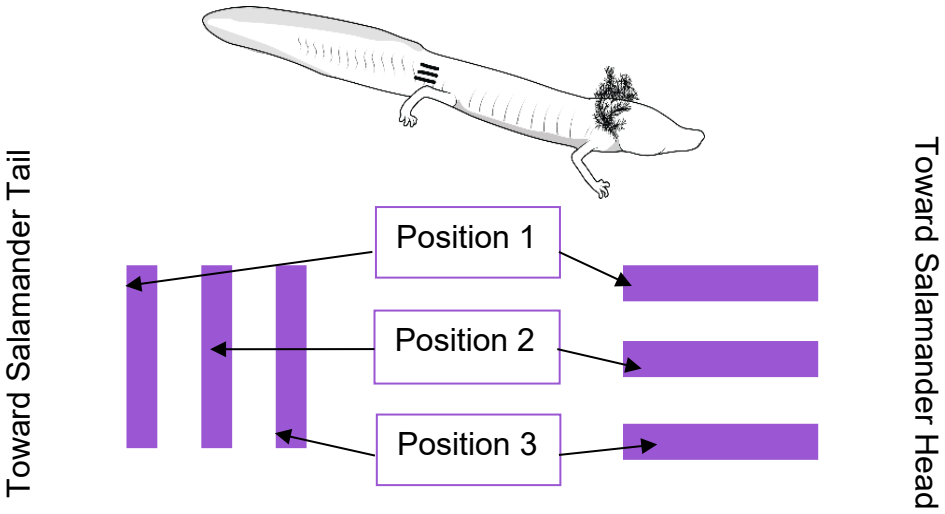


Figure 1 Vertical and horizontal lines depicting the two different visible implant elastomer tag compositions. Tags are read left to right or top to bottom.

Tag Readability and Retention

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

Readability Index:

0: Tag not visible or not present

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

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

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

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

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

During the study, we noticed that VIE tags would sometimes develop breaks in their lines. This breakage was not taken into account in the Readability Index defined prior to the experiment. Thus, we went back to the photographs of the tags and estimated breakage in two ways: Percentage of tag remaining intact and numbers of breaks in a line (Table 1). Each line position was given a rating and the average of all three was taken for an overall Percentage rating for the tag at the given time of the photo. Each line position was given a rating for number of breaks and the total of the three lines summed to give an overall Number of Breaks for the tag at the given time of the photo.

Table 1 Categorical ratings of the two different ways tag breakage was evaluated.

VIE TAG INTEGRITY CATEGORY						
Category Number	**0**	**1**	**2**	**3**	**4**	**5**
Percentage (%) tag remaining	0	1-24	25-49	50-74	75-99	100
Beakage: Number of breaks	N/A	10+ breaks	7 to 9	4 to 6	1 to 3	No breakage

PIT tags were evaluated based on whether or not they were retained, if the Radio Frequency Identification (RFID) could be read through air or through water, and the time required to read the RFID. We tested the distance tags could be read through water, both in the tank and above the water surface. We also tested if the tag-reader could distinguish multiple RFID tagged individuals in a tank; we started with two individuals and added individuals until the tag-reader could not distinguish tags.

Statistical Analysis

All statistical analyses were performed in Rstudio (R version 4.0.2). Packages used for these analyses were Surv, lme4, multcomp, MuMIn, emmeans, betareg, and Matrix. Tag retention rates and comparisons of tag retention within and among species was done with a Cox proportional hazards model. We used a Cox proportional hazards model to evaluate salamander survival within the experiment compared to salamanders not within the tagging experiment. Tag readability was analyzed by a linear mixed model after using a second-order Akaike Information Criterion (AIC_C) to assess goodness of fit and determine the best model effects. Tag Percent Remaining and Number of Breaks was analyzed by a linear mixed model after using AIC_C to determine the best model effects.

Results

Injection observations

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

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

Figure 3 Loading VIA tag into injector needle.

Researchers also found that the VIA tags themselves easily pierced the skin during injections, creating an exit wound in some salamanders. However, Texas blind salamanders have thicker skin that did not tear when the VIA needle was inserted. It was impossible to see or obtain a clear picture of the fresh tags in salamanders with injection sites closed with surgical glue, which formed an opaque layer over the site. The plunger of the flat VIA injection needle tended to stick to the tag or go over the top of the tag instead of pushing it out, forcing us to disassemble the needle before continuing. The alphanumeric code is written only on one face of the tag (Figure 3). Thus, we could not adjust the orientation that the tag was loaded into the needle. For safety of the animal, all injections were done with the needle facing away from the body cavity. For consistency of tag orientation, we adjusted salamander body orientation to correspond with the handedness of the person tagging; one tagger was left-handed, the other, right-handed (Figures 4 & 5).

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

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

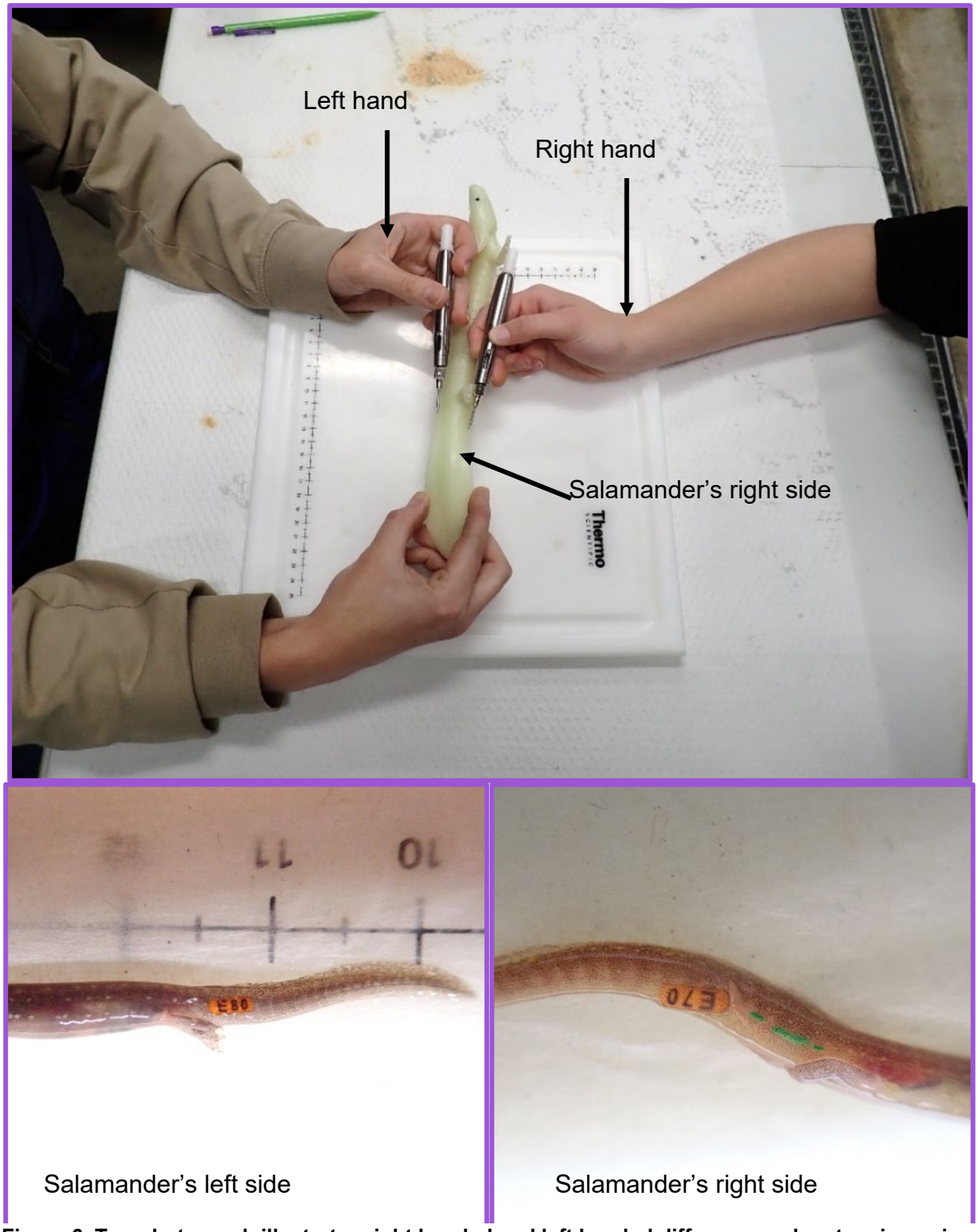


Figure 6 Top photograph illustrates right handed and left handed differences when tagging using a salamander model for demonstration purposes. Researchers wear gloves and have a surgery set up during actual tagging of live organisms. Bottom photographs showcasing the difference in tag orientation depending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

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

We first injected 20 Comal Springs salamanders with VIA tags and did not close the injection site with surgical glue because of concern that their back limb would become stuck in the glue. However, within one week, more than half of the salamanders shed their tags, many within the first few days after injection. Thus, when injecting the San Marcos salamanders, taggers closed the injection sites with surgical glue, taking care to keep the back leg away from the glue and dabbing off excess glue with a clean Kim wipe. Glue was given time (under 1 min needed) to dry to the touch before the salamander was placed in recovery. Because all but two of the Comal Springs salamanders shed their tags during the first month, we decided to tag a second group of 20 Comal Springs salamanders, this time closing the injection sites with glue.

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

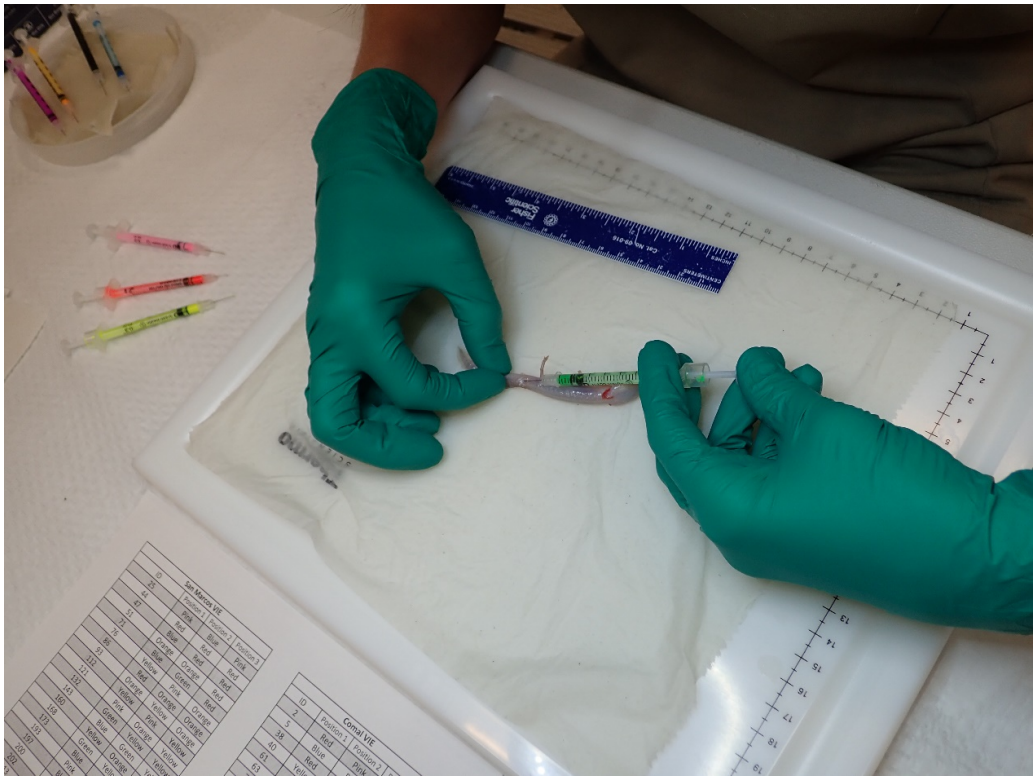


Figure 7 Horizontal injection of triple VIE tags into a Texas blind salamander.

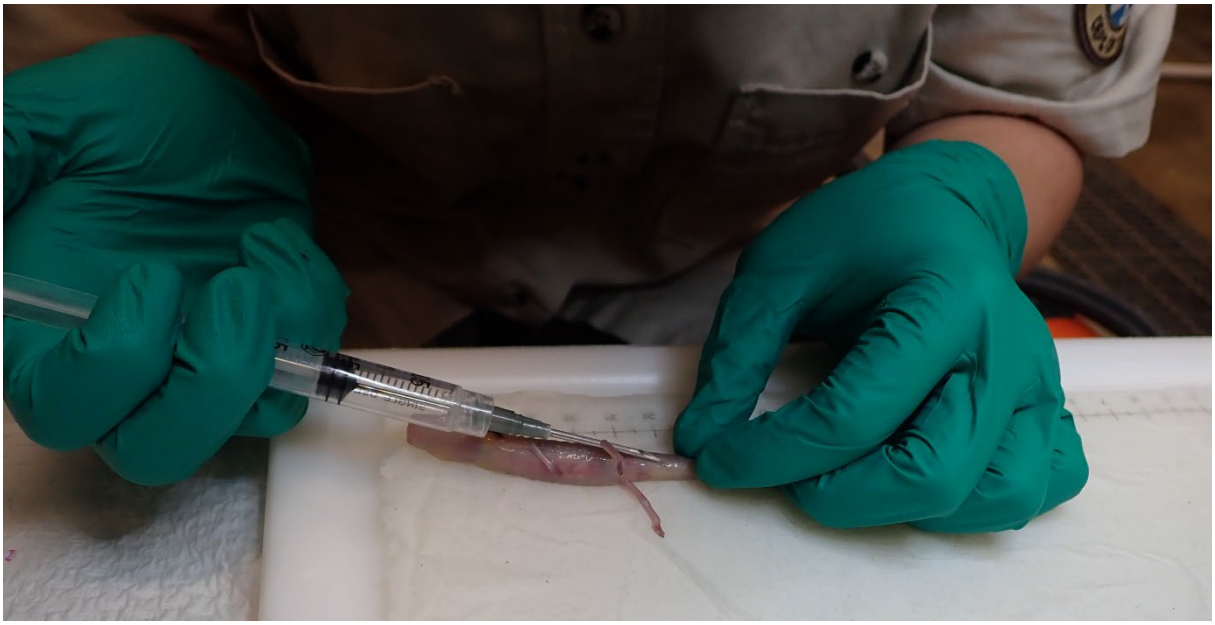


Figure 8 Injection of PIT tag into a Texas blind salamander.

Tag Retention

Retention rates of tags ranged from zero (Comal Springs VIA) to 1 (all species VIE) (Table 2). There was no difference in retention between the Comal Springs with non-glued injection sites versus salamanders with glued injection sites, as all tags were shed by the second month. Therefore, all Comal Springs salamander VIA individuals were grouped in further analysis. Retention of VIA tags was significantly different among species (Likelihood ratio test = 60.23, df =2, $p << 0.001$), with Texas blind salamander VIA retention significantly better than San Marcos or Comal Springs salamanders (Coxph Texas blind $z = -4.686$, $Pr(>|z|) << 0.001$). Ninety percent of Texas blind salamanders retained their VIE tag, whereas only one San Marcos salamander retained its tag the for 12 months and no Comal Springs salamanders retained their VIE tags. Of the shed VIE tags, 95% were shed during the first month.

Only six of the eleven PIT tags in Texas blind salamanders were retained a month after their injections. Due to this low retention rate (0.545%, SE = 0.15), the relative size of the tag to smaller salamanders, and the high performance of the other tag types in Texas blind salamanders, we decided to end further injections of PIT tags. One of the directives for research done within the Refugia Program is that the research must benefit the refugia. We did not see any benefit to the refugia or the salamanders to continue with PIT tags that ultimately would not be used in refugia. Tag retention of PIT tags was significantly worse than that of VIA and VIE (Likelihood ratio test = 18.93, df = 2, $p << 0.001$; no difference between VIA and VIE, z = -0.002, Pr(|z|) = 0.9987).

Table 2 Retention rates of the different tag types by species with upper and lower confidence interval values.

Tag Type	N value	Retention Rates	Std. Err	Lower 95%	Upper 95%
Texas blind VIA	20	0.9	0.0671	0.778	1
San Marcos VIA	20	0.05	0.0487	0.0074	0.338
Comal VIA	40	0.00	NA	NA	NA
Texas blind VIE	50	1	0	1	1
San Marcos VIE	20	1	0	1	1
Comal VIE	20	1	0	1	1
Texas blind PIT	11	0.545	0.150	0.318	0.936

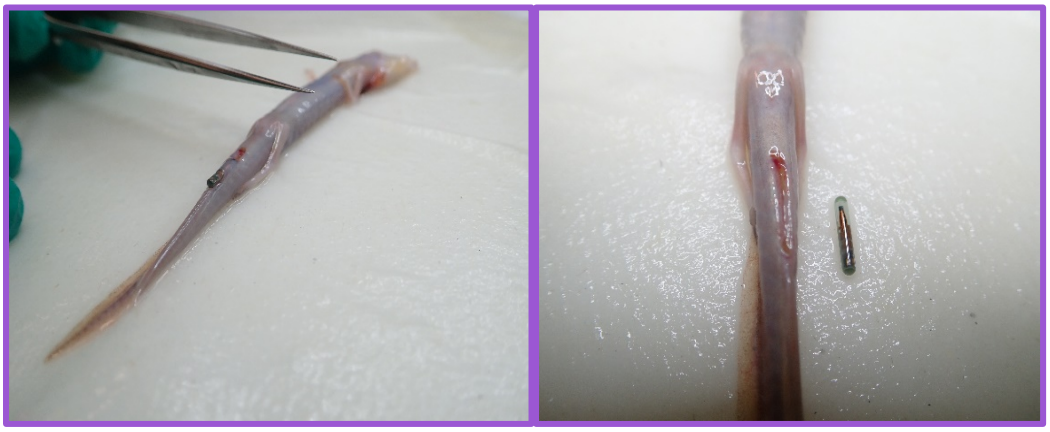


Figure 9 Texas blind salamander with tag protruding from skin (left) and residual wound after tag was removed (right).

Texas blind salamanders that shed their PIT tags had notable scars, which diminished over time. No signs of infection occurred after injection or when tags were shed. In one case, an individual was found to have a PIT tag protruding from the opposite side of the initial injection site during first tag check (Figure 9). To reduce the chances of infection or further complications, the tag was removed by grasping the tag with forceps and pulling it free. The salamander was isolated in a holding tank for a week until the open wound healed and recovered without further complications.

Survival

In all groups, survival of the salamanders was higher in the tagging experiment than those not in the tagging experiment (Table 3). Of those not in the tagging experiment, we censored the heritage group of San Marcos salamanders that have lower survivorship and very small Texas blind salamanders that were a part of a large group (167) of recently hatched juveniles that came in over a three month period. The slight difference in survival of the Control group in Texas blind salamanders was influenced by the start and end of the 12 month periods for the groups, which were different, changing mortality accounting during each period. Cox proportional hazard analysis found no significant differences between any of the experimental groups that had less than 100% survival and their control.

Table 3 Survival percentages of salamanders in the tagging experiment by species and tag type and that of the Control group of salamanders of that species not in the tagging experiment.

Tag Group	In Experiment Survival	Control Survival
San Marcos VIE	70.0%	67.4%
San Marcos VIA	100%	67.4%
Comal VIE	95.0%	84.2%
Texas blind VIE	96.0%	88.1%
Texas blind VIA	100%	89.6%
Texas blind PIT	100%	89.5%

Readability

Expert readability was significantly different by tag type (linear regression model, df = 1307, p << 0.001) (Figure 10). No animals scored readability values of 0 or 1 across all tag types. Only two VIA individuals scored a 2 and only two VIE individuals scored a 2. Since only one VIA tag remained in San Marcos salamanders and none in Comal salamanders, these groups were excluded from the analysis. Novel readers were used at the three, six, nine, and twelve month tag checks. A different novel reader or readers who had not had experience reading tags were used each month. Expert Readability for San Marcos tags was better than novel readers in later months (linear mixed-effects model, Reader and month effects, z = 1.725, Pr(>|z|) = 0.0845, significant code = 0.05). There was no difference in Readability by the Expert reader between taggers for San Marcos salamanders (pairwise comparison, df (Kenward-Roger method) = 22.7, t-ratio = -1.496, p = 0.1483). For Texas blind salamanders, we found no statistical difference between Readability of all groups by the Expert and Novel. Tagger was significant with tags done by Linda Moon rated higher by the Expert reader (Tukey Contrasts, z = 2.907, Pr(>|z|) = 0.00365, significant code = 0.001). For Comal Springs salamanders, there was no significant difference between Expert or Novel reader (cf test, z = -0.382, Pr(>|z|) = 0.7024), nor did the tagger have an effect (Tukey pairwise differences, df Kenward-roger method = 21.2, t-ratio = -0.412, p = 0.6841).

Figure 10 Readability Score for the different type of tags over the 12 months as scored by our Expert reader. Bars are averages with standard deviation.

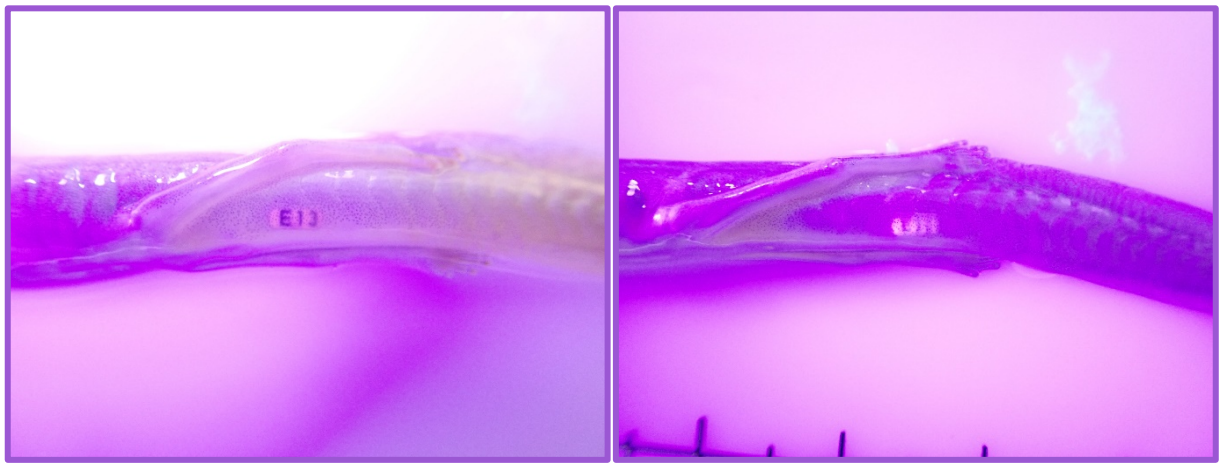


Figure 11 VIA tag "E13" (left) illustrates an ideal VIA tag under salamander skin. "E22" (right) illustrates a poor quality VIA tag, with the code being blurred beyond legibility.

Readability of VIA tags depends on the depth and angle of tag injection and the presence of melanophores, which can obscure the printed number (Figure 10). All the VIA tags started with the same letter ("E") so this letter was not assessed by the readers.

In VIE tags, we noted several occurrences of breakage and migration of individual tag lines (Figure 11). VIE tag lines were injected vertically for San Marcos and Comal Springs salamander groups due to limited space on their tails for horizontal lines. Two groups of Texas blind salamanders were given VIE tags: 20 sub-adult/adults were given horizontal tags and 29 younger, smaller juveniles were given vertical tags. We increased number of vertical VIE tags because this whole group had reached the individual tagging size; so we followed all of them instead of just a smaller subset.

VIE tags tended to break at costal grooves, which was not surprising since the elastomer lines were observed separating at initial injection (Figure 12). Percent of tag remaining decreased the most between initial tagging and the three month check, with little change thereafter (Table 4). In the same pattern, the number of breaks in the tags increased the most between the initial tagging and month three (Table 5). The majority of breaks were equal or less than two, 60.4% (Figure 13). Only nine of the 86 VIE tagged salamanders had more than ten breaks in a single line; these were all Texas blind salamanders with vertical tags. There was a correlation with both Percentage and Breaks with growth. The Texas blind VIE group were the youngest salamanders, thus experiencing the highest growth rates as compared to the adult San Marcos and Comal Springs salamanders and the sub-adult/adult Texas blind salamanders. Higher growth rate translates into expanding muscles and skin tissues which would be consistent with higher breaks in tag lines.

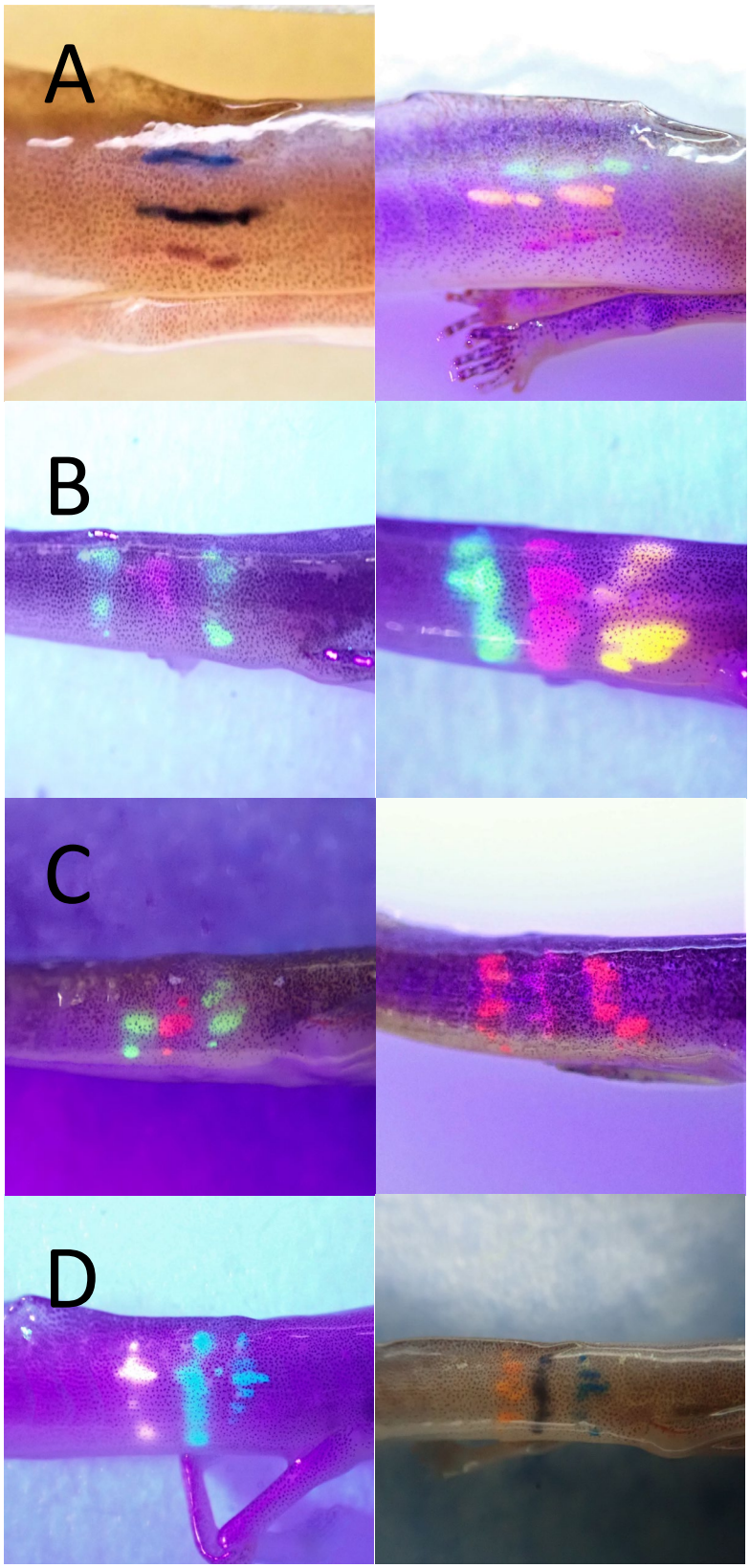


Figure 12 Typical tag clarity at the end of 12 months for Texas blind salamander horizontal (row A), San Marcos salamanders (row B), Comal salamanders (row C), and Texas blind salamanders vertical (row D).

Table 4 Average Percentage of Tag Remaining by treatment group of the course of the study.

	Comal VIE		San Marcos VIE		Texas VIE Horizontal		Texas blind VIE Vertical	
Month	**Mean Percent Remaining**	**StD**	**Mean Percent Remaining**	**StD**	**Mean Percent Remaining**	**StD**	**Mean Percent Remaining**	**StD**
At Tagging	84.87	15.66	91.90	13.59	94.79	10.01	99.86	0.77
3	62.06	21.06	76.39	15.85	86.67	18.61	84.48	11.13
6	61.62	20.58	74.77	15.94	86.04	18.35	80.46	11.84
9	61.18	20.27	72.40	18.75	85.00	19.28	77.59	13.38
12	61.18	20.27	74.40	17.44	83.13	21.31	77.83	13.37

Table 5 Average number of breaks and standard deviation by groups over the course of the study.

Month	Comal VIE Average Breaks	StD	San Marcos VIE Average Breaks	StD	Texas VIE Horizontal Average Breaks	StD	Texas blind VIE Vertical Average Breaks	StD
At Tagging	2.42	2.27	0.67	1.68	1.90	1.77	0.90	1.37
3	6.47	2.72	7.11	3.76	5.55	2.96	13.83	4.77
6	6.63	2.95	7.61	3.33	5.75	3.06	14.28	4.65
9	6.79	2.99	7.63	3.50	5.75	3.06	14.48	4.56
12	6.79	2.99	8.29	3.20	5.60	3.07	14.68	4.74

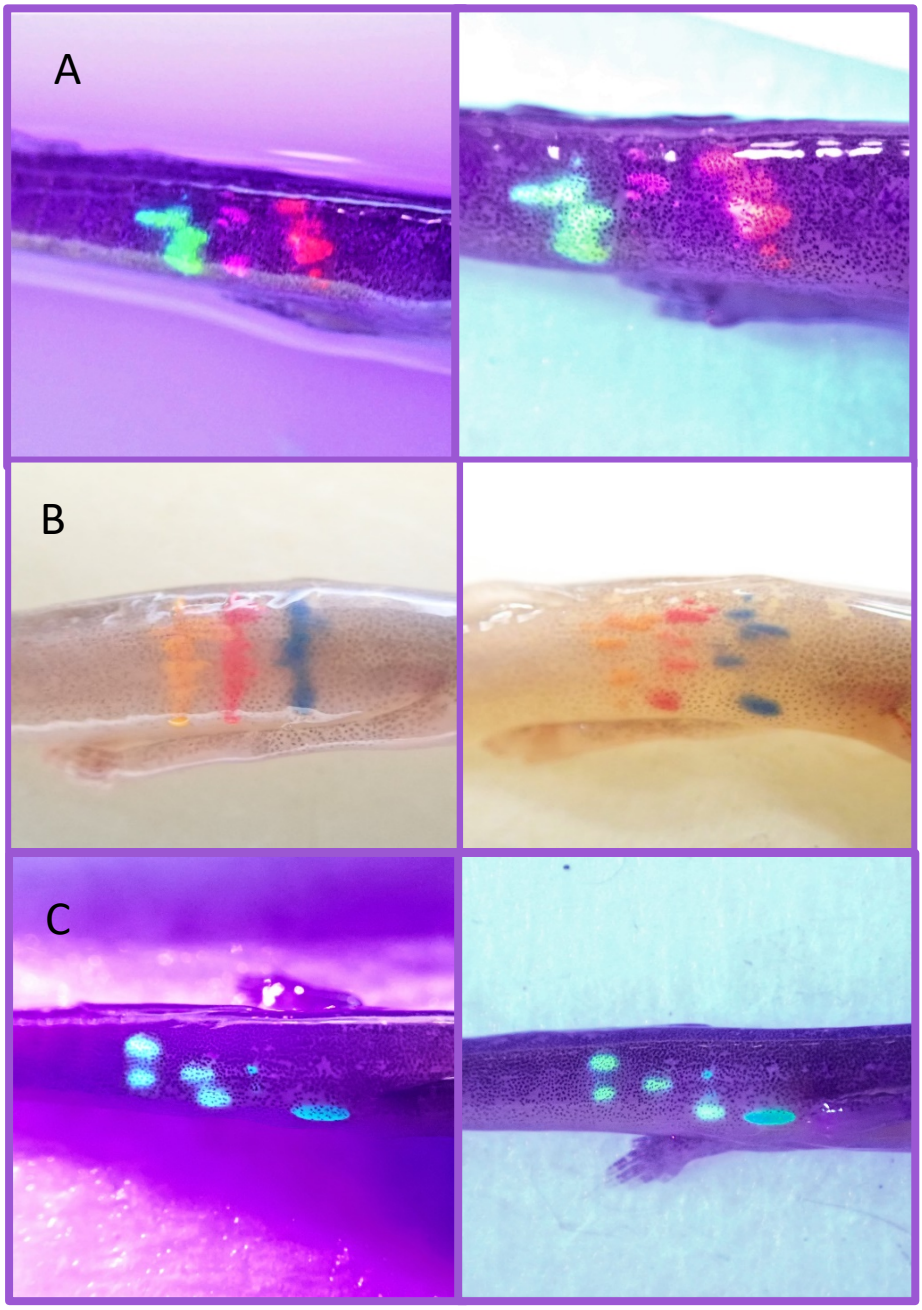


Figure 13 Three examples of tag breakage. (A) Elastomer bleeding along the costal groves and a portion of the tag in position 2 disappears. (B) Common striation breaking in Texas blind salamanders, but tag still scoring High on the Readability scaled used. (C) Extreme case of tag breaking, migrating, and disappearing in a San Marcos salamander with the position 2 line of pink only have one small dot.

PIT tags were easily read using a Biomark reader. We were able to read all tags during monthly checks in under 15 seconds by waving the wand over the animal. Most PIT tags are not notable to the naked eye, but can been seen with close inspection (Figure 14).

Figure 14. Passive integrated transponder 6 month after initial injection in a Texas blind salamander. A light injection scar can be seen to the left of the PIT tag.

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

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

Discussion

In general, individually marking salamanders with vertical VIE color combinations resulted in the highest readability and retention scores in all three species of aquatic salamanders verses VIA and PIT tags. Skin texture and thickness of each species affected the retention and readability scores of the three different tagging methods used.

We recommend future VIA tag studies with Texas blind salamander use multiple fluorescent colors along with codes that begin with varying letters. VIA tags come in different sizes, and the larger sized VIA tags could be used on larger Texas blind salamanders to increase readability. Insertion accuracy of VIA tags greatly effects readability, as the tags need to be shallow enough under the skin and without a tilted angle to be seen clearly. Retention rate of VIA tags in Texas blind salamanders was high and not significantly different from VIE tags, but should be noted if these tags were used in a mark-recapture study. We have found that the skin of Texas blind salamanders thickens and becomes increasingly difficult to see through (they are not transparent, rather light pigmented) as they age. This might decrease the ability to read a tag as the organism ages, but would likely take serval years to see if this occurs. One drawback of VIA tags is that they could not be used on small juvenile Texas blind salamanders. Difficulties in injections tearing the skin and low retention rates discouraged us from recommending VIA tag use for individually marking San Marcos or Comal Springs salamanders. Biologists should consider skin thickness and fragility when considering VIA tags, plus the tendency of a species to reject foreign objects from their skin.

PIT tags were not injected on any of the smaller Texas blind salamanders and are not recommended for San Marcos or Comal Springs salamanders due to the animals' small size. Should a smaller PIT tag be developed in the future, additional studies are warranted with caution. Retention might be higher if we had injected the PIT tags into the body cavity as is traditionally done. However, the small size of these salamanders makes it difficult to avoid piercing internal organs when injecting into the body cavity. The value of PIT tags would be in conjunction with submerged detection arrays in caves/wells for movement or population analysis studies. Low retention rates and concerns about injection site infection in the wild should be considered.

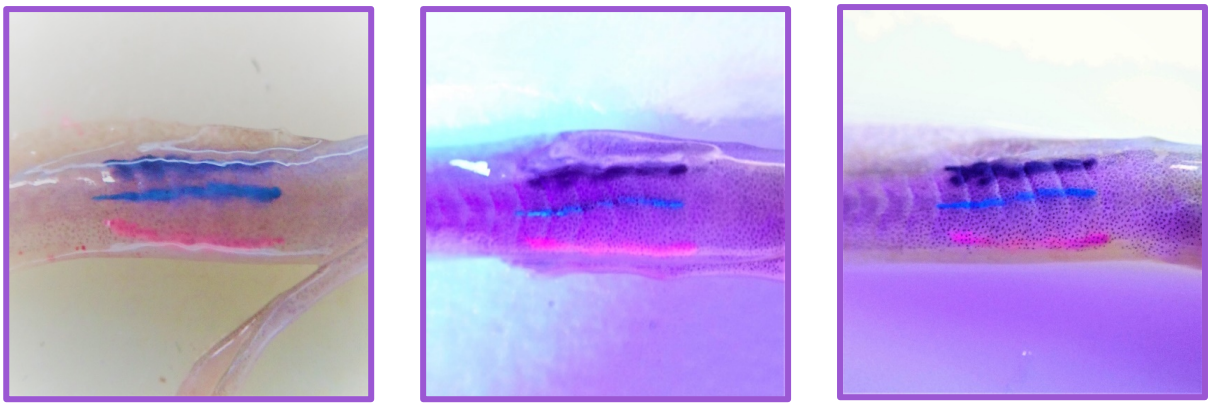


Figure 15 Texas blind salamander with horizontal color combination black, blue, pink. Initial tag (right), 3 month (center) and 6 month photos (left). This time-sequence reveals what an ideal visible implant elastomer tag should look like with no breakage and straight lines.

Of the three tags tested, we found VIE tags best for our purposes of quick, tank-side identification, ease of insertion, retention, and readability (Figure 15). Elastomer lines breaking from solid lines did reduce the clarity of some tags, but the majority of the tags could be accurately read and distinguished even with the breaks. Vertical VIE tags in Texas blinds appeared more vibrant in photographs, but these were also in younger salamanders with thinner, less pigmented skin than older salamanders tagged horizontally. The darker pigment in San Marcos and Comal Springs salamanders reduced the vividness of some colors and required an ultraviolet light to clearly see the tags. We do not recommend using non-fluorescing colors on darker pigmented salamanders, though the purple VIE is easily read in our two darker species. Pink and red VIE were difficult to distinguish, as were green and yellow. Our tagger that used longer elastomer lines had higher expert readability scores. It should be noted that the expert reader was also the tagger with the higher score, so the ability to read one's own tagging technique might increase recognition of tagging.

In general, we found that un-shed VIE tags and VIA tags could easily and accurately be identified by both expert and novel readers among the three salamander species. These tags would be useful in a variety of situations: individual tagging, batch tagging, cohort identification, gender identification, and mark-recapture studies. Our research lays the foundation for using these tag types for mark-recapture studies as it quantifies retention rates. This study was the first of its kind to compare three different tagging types on three species of salamanders simultaneously. It is one of the few studies to evaluate tags on salamanders that do not metamorphose and remain aquatic in all life stages. In addition, this study covers some of the smallest salamanders in the literature.

References

Appleby, B.W. 2015. Analysis of persistent marking techniques using passive integrated transponders and visible implant elastomer through metamorphosis in *Ambystoma mavortium*. Environmental Studies Undergraduate Student Theses. 175. http://digitalcommons.unl.edu/envstudtheses/175

Bailey, L.L. 2004. Evaluating Elastomer Marking and Photo Identification Methods for Terrestrial Salamanders: Marking Effects and Observer Bias. Herpetological Review 35(1)38-41.

Davis, T.M., Ovaska K. 2001. Individual Recognition of Amphibians: Effects of Toe Clipping and Fluorescent Tagging on the Salamander *Plethodon vehiculum*. Journal of Herpetology 35(2) 217-225.

Heemeyer, J.L., Homyack, J.A. 2007. Retention and readability of visible implant elastomer marks in eastern red-backed salamanders (*Plethodon cinereus*). Herpetological Review 38(4) 425-428.

Lunghi, E., Veith, M. 2017. Are Visual Implant Alpha tags adequate for individually marking European cave salamanders (genus Hydromantes)?. SALAMANDRA 53 (4) 541-544. ISSN 0036-3375.

Mitchell, M.S., Ennen, J.R., Cecala, K.K., Davenport, J.M. 2017. Ex-Situ PIT-tag Retention Study in Two Desmognathus Species. Herpetological Review 48(2) 313-316.

Osbourn, M.S., Hocking, D.J., Conner, C.A., Peterman, W.E., Semlitsch, R.D. 2011. Use of Flourescent Visible Implant Alphanumeric Tags to Individually Mark Juvenile Ambystomatid Salamanders. Herpetological Review 42(1) 43-47.

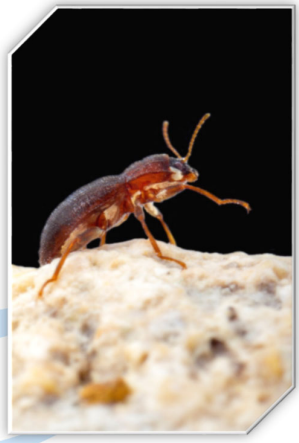
Phillips, C.T., Fries, J.N. 2009. An Evaluation of Visible Implant Elastomer for Marking the Federally Listed Fountain Darter and the San Marcos Salamander. North American Journal of Fisheries Management, 29: 529-532.

Whiteman, H.H., Doyle, J.M., Aubee, C., Brown, R., Thomason, S., Schoborg, T. 2016. A PIT Tagging Technique for Amystomatid Salamanders. Herpetological Review 47(1) 32-34.

Continuation of Captive Population Nutrition of the Comal Springs Riffle Beetle (*Heterelmis comalensis*)

2020 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program



Prepared by Lindsay Campbell, PhD with contributions from Amelia Hunter

San Marcos Aquatic Resources Center

U.S. Fish and Wildlife Service

Table of Contents

Background

The Comal Springs riffle beetle (*Heterelmis comalensis,* CSRB) was a species selected for focused research in 2020; highlighting its importance not only to the EAHCP, but also for the conservation of this species within the focus of the USFWS. Targeted research with captive standing stock was needed to successfully maintain healthy individuals and for the overall success of our refugia.

In 2018, CSRB in captive refugia at SMARC tracked by groups delimited on date collected, showed a sharp decrease in survival after 5 to 7 months in captivity. CSRB collected in the wild were of unknown age; therefore observed captive mortality could be due to natural senescence. We are seeking further opportunities to increase survival rates of adult CRSB through nutrition studies. Long-term survival of captive wild-stock CSRB–even after implementation of previous applied research studies on *H. comalensis* captive holding techniques (Nowlin *et al*. 2017; Worsham *et al*. 2017)–have been low. We suspect that the standard food items offered in captivity may not be adequate in macro- or micro-nutrients that could affect CSRB long-term survival. These deficiencies could be potentially supplemented through manufactured feed.

Previously awarded funding through USFWS was used to develop a series of diet formulations to test ingredient combinations and differing feed presentation types. The capabilities of the Fish Nutrition and Diet Development Research programs at Bozeman Fish Technology Center (BFTC) were utilized to provide scientific expertise and technical support to the SMARC. Feeds were formulated and manufactured with this funding in 2019. During 2020, we offered these feeds to the CSRB to test their efficacy with the beetles.

Objectives

The goal of this research project was to improve survival rates of adult CSRB in refugia through nutritional experimentation. This research had three parts:

1. Compare pellet types manufactured by BFTC for use in the Refugia.

    a. Use stable isotope analysis to determine which pellet(s) are consumed and utilized within the guts of wild caught adults in captivity.
    b. Determine which, if any, pellet in contrast to current diet given in captivity improves longevity and colonial fecundity of adults.
2. Asses the efficacy of 3D printed feed extrusions
    a. Test plastic and wood (30% wooden fibers: 70% Polylactic Acid) filaments for use as adherence structures for pastes, ideally a conditioned wood-like shape mimicking natural structures.
    b. Test if manufactured dry meal diet can be mixed and extruded by the 3D printer

Our hypothesis is that at least one of the four feeds manufactured by BFTC will serve as a more nutritious diet, and hence increased survival of the endangered CSRB in refugia at SMARC than the standard offerings in captivity.

Methods

Diet Development at Bozeman Fish Technology Center

Diet Formulation

Four diet formulations (USFWS 2019) were utilized to assess the ingredient preference of CSRB: a single-cell, protein (Protein) based diet, a plant-based (Plant) diet, an animal-based (Animal) diet, a bacteria/yeast-based diet (Bacteria), and log-shaped (Artificial Log) diet. Ingredients for Protein diet included a blend of single cell proteins from bacterial (Proplex-T), yeast (Proplex DY), and algal (Earthrise spirulina), which contributed most of the dietary protein (Table 1). Ingredients for the Plant diet consisted of primary protein ingredients of alfalfa meal, soy protein concentrate, spirulina, and corn protein concentrate. Ingredients for the Animal diet were chosen due to their common use in fish and shrimp feeds and general acceptance for most aquatic species studied at BFTC. The fourth formulation, Artificial Log, was utilized to provide a substrate for natural biofilm growth on which CSRB could graze. This diet substrate was extruded in a different shape and consistency than the other diets. Because we lack of knowledge of specific nutritional requirements of CSRB, all manufactured diets were supplemented to nutritional targets for fish, with respect to vitamins, minerals and amino acids. Wheat gluten and guar gum were used primarily for their binding capacity for formation of both sticky film and a water-stable pellets.

Feed Manufacture

Diets were produced in 2019 utilizing a commercial style manufacturing process (USFWS 2019). In brief, sinking pellets (Figure 1) and the artificial log were manufactured by cooking extrusion (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18 second exposure to an average of 127 °C in the sixth extruder barrel section. Pellets of 6 by 23 mm were produced for the bacterial, plant and animal diets. The artificial log pellets were pressed through an 8 mm die and cut to various lengths. The diets were dried and placed on a forced-air cooling table to reach room temperature.

Table 1 Ingredient list for comparison of the different artificial diets. Ingredients found primarily in one diet are color coded to that diet as listed in the headings. Ingredients highlighted in bright yellow were contained in all formulations. Ingredients in white were found in the first three diets.

Ingredient index	**Single Cell Protein** % of diet dry	**Plant** % of diet dry	**Animal** % of diet dry	**Artificial Log** % of diet dry
Alfalfa Nutrient Concentrate - Desalis	3	- -	- -	- -
Proplex DY - Ethanol Yeast	9	- -	- -	10
ProPlex-T ADM Threonine Biomass	9	- -	- -	10
Spirulina - Earthrise	9	3	3	- -
Alfalfa meal DWB	- -	10	- -	5
Corn Protein Concentrate	- -	10	- -	- -
Soy Protein Concentrate	- -	10	- -	- -
Blood meal- AP301	- -	- -	3	- -
Chicken 42 - ADF	- -	- -	8	- -
Fresh trimings fishmeal, Bio-Oregon Proteins	- -	- -	8	- -
BioMos bacteria	- -	- -	- -	5
Brewer's yeast	- -	- -	- -	10
Cellufil	- -	- -	- -	15
Rice hulls	- -	- -	- -	15
Wheat flour	53.43	49.17	61.78	15
Guar gum	5	5	5	10
Wheat gluten meal	5	5	5	10
Lysine HCl	0.89	1.04	0.17	- -
Monocalcium Phosphate	0.76	1.65	1.1	- -
Threonine	0.11	0.33	0.21	- -
Astaxanthin	0.08	0.08	0.08	- -
Choline Cl 50%	1	1	1	- -
DL-Methionine	0.08	0.08	0.01	- -
Lecithin - Yelkinol AC dry lecithin	1	1	1	- -
Magnesium Oxide	0.06	0.06	0.06	- -
NaCl	0.28	0.28	0.28	- -
Potassium chloride	0.56	0.56	0.56	- -
Stay-C 35	0.15	0.15	0.15	- -
Taurine	0.5	0.5	0.5	- -
TM ARS 1440	0.1	0.1	0.1	- -
Vitamin premix ARS 702	1	1	1	- -

Figure 1 The different pellet types undergoing buoyancy test and observation period for water clarity at BTC. From left to right, plant-based pellet, animal-based pellet, and single-cell-bacteria based pellet.

Experimental Design at the San Marcos Aquatic Resources Center

We offered the four manufactured feeds along with leaves, and cloth for biofilm to replicate groups of CSRB to test if CSRB consumed the feeds. We used ^{13}C:^{15}N isotopic analysis to assess diet signatures. Before the experiment with CSRB, the manufactured feed, leaves, cloth, and reference sample of CRSB were analyzed for discernible signatures. At the conclusion of the diet preference experiment, CSRB were sacrificed and sent for isotopic analysis to compare with reference samples.

Experimental Diet Preference Design

Six circular tanks (12 inch diameter; 8 inch height, 3 inch water depth) were set up to serve as replicates for each treatment (Figure 2). We designed a system for diet preference experiments as a partial recirculation system with a small heater in the sump to maintain optimal temperature for the beetles.

Figure 2 Tank set up for the diet preference experiment. Each tank had water input from above via black tubing.

Prior to experimentation with CRSB, we assessed the pellets in experimental tanks to estimate duration in which pellets retained structural integrity before dissolving. Manufactured food pellets, a cloth for biofilm, and leaves were placed in a circle in the tanks. Initially, four pellets of Protein diet, Plant diet, and Animal diet, and one Artificial Log were used in each tank, held in wire cages to keep types grouped together (Figure 3).

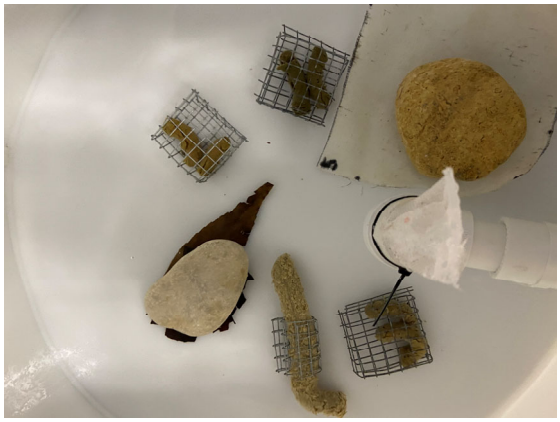


Figure 3 Configuration of food items offered to the beetles.

Adults collected from the wild on February 13, 2020, were placed in a transportation container for two hours to evacuate gut contents before we started the experiment (Nair, P. *pers. comm.*). Before animal placement, all collected beetles were sexed at the SMARC using an Olympus® SZX16 microscope to examine internal reproductive structures (Kosnicki 2019). Twelve beetles (6♀ + 6♂) were placed in each of the six tanks in the middle of the food item circle. CRSB grazed at will for 48 days. The experiment duration was determined based on the amount of time needed for the beetles to fully incorporate diet items (Nair, P. *pers. comm.*). During this time, pellets and traditional diet items were refreshed or replaced as needed based on visual degradation.

By the end of the first week, all the pellet types were fungused and the drains were clogged. We removed all fungused material and any floating debris and cleared the drains. Some beetles were observed trying to climb out of the tank, a sign of stress. We decided to cease the experiment and remove what beetles were still alive on February 18, 2020. Water quality parameters were recorded upon ending of the experiment; all measurements we nominal.

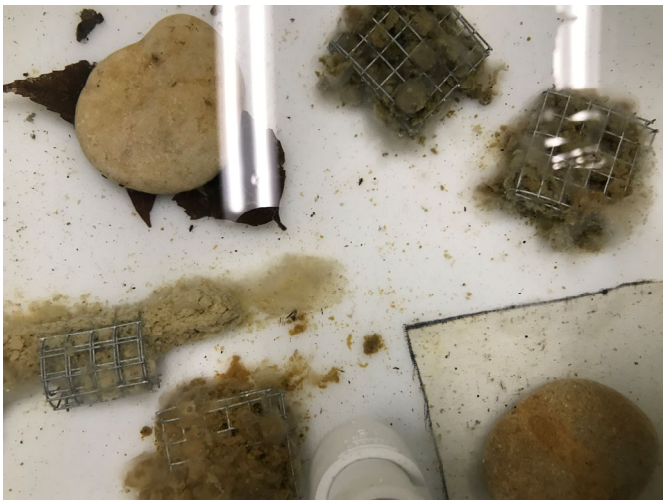


Figure 4 Overly fungused and degraded pellets.

We surmised that elevated temperature, 21 °C and above, might have increased fungal growth, exacerbated with too many pellets within the whole system, increasing biological input. We installed a UV sterilizer in the recirculating system and decreased the water temperature to 20 °C, still within a safe range for CSRB. We then tested the pellets for stability again with two pellets each. This setup ran for 48 hours and showed minimal signs of fungal growth. We re-started the experiments on February 25, 2020, with beetles from the same collection date, but did not use any previously used beetles. We monitored water quality and checked for signs of fungus. Pellets were replaced every 48 hours. The experiment ended on April 13, 2020.

Stable Isotope Analysis

In order to compare which (if any) manufactured pellet a riffle beetle digested, stable isotopes ^{13}C and ^{15}N and delta values of each were analyzed by scientists at University of California–Davis at the Stable Isotope Facility (UCD-SIF). Initial analysis of all current food offerings in captivity, the new pellet types, and a reference sample of Refugia CSRB were homogenized and sent to UCD-SIF for analysis before the start of the diet preference experiment.

At the experiment end, remaining beetles were prepared for analysis at UCD-SIF. Beetles were pooled by replicate container. Organisms were euthanized, rinsed with distilled water, and put into a centrifuge tube and stored at -80 ˚C. Before homogenization, samples were freeze dried for 72 hours. After the samples were completely dry, we homogenized the samples using mortar and pestle (Nair 2019). CSRB were then placed into a pre-weighed tin capsule and weighed to ensure they within detection range in UCD-SIF's analyzers, 0.8 to 1.2 mg.

Statistical Analysis

We used a Bayesian mixing model to analyze stable isotope data from the food items and the CSRB, allowing estimation of the proportion of each food item eaten by CSRB. We used the MixSIAR package in R (version 4.0.3) to run a three chain Monte-Carlo Mixing model (uninformed prior) with 100,000 chain length, a 50,000 burn-in, and 50 thinning. Trophic enrichments factors for $\delta^{13}C$ and $\delta^{15}N$ were 0.75 ± 1.87 and 2.75 ± 1.64 respectively.

Diet Performance

Food preference does not necessarily indicate high nutritional benefit or improve fitness of the individuals. Therefore, after the completion of the stable isotopes, a final experiment will be conducted to determine how each pellet impacts adult longevity in captivity and larval production.

Nutritional 3D Printing

In the original 2020 proposal, we intended to use an adapted 3D printer to extrude developed mash to test adhering the nutritional mash to 3D printed, rough textured models resembling wooden log pieces. We were unable to complete this task due to CoVID-19 related delays in the other prerequisite experiments.

Results and Discussion

Stable isotope analysis of reference samples of food items, pellets, and CSRB showed differences among the items (Figure 5). Out of the 72 adults at the start of the first diet preference experimental run, only 34 adults were alive on February 18, 2020. By this point, many dead adults were covered in fungus and could not be used for stable isotope analysis. At the conclusion of the second experimental run a total of 53 of the 72 beetles remain. These were pooled by replicate container for stable isotope analysis. The number of beetles per replicate were 9, 9, 10, 10, 8, and 7. Recorded weight of each homogenized sample were submitted with the samples for standardizing stable isotope values. Samples of cloth with biofilm from the experimental containers were also sent for analysis. UCD closed due to CoVID-19 until May 2020, and had a back-log of samples to process. Thus, our project was delayed. The experimental samples were sent May 26, 2020, and results returned August 10, 2020.

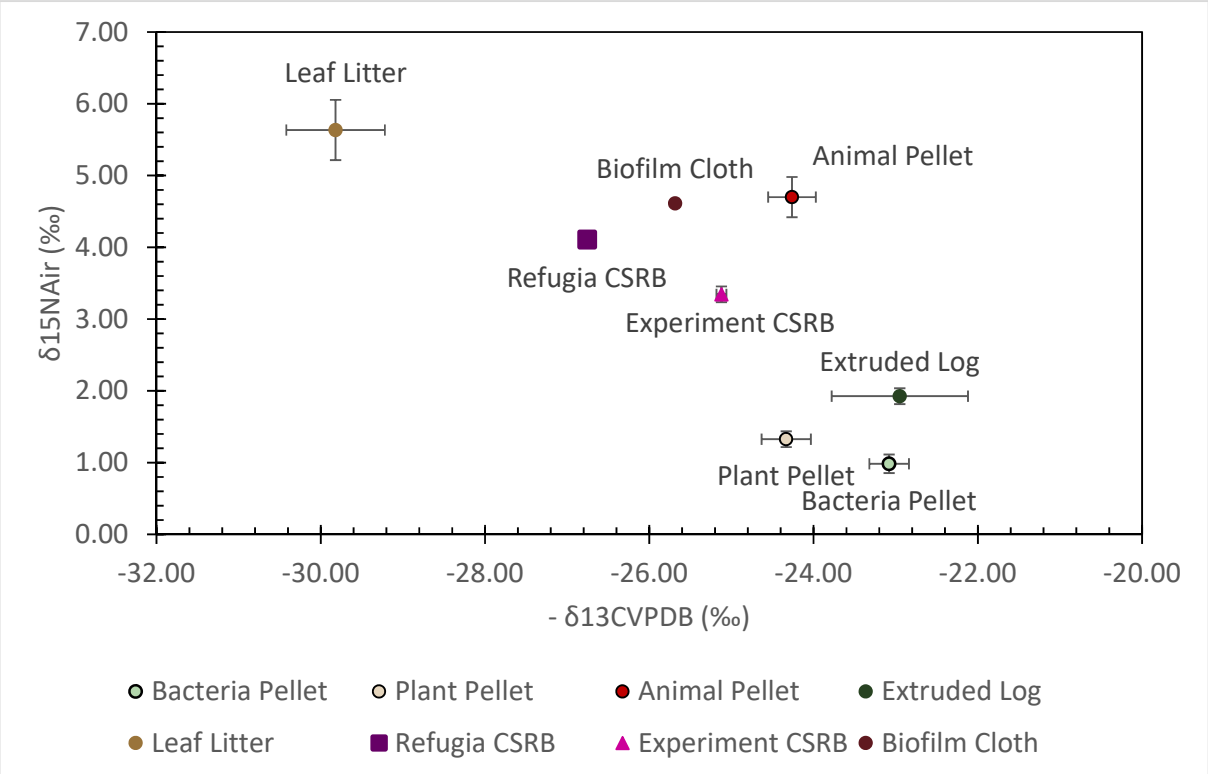


Figure 5 Biplot of delta Nitrogen and Carbon stable isotope analysis. Bars around each point are the standard error for each value. Each point is the average of replicates, except for the Refugia CSRB as only one replicate was analyzed (no error bars on this point). The error bars of the Biofilm Cloth point were too small to extend past the marker.

The stable isotope signature of CSRB shifted during the experiment when compared to the reference sample of CSRB offered standard food in the Refugia (Figure 5). The Bayesian mixing model estimated that leaves were the main source of the CSRB diet (Table 2). Of all manufactured diets, the Plant pellet was consumed the most. Posterior density distributions of proportional contributions of food items estimated using MixSIAR are presented visually in Figure 6, which sums proportions across the model iterations. The matrix plot (Figure 7) shows the posterior probability distribution histograms on the diagonal, correlation between the sources to the right of the diagonal, and contour plots of the relationship between the sources to the left of the diagonal. The histograms represent the proportions simulated by the model. Bars indicate the likelihood of that proportion of the diet. For example, the histogram for the Animal diet peaks close to the 0.0 proportional range, indicating this diet is less likely to be a component of the food ingested by CSRB. Large negative correlations (numbers in boxes to the left of the diagonal) indicate that the model cannot discern between the two sources. Our items do not have negative correlations greater than -0.50, indicating the model could discern between sources. This analysis gives us a basis of potential diet elements and nutrients to introduce to captive CSRB in the future.

Table 2 Proportion of diet for each food type offered as estimated by the Bayesian Mixing Model. The mean value, median value, standard deviation (SD), and lower (2.50%) and upper (97.50%) bounds of the credible interval are listed.

Diet Item	Mean	Median	SD	2.50%	97.50%
Animal Pellet	10.7	7.8	10.1	0.3	37.1
Single Cell Pellet	16.0	13.2	13.0	0.7	46.7
Plant Pellet	19.3	16.6	14.4	0.6	52.1
Artificial Log	13.3	14.4	15.2	0.5	55.2
Leaves	22.6	24.9	10.9	0.5	40.3
Biofilm Cloth	13.3	10.3	11.6	0.4	43.8

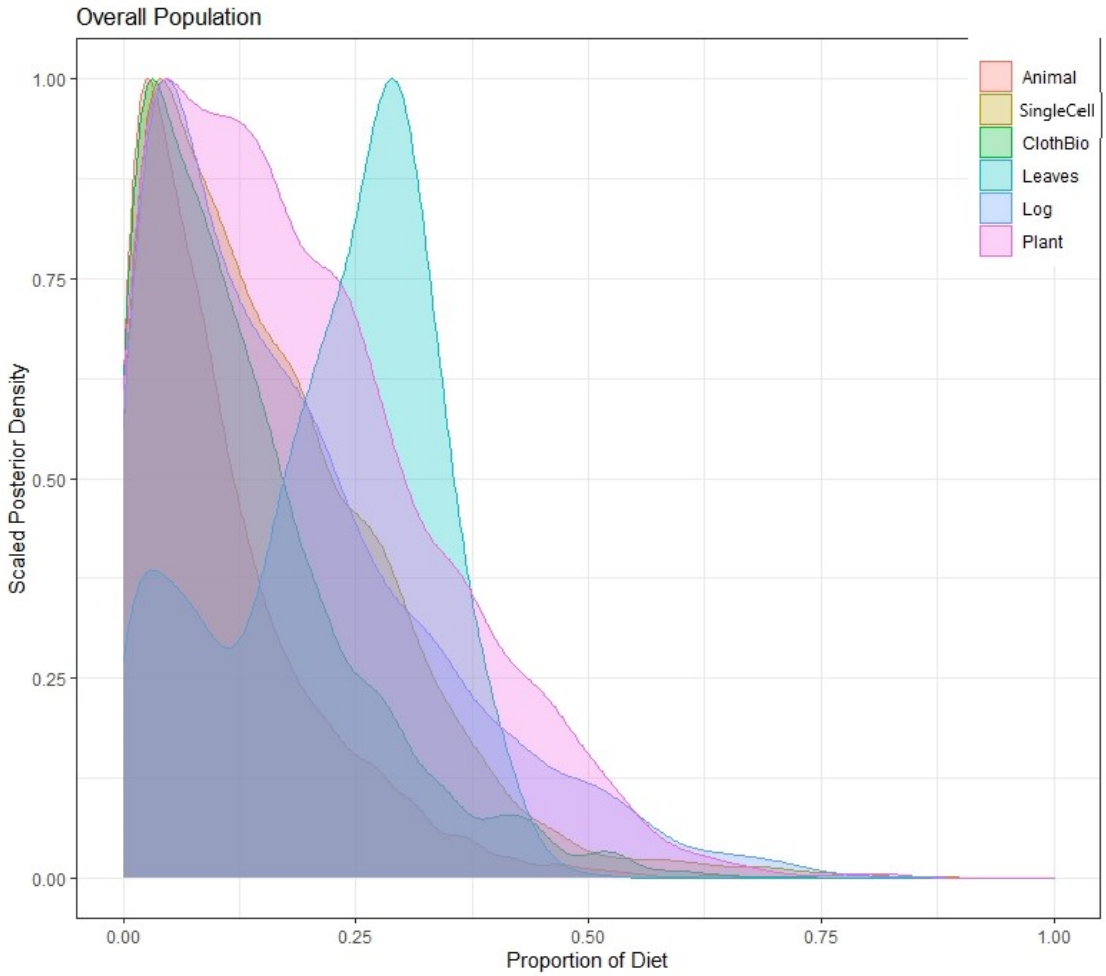


Figure 6 Combined distributions of each food item in the diet of CSRB from food preference experiment.

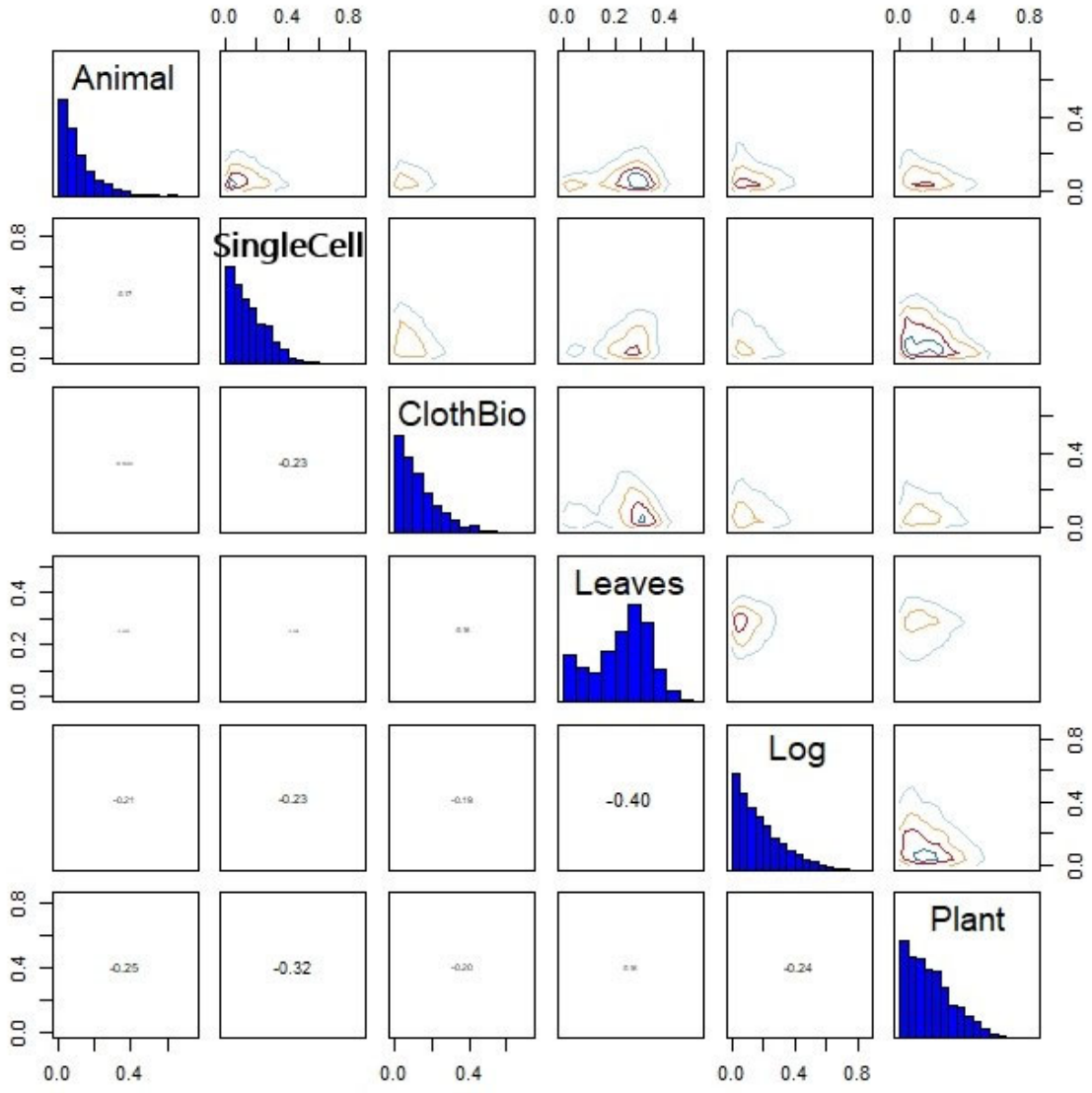


Figure 7 Matrix plot of individual food item distributions as determined by MixSIAR Bayesian mixing model. Diagonal cells show posterior probability distribution histograms for each item. Correlation values between items are to the left of the diagonal. Increased font size indicates increased correlation. Boxes to the right of the diagonal show contours of the joint probability distribution for contribution for pairs of food sources.

The isotopic analysis of the food preference experiment arrived later than anticipated and results suggested that none of the manufactured diets were equivalent or better than a leaf-based diet (a standard food offering for CSRB). We decided not to pursue the diet performance experiment with the top consumed manufactured feed. Our tests revealed that none of the pellets nor the log in their current forms could be realistically used in standard Refugia CSRB containers. The pellets required replacement every other day due to fungal growth, fouling the water. On a Refugia scale, this would decrease the efficiency of CSRB refugia operations. Additionally, we have observed CSRB entrapped, dead, in fungus; thus the pellets would not be conducive to increasing survival rate. In the future a smaller Plant pellet might be worth testing as an occasional addition to CSRB holding containers. Refugia staff plan on pursuing the 3D printing of habitat structure in 2021.

References

Balasubramanian, S, M. Aubin-Tam, and A.S. Meyer. 2019. 3D Printing for the Fabrication of Biofilm-Based Functional Living Materials. ACS Synth. Biol. 8: 1564-1567.

Kosnicki, E. 2019. Determining Sexual Dimorphism of Living Aquatic Beetles, *Stygoparnus comalensis* (Coleoptera: Dryopidae) and Heterelmis comalensis (Coleoptera: Elmidae), Using Internal Abdominal Structures. Journal of Insect Science 19(4). DOI: 10.1093/jisesa/iez075.

Ning, E., G. Turnbull, J. Clarke, F. Picard, P. Riches, M. Vendrell, D. Graham, A.W. Wark, K. Faulds, and W. Shu. 2019. 3D bioprinting of mature bacterial biofilms for antimicrobial resistance drug testing. Biofabrication, 11: 045018.

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

Nowlin, W. H., D. Hahn, P. Nair, and F. Alfano. 2017. Evaluation of the trophic status and functional feeding group status of the Comal Springs riffle beetle. Edwards Aquifer Habitat Conservation Plan (EAHCP) (EAHCP Project No. 148-15-HCP) Applied Research Program. https://www.edwardsaquifer.org/doc_publications/evaluation-of-the-trophic-status-and-functional-feeding-group-status-of-the-comal-springs-riffle-beetle. 30pp.

Parnell, A. C., D. L. Phillips, S. Bearhop, B. X. Semmens, E. J. Ward, J. W. Moore, A. L. Jackson, J. Grey, D. J. Kelly, and R. Inger. 2013. Bayesian stable isotope mixing models. Environmetrics, 24:387-399.

Spiesz, E.M., K. Yu, B.A.E. Lehner, D.T. Schmieden, M. Aubin-Tam, A.S. Meyer. 2019. Three-dimensional Patterning of Engineered Biofilms with a Do-it-yourself Bioprinter. J. Vis. Exp. (147), e59477, doi:10.3791/59477.

Stock BC and Semmens BX. 2016. MixSIAR GUI User Manual. Version 3.1. https://github.com/brianstock/MixSIAR. doi:10.5281/zenodo.1209993.

Stock BC, Jackson AL, Ward EJ, Parnell AC, Phillips DL, Semmens BX. 2018. Analyzing mixing systems using a new generation of Bayesian tracer mixing models. PeerJ 6:e5096 https://doi.org/10.7717/peerj.5096

United States Fish and Wildlife Service (USFWS). 1980. Endangered and Threatened Wildlife and Plants; Listing of the San Marcos Salamander as Threatened, the San Marcos Gambusia as Endangered, and the Listing of Critical Habitat for Texas Wild Rice, San Marcos Salamander, San Marcos Gambusia, and Fountain Darter. Federal Register 45:47355-47364.

United States Fish and Wildlife Service (USFWS). 2019. System Wide 1311 Project Region 2 Report for Diet Development for Threatened and Endangered Species: Comal Springs riffle beetle.

Worsham, M., E. Julius, A. H. Everett, W. H. Nowlin, P. Nair, D. Sada, E. Koznicki, and E. Oborny. 2017. Comal Springs riffle beetle (*Heterelmis comalensis*): Life history and captive propagation techniques. EAHCP Applied Research Final Report. 36 pp.

TEXAS PARKS & WILDLIFE

Receipt for Donation of Fish or Wildlife Specimens

Source *(Please place an "X" in one box and provide a valid permit number and the permit period of validity.)*

☐ Educational _____ ☒ Scientific SPR-0616-153

☐ Zoological _____ ☐ Rehabilitation _____

Permit Effective Period: 06/23/2016 through 06/23/2019

Permittee Name: Kenneth Ostrand Daytime Telephone: (512) 353-0011

Facility Name: San Marcos Aquatic Resources Center AZA Accredited? ☐ Yes ☒ No

Address: 500 East McCarty Lane City: San Marcos State: TX Zip: 78666

Destination *(Please place an "X" in one box and provide a valid permit number and the permit period of validity.)*

☐ Educational _____ ☒ Scientific _____ ☐ Zoological _____

Permit Effective Period: _____ through _____

Permittee Name: Dave Hampton Daytime Telephone: (575) 734-5910

Facility Name: Southwestern FIsh Health Unit AZA Accredited? ☐ Yes ☒ No

Address: 7116 Hatchery Rd City: Dexter State: NM Zip: 88230

Specimens: (**Live** refers to live healthy specimens; **NR** refers to live specimens deemed as non-releasable; **Dead** refers to non-living specimens to be used for research, as voucher specimens or preserved/mounted specimens for display.)

Common Name	Scientific Name	Quantity	Live	N/R	Dead
Fountain Dater (San Marcos)	*Etheostoma fonticola*	60	☒	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

Source Signature: I certify that I, (place an "X" in one) ☐ permittee or ☒ sub-permittee, am authorized by my permit to donate the above referenced specimens to other Scientific, Educational and/or Zoological permit holders who are authorized to receive such specimens.

Signature of Donor: Linda Moon **Date:** 10/21/2019

Destination Signature: I certify that I, (place an "X" in one) ☐ permittee or ☐ sub-permittee, am authorized by my permit to receive the above referenced specimens.

Signature of Recipient: [signature] **Date:** 10/22/19

NOTE: This form may be reproduced as necessary.

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

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

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

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

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

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

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

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

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

FOD Parasite Data Sheet - Form P-03

Case History No. 20-05

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

Collection site: San Marcos River, TX

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

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

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

Examiner signature HK, MB, JW

Revised on 9/20/2017

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

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

Memorandum: October 12, 2019

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

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

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

Results:

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

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

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

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

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

AQUATIC HEALTH ACCESSION FORM

Washington Animal Disease Diagnostic Laboratory

College of Veterinary Medicine, Washington State University

Mailing address:
PO Box 647034
Pullman, WA. 99164-7034

Web Site: http://waddl.vetmed.wsu.edu
Shipping address:
Bustad Hall, Rm.155-N
Pullman, WA. 99164-7034

Phone: (509) 335-9696
FAX: (509) 335 7424
E-Mail: waddl@vetmed.wsu.edu

Please type or use black ink and print clearly.

Veterinarian or Case Coordinator: Allan Pessier
WADDL VET CLIENT #:
WADDL CLINIC CLIENT #:

Clinic:

Street:

City: State: Zip:

Phone: Fax:

Date Shipped: E-mail:

WADDL USE ONLY

Owner: San Marcos ARC
WADDL OWNER CLIENT #:

Street: 500 E Mccarty Lane

City: San Marcos State: TX Zip: 78640

Phone: 512-353-0011 Fax/E-mail:

Please fill out completely as possible:

Specimen(s) Submitted: 36 whole salamanders live in freshwater

Sampling Date: 7/2/19

Aquatic Tests Requested:

- [x] *Necropsy*
- [x] *Histopathology*
- [x] *Toxicology*
- [x] *Virology*
- [x] *Fungal culture*
- [x] *Mycobacteria culture*
- [x] *Bacteriology*
- [x] *Parasitology*
- [ ] *Whirling Disease*
- [ ] *PCR*
- [ ] *Antibiotic Sensitivity* — *Antibiotic of interest:*
- [x] *Other* Trace minerals, vitamin A, microsporidia

Note: WADDL reserves the right to modify the tests requested for more efficient case work-up and / or send specimens to outside laboratories to perform tests not done at WADDL.

Species	*Animal ID (name/tag#) or Lot #*	*Water Temperature*	*Animal Weight*	*Age*
Eurycea nana		21C		

Location of Lesion(s)	*No. in group*	*No. Dead*	*No. Sick*	*No. on Premises*	*Duration of Problem*
Skull, limbs, spine, tail					

** Was animal euthanized? If so, what method?*

Water:
- [ ] *Marine / Brackish*
- [x] *Freshwater*

System:
- [x] *Flow-through*
- [ ] *Recirculating*
- [ ] *Net pen*
- [ ] *Other* _____

- [x] *Health Testing*
- [x] *Diagnostic Testing*

Pathogen(s) of interest:

Additional History: *Vaccinations, signs, stress factors, treatments, post mortem findings, pertinent feed or feed additives, clinical lab results, previous WADDL Case Numbers. (Attach additional sheets as necessary.)*

15 gravid females freshly collected from the wild population, 10 females from younger/recently collected (2018-2019), and 10 females from the historic (older, sickly) population in refugia are included in submission to compare disease, analyze trace minerals, conduct vitamin A tests and histopathology, and to look for differences between populations and possible explanations for females rupturing eggs and/or a lack of reproduction in captivity.

Also included is one sick male (live, freshly collected) exhibiting swelling similar to what we have seen in refugia. This animal was collected in this condition from the wild.

SAMPLE COLLECTOR:

Print Collector's Name _____ Collector's Signature _____

Veterinarian's or Clinician's Signature:

Condition(s) Suspected:

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/963

Memorandum: February 11, 2020

To: Mark Yost, Uvalde National Fish Hatchery
From: Jason Woodland, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the Fountain Darters (SNARRC Case Number 20-13)

On January 9, 2020, staff at the Southwestern Fish Health Unit (SFHU) received five fountain darters (*Etheostoma fonticola)* from Uvalde National Fish Hatchery for diagnostic examination. Hatchery staff reported that the fish displayed lethargy and showed reduced feeding. Hatchery staff also reported slight presence of monogenetic trematodes on the mortalities. A total of seven fish mortalities were observed on the Sunday prior to submitting fish to the Fish Health Unit for testing.

Fish stocking densities were reported at 1 fish per 6–7 liters of water. These tanks reportedly receive three exchanges of well water each day. At the time of sampling, tank water temperature was 20.7°C, DO concentration was 7.11 mg/L, total ammonia and nitrate were measured as 0.00 ppm, and 0.06 ppm, respectively, salinity was 0.0 ppt, and the pH value was recorded as 8.1. Fish were treated with a static 24-hour salt bath and formalin at a concentration of 0.5% and 15 ppm, respectively, on January 2. They also had received a 24-hour salt bath (0.5%) on January 8 prior to being shipped to the SFHU.

Upon arrival at the SFHU, fish were euthanized in sodium bicarbonate buffered MS-222. Standard light microscopy of wet-mount preparations of skin and gill recorded no significant finding. No external parasites were observed. A total of three fish were sampled for bacteriology, sampling the kidney and plating on brain heart infusion agar medium. Whole bodies of these three fish were also sampled for virology. The remaining two fish were sampled whole in Z-fix for histopathology. All clinical testing was conducted per the American Fisheries Society-Fish Health Section Bluebook (2016 Edition) and standard SFHU protocols. Fixed fish were submitted to the Washington Animal Disease Diagnostic Lab for histopathological evaluation.

Results:

Virology cell culture testing did detect presence of any virus. Likewise, no bacterial cultures were isolated from the kidneys. Histopathology identified proliferative bronchitis, histiocytic dermatitis, and coelomitis. A mild myxozoan infection of spores was also observed in the brain cavity.

Final Diagnosis: Proliferative bronchitis, histiocytic dermatitis, and coelomitis.

A mild myxozoan infection was detected in the brain cavity; however, the infection was noted as mild. Notes from histopathology report indicate that these myxozoan infections are not abnormal for these fish. Previous parasite infections or particulate matter in the water may be responsible for the proliferative bronchitis. Reports by hatchery staff of previous parasite infections support this finding. A cause for the histiocytic dermatitis and coelomitis was not determined.

Staff from the Southwestern Fish Health Unit made the recommendation to try 0.5% salt bath treatment every other day or every third day due to reported success by hatchery staff. It was also recommended to increase water flow. Hatchery staff planned to make these changes for a couple of weeks to see if they would reduce or eliminate fish mortalities.

Please let us know if there is need for additional assistance. If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 20-13 for any follow up correspondence.

cc: Patricia Duncan, Uvalde NFH
Huseyin Kucuktas, Southwestern Fish Health Unit – SNARRC

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 23, 2020

In Reply Refer To:
FWS/R2/FR-SFHU/968

Memorandum

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

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

Subject: Final report for the fountain darters from the Comal River, TX (Case Number 20-26).

On February 25, 2020, the Southwestern Fish Health Unit (SFHU) received a total of 11 fountain darters (*Etheostoma fonticola*) from the Comal River, TX. The receipt for donation stated that a total of 10 fish were submitted from this location. These fish were collected by staff at the San Marcos Aquatic Resource Center and shipped live to the SFHU. Location of fish collection was recorded at latitude 29.7107° and longitude -98.1276° in Comal County, Texas.

The fish were examined for *Centrocestus formosanus* parasite enumeration. Screening for *C. formosanus* was conducted by examining the left gill arches for each fish under light microscopy. Eight out of ten fish examined had *C. formosanus* on their gills. The final numbers are reported on the following page.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to the case number 20-26 for any follow-up correspondence.

cc: Lindsay Campbell, San Marcos Aquatic Resource Center
Linda Moon, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 20-26

Date examined: 02/25/2020 Date Collected: 02/24/2020

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)	535	331	265	187	221	323	317	300	131	163
Total Length (mm)	30	33	31	25	26	35	30	31	24	27

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	1,5,4,2	1,4,2,1	1,1,2,3	0,0,0,0	0,1,3,0	1,5,2,1	0,3,4,4	0,1,3,0	0,0,0,0	2,2,1,0
Monogenea	L	0,0,1,0	0,4,0,1	0,1,0,0	1,1,0,1	0,1,0,0	2,1,2,1	0,1,1,0	1,2,0,1	1,0,1,0	0,0,0,0
Myxobolus sp.	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Other	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0

Examiner signature Huseyin Kucuktas

Revised on 9/20/2017

FOD Parasite Data Sheet - Form P-03

Case History No. 20-27

Date examined: 02/25/2020 Date Collected: 02/24/2020

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)	489	410	243	294	292	556	264	495	280	187
Total Length (mm)	39	36	31	32	32	40	30	37	32	29

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	1,1,0,0	0,2,5,1	0,0,0,0	0,0,0,0	0,0,2,2	1,1,1,1	0,0,2,0	0,0,1,2	0,0,0,0	0,0,1,0
Monogenea	L	0,0,0,0	0,2,1,0	2,4,0,1	0,0,1,0	1,1,1,0	0,0,1,0	1,0,0,0	2,0,2,0	2,0,2,1	0,0,0,0
Myxobolus sp.	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Other	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0

Examiner signature Huseyin Kucuktas

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 23, 2020

In Reply Refer To:
FWS/R2/FR-SFHU/969

Memorandum

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

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

Subject: Final report for the fountain darters from the San Marcos River, TX (Case Number 20-27).

On February 25, 2020, the Southwestern Fish Health Unit (SFHU) received a total of 10 fountain darters (*Etheostoma fonticola*) from the San Marcos River, TX. These fish were collected by staff at the San Marcos Aquatic Resource Center and shipped live to the SFHU. Location of fish collection was recorded at latitude 29.8754° and longitude -97.9319° in Hays County, Texas.

The fish were examined for *Centrocestus formosanus* parasite enumeration. Screening for *C. formosanus* was conducted by examining the left gill arches for each fish under light microscopy. Seven out of ten fish examined had *C. formosanus* on their gills. The final numbers are reported on the following page.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to the case number 20-27 for any follow up correspondence.

cc: Lindsay Campbell, San Marcos Aquatic Resource Center
Linda Moon, San Marcos Aquatic Resource Center

20-26 & 20-27

TEXAS PARKS & WILDLIFE

Receipt for Donation of Fish or Wildlife Specimens

Source *(Please place an "X" in one box and provide a valid permit number and the permit period of validity.)*

☐ Educational _____ ☒ Scientific SPR-0616-153

☐ Zoological _____ ☐ Rehabilitation _____

Permit Effective Period: 06/23/2016 through 06/23/2019

Permittee Name: Kenneth Ostrand Daytime Telephone: (512) 353-0011

Facility Name: San Marcos Aquatic Resources Center AZA Accredited? ☐ Yes ☒ No

Address: 500 East McCarty Lane City: San Marcos State: TX Zip: 78666

Destination *(Please place an "X" in one box and provide a valid permit number and the permit period of validity.)*

☐ Educational _____ ☐ Scientific _____ ☐ Zoological _____

Permit Effective Period: _____ through _____

Permittee Name: Dave Hampton Daytime Telephone: (575) 734-5910

Facility Name: Southwestern Fish Health Unit AZA Accredited? ☐ Yes ☒ No

Address: 7116 Hatchery Rd City: Dexter State: NM Zip: 88230

Specimens: (**Live** refers to live healthy specimens; **NR** refers to live specimens deemed as non-releasable; **Dead** refers to non-living specimens to be used for research, as voucher specimens or preserved/mounted specimens for display.)

Common Name	Scientific Name	Quantity	Live	N/R	Dead
Fountain Darter (San Marcos)	*Etheostoma fonticola*	10	☒	☐	☐
Fountain Darter (Comal)	*Etheostoma fonticola*	~~10~~ 11 OH	☒	☐	☐
			☐	☐	☐
			☐	☐	☐

Source Signature: I certify that I, (place an "X" in one) ☐ permittee or ☒ sub-permittee, am authorized by my permit to donate the above referenced specimens to other Scientific, Educational and/or Zoological permit holders who are authorized to receive such specimens.

Signature of Donor: Linda Moon **Date:** 2/24/2020

Destination Signature: I certify that I, (place an "X" in one) ☐ permittee or ☐ sub-permittee, am authorized by my permit to receive the above referenced specimens.

Signature of Recipient: _____ **Date:** 2/25/2020

NOTE: This form may be reproduced as necessary.

PWD 1143 – W7000 (11/06)

Lower comal (schlitterbahn employee parking lot)	29.71069	-98.1276	10
Lower San marcos (ramon lucio park)	29.87544	-97.9319	10

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/973 June 25, 2020
Memorandum

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

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 20-57).

On May 27, 2020, Southwestern Fish Health Unit (SFHU) staff received 49 fountain darters (*Etheostoma fonticola*) from the San Marcos River (GNIS ID: 1375972), Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for fish health testing. San Marcos ARC staff recorded collection of fountain darters from three locations on the San Marcos River as follows: below Spring Lake Dam at latitude 29.8901° and longitude -97.9340°; Middle/City Park at latitude 29.8860° and longitude -97.9358°; and lower/HI35 at latitude 29.8754° and longitude -97.9319° in Hays County, Texas.

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

Results:
Centrocestus formosanus was observed in 8 of 10 fish examined. Aquareovirus was also isolated in cell culture and confirmed by PCR testing. 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 20-57 for all follow up correspondence.

FOD Parasite Data Sheet - Form P-03

Case History No. 20-57

Date examined: 5/27/2020

Date Collected: April 21-23,2020

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)	531	548	202	310	265	458	448	222	209	154
Total Length (mm)	290	310	220	270	260	290	280	230	240	190

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

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

Examiner signature 

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

7/15/2020

In Reply Refer To:
FWS/R2/FR-SFHU/975

Memorandum

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

From: Trista Becker

Subject: Diagnostic case 20-55, Fountain darter

Diagnosis: verminous gastritis, systemic inflammatory disease, nephrocalcinosis
Agent/Cause: Cryptobia sp.

A single fountain darter was submitted directly to Washington State Aquatic Diagnostic lab from the population in quarantine at San Marcos Aquatic Resources Center. There has been a recent history of bloating and moribund fish in some tanks, and due to the ongoing pandemic travel restrictions fish health has not been able to attend to the issue on site directly.

The histopathology results from this fish indicated a severe systemic (widespread) inflammatory disease due to high numbers of a flagellated parasite. Also noted was a severe nephrocalcinosis (calcifications in the kidneys) with granuloma formation. The granulomas indicate a long-term issue, and sometimes this can be related to water quality such as high CO2, metals, and/or mineral deficiencies. This may have predisposed the groups to having larger amounts of parasite, as alone it is often considered incidental in wild fish. Also, the lesions suggest that the parasitism was a more recent occurrence than the kidney issues. The presumptive parasite identification based on what is known was *Cryptobia iubilans*.

Communication with a local veterinarian (Dr. Doll) and an extensive search by Dr. Campbell enabled an in-water treatment with dimetridazole at 80ppm for 24h as a static bath for 3 days. The dose and drug chosen was based on previously published studies and communications with researchers at UFl. Variable tolerance of this dose was noted, so further treatments that needed to be carried out were recommended to be attempted following a “step down” bioassay, starting at 60ppm. Some tanks of fish more recently captured from the wild showed signs of toxicity quickly after administration (twitching and rolling). It is unsure whether the toxicity noted could be related to the

underlying kidney issues in some fish. Salt was also applied at 1% to provide some osmotic stress relief to the fish.

It has been hypothesized that the fountain darters may be picking up this parasite in the wild, so further discussions to treat as fish are captured for the refugia population would be warranted. Also, the patterns seen with the long-term kidney calcifications would suggest a water quality/toxin issue at the site of capture.

Further definitive identification of the parasite through molecular analysis is being pursued at WADDL currently. Additional cases of fish submitted for histopathology are still pending and may give further details.

Thank you for letting us care for your fish!

Sincerely,

Trista Becker, DVM, MS, CertAqVet

cc: Lindsay Campbell, Linda Moon

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

7/22/2020

In Reply Refer To:
FWS/R2/FR-SFHU/977

Memorandum

To: Ken Ostrand

From: Trista Becker

Subject: fountain darter case 20-47 from WADDL submission April 10

Diagnosis: nephrocalcinosis, hepatic necrosis and moderate skeletal parasitism
Agent/Cause: mineral deposits in kidney, unknown type; unidentified myxozoan parasites in cranium and branchial cavity (possibly incidental)

Throughout the spring months (roughly March through June), signs of dropsy have been noted in some fish from the San Marcos fountain darter tanks. On April 10, one fountain darter with dropsy was submitted to WADDL as a whole fish for histopathology. Similar lesions associated with granular mineral deposits were seen in the kidneys as are often seen in other fountain darter submissions. This nephrocalcinosis, though often mild to moderate and singly not determined severe enough to cause mortality, it is often associated with other findings such as parasitism by various organisms and generalized inflammation that – when combined – may lead to the chronic, slow, mortality with associated dropsy. Renal (caudal kidney) lesions in this case are chronic, and may have led to a slow and progressive coelomic fluid buildup due to loss of osmotic function.

Significant autolysis was reported by the histopathologist (Dr. Lori Bedient), but some lesions could still be observed in organ systems. Autolysis will mask any lesions potentially present in the cells due to breakdown of cellular material and organization. Speedy immersion of samples into 10% neutral buffered formalin (or other appropriate preservative) after opening the body cavity and removing the opercula from the gills will improve preservation of cells and thus the diagnostic quality of the specimens. Unfortunately, there was also a lengthy delay in the reporting of histopathology results. This was discussed with the senior pathologist at WADDL.

Please also refer to other reports for cases related to this case – case # 20-55 (report # 975 from 7/15) and 20-60 (report # 978 from 7/22). There have been many submissions of fountain darters over the years, with variable lesion descriptions. As one commonality appears to be nephrocalcinosis, this issue should be pursued in the future and efforts to evaluate water hardness and reduce mineral

levels in the tank water source should be considered. A large-scale summary report of the issues seen in fountain darters may help to further identify common themes. Site visits to evaluate fish in their systems once COVID-19 concerns have passed will also be essential. Occasional submission of samples to WADDL followed by sporadic diagnostic reports from different pathologists with each submission make it difficult to evaluate the full case picture over time.

Thank you for letting us care for your fish!

Sincerely,

Trista Becker, DVM, MS, CertAqVet

cc: Lindsay Campbell, Linda Moon, Jason Woodland

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

7/22/2020

In Reply Refer To:
FWS/R2/FR-SFHU/978

Memorandum

To: Ken Ostrand

From: Trista Becker

Subject: fountain darter case 20-60 from WADDL submission June 5

Diagnosis: steatitis, with probable associated multi-systemic inflammation
Agent/Cause: undetermined, possible nutritional and/or toxin exposure/water quality issues

Throughout the spring months (roughly March through June), signs of dropsy have been noted in some fish from the San Marcos fountain darter tanks. On June 5, one fountain darter with dropsy was submitted to WADDL as a whole fish for histopathology. The main issue noted was hemorrhage from the liver filling the coelom. With the absence of a known significant trauma, it may be that the liver was friable and and more fragile in this fish. Toxin exposure can lead to liver damage, as can lipid peroxidation due to steatitis. There was mild nephrocalcinosis, as has been noted in many other case submissions to date, and other mild, nonspecific signs of inflammatory process. Also, a mild steatitis (fat oxidation/breakdown) was also noted which may be related to a dietary issue compounded by vitellogenesis in this female producing eggs.

Please also refer to other reports for cases related to this – case # 20-55 (report # 975 from 7/15) and 20-60 (report # 977 from 7/22). As noted recently in report 977, there have been many submissions of fountain darters over the years, with variable lesion descriptions, and the nephrocalcinosis continues to reappear at varying degrees of severity. The lack of pathogenic organisms identified to explain the mild systemic inflammatory signs may place toxin exposure higher on the list of underlying issues, though the lesions noted in this fish are more likely associated with a nutritional steatitis issue.

Thank you for letting us care for your fish!

Sincerely,

DEPARTMENT OF THE INTERIOR
U.S.Fish and Wildlife Service

FISH HEALTH INSPECTION REPORT[1]

This report is NOT evidence of future disease status. To determine status, contact the inspecting biologist below.

Fish Source & Facility Contact	Fish Examined	Water Supply[2]	5 Year facility classification	
San Marcos Aquatic Resource Center 500 East McCarty Lane San Marcos, TX 78666 (512) 353-0011 Ken Ostrand: Center Director	☑ Hatchery ☐ Wild	☐ Unsecured: Open Spring, Stream ☑ Secured: Well, Sterilized	Last Sample Date	Classification
			06/22/20	A
			06/25/19	A
			06/26/18	A
			06/27/17	sLMBV
			06/14/16	LMBV+

Species[3]	Lot Identity	Age[4]	# in lot	(E) Eggs or (F) Fish obtained from	Pathogens inspected[3] & results'[5]													
					EI	AS	YR	RS	MC	IH	IP	IS	LM	OM	SV	VH	A	B
BBG	2017-2019	Varies	50	(F): SNARRC-captive						24	24		24			24	5	
										-	-		-			-	-	
DEV	2017-2019	Varies	500	(F): SMARC-captive		21	21			21	21		21			21	21	
						-	-			-	-		-			-	-	
BTS	2017-2020	Varies	1,500	(F): SMARC-captive		10	10			30	30		30			30	15	
						-	-			-	-		-			-	-	
GSF	2017-2020	Varies	2,000	(F): SMARC-captive		30	30			30	30		30			30	30	
						-	-			-	-		-			-	-	
.	.	.		. .														
.	.	.		. .														
.	.	.		. .														
.	.	.		. .														
.	.	.		. .														
.	.	.		. .														

Remarks[6]: A= Asian Tapeworm , B = Edwardsiella tarda

Inspecting Biologist Signature	Concurred (signature and title)	
Print: Huseyin Kucuktas **Date:** 7/22/2020	**Print:** Trista Welsh-Becker **Date:** Fish Health Unit Lead	**Southwestern Fish Health Unit** **7116 Hatchery Road** **Dexter, NM 88230** **(575) 734-5910**

[1]Done in accordance with the AFS Fish Health Section Bluebook *Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens* and the U.S. Fish and Wildlife Service Fish Health Policy 713 FW 1-5. [2]Secure = free of all aquatic pathogens, or sterilized. Unsecured = aquatic pathogens may be present.

[3]FWS abbreviations (see back of this page). [4]For hatchery fish give age in months; for feral fish, use symbols: e=eggs or fry; f=fingerling; y=yearlings; b=older fish.

[5] Findings reported as number examined over results; (-) = undetected, (+) = positive, and NT= not tested, A,B = other pathogens as listed in results.

[6]Additional remarks can be made on back page.

DEPARTMENT OF THE INTERIOR
U.S.Fish and Wildlife Service

FISH HEALTH INSPECTION REPORT[1]

Additional Inspection Information
Laboratory Case Number (CHN): 20-62

BTS= Blacktail Shiner

Please note that all lots were not tested to the presumed 95% confidence level according to USFWS Aquatic Animal Health Policy.

PATHOGEN ABBREVIATIONS

AS	Aeromonas salmonicida
EI	Edwardsiella ictaluri
RS	Renibacterium salmoninarum
YR	Yersinia ruckeri
MC	Myxobolus cerebralis
IH	Infectious Hematopoietic Necrosis Virus
IP	Infectious Pancreatic Necrosis Virus
IS	Infectous Salmon Anemia Virus
LM	Largemouth Bass Virus
OM	Oncorhynchus masou Virus
SV	Spring Viremia Carp Virus
VH	Viral Hemorrhagic Septicemia Virus

SPECIES ABBREVIATIONS

ALG	Alligator gar
APT	Apache trout
AXR	Apache x Rainbow trout
ARS	Arkansas River shiner
BES	Beautiful shiner
BBG	Big Bend gambusia
BLB	Black bullhead
BLC	Black crappie
BCF	Blue catfish
BLG	Bluegill
BTC	Bonytail
BON	Bowfin
BKS	Brook silverside
BKT	Brook trout
BRB	Brown bullhead
BNT	Brown trout
CCF	Channel catfish
CCH	Chihuahua chub
CCG	Clear Creek gambusia
CPM	Colorado pikeminnow
CSP	Comanche Springs pupfish
CAP	Common carp
CXM	Cutbow hybrid
CUT	Cutthroat trout
DEP	Desert pupfish
DSK	Desert sucker
DHP	Devils hole pupfish
DEV	Devils River minnow
FHM	Fathead minnow
FMS	Flannelmouth sucker
FCF	Flathead catfish
FHC	Flathead chub
FOD	Fountain darter
FRD	Freshwater drum
GIC	Gila chub
GTM	Gila topminnow
GIT	Gila trout
GIS	Gizzard shad
GDE	Goldeye
GOF	Goldfish
GRC	Grass carp
GSF	Green sunfish
GUB	Guadalupe bass
HBC	Humpback chub
KOE	Kokanee salmon
KOI	Koi
LMB	Largemouth bass
LSP	Leon Springs pupfish
LCD	Little Colorado spinedace
LOM	Loach minnow
LSF	Longear sunfish
LFD	Longfin dace
LNG	Longnose gar
MZT	Mozambique Tilapia
SNK	Northern snakehead
PBS	Pecos bluntnose shiner
PAH	Paddlefish
PRC	Pahranagat roundtail chub
PLS	Pallid sturgeon
PEG	Pecos gambusia
PPF	Pecos pupfish
PSS	Pumpkinseed
RBT	Rainbow trout
RBS	Razorback sucker
RES	Red shiner
RDS	Readbreast Sunfish
RSF	Redear sunfish
RGC	Rio Grande chub
RGT	Rio Grande cutthroat trout
RGSM	Rio Grande silvery minnow
RCS	River carpsucker
RKB	Rock bass
RTC	Roundtail chub
WXS	Saugeye
SNG	Shortnose gar
SSN	Shortnose sturgeon
SMB	Smallmouth bass
SAB	Smallmouth buffalo
SDC	Speckled dace
SOS	Sonora Sucker
SPE	Spikedace
SPB	Spotted bass
SPG	Spotted gar
STB	Striped bass
SBH	Striped bass hybrid
TFS	Threadfin shad
VRC	Virgin River chub
WAE	Walleye
WMS	Warmouth
WMF	Western mosquitofish
WHB	White bass
WCF	White catfish
WHC	White crappie
WHS	White sucker
WDF	Woundfin
YCF	Yaqui catfish
YAC	Yaqui chub
YAS	Yaqui sucker
YTM	Yaqui topminnow
YLB	Yellow bass
YEB	Yellow bullhead
YEP	Yellow perch

AQUATIC HEALTH ACCESSION FORM

Washington Animal Disease Diagnostic Laboratory

College of Veterinary Medicine, Washington State University

Mailing address:
PO Box 647034
Pullman, WA. 99164-7034

Web Site: http://waddl.vetmed.wsu.edu
Shipping address:
Bustad Hall, Rm.155-N
Pullman, WA. 99164-7034

Phone: (509) 335-9696
FAX: (509) 335 7424
E-Mail: waddl@vetmed.wsu.edu

Please type or use black ink and print clearly.

Veterinarian or Case Coordinator: Allan Pessier
WADDL VET CLIENT #:
WADDL CLINIC CLIENT #:

Clinic:

Street:

City: State: Zip:

Phone: Fax:

Date Shipped: E-mail:

WADDL USE ONLY

Owner: San Marcos ARC
WADDL OWNER CLIENT #:

Street: 500 E Mccarty Lane

City: San Marcos State: TX Zip: 78640

Phone: 512-353-0011 Fax/E-mail:

Please fill out completely as possible:

Specimen(s) Submitted: 36 whole salamanders live in freshwater

Sampling Date: 7/2/19

Aquatic Tests Requested:

- [x] *Necropsy*
- [x] *Histopathology*
- [x] *Toxicology*
- [x] *Virology*
- [x] *Fungal culture*
- [x] *Mycobacteria culture*
- [x] *Bacteriology*
- [x] *Parasitology*
- [ ] *Whirling Disease*
- [ ] *PCR*
- [ ] *Antibiotic Sensitivity*
- [x] *Other* Trace minerals, vitamin A, microsporidia

Antibiotic of interest:

Note: WADDL reserves the right to modify the tests requested for more efficient case work-up and / or send specimens to outside laboratories to perform tests not done at WADDL.

Species	*Animal ID (name/tag#) or Lot #*	*Water Temperature*	*Animal Weight*	*Age*
Eurycea nana		21C		

Location of Lesion(s)	*No. in group*	*No. Dead*	*No. Sick*	*No. on Premises*	*Duration of Problem*
Skull, limbs, spine, tail					

** Was animal euthanized? If so, what method?*

Water:
- [ ] *Marine / Brackish*
- [x] *Freshwater*

System:
- [x] *Flow-through*
- [ ] *Recirculating*
- [ ] *Net pen*
- [ ] *Other* _____

- [x] *Health Testing*
- [x] *Diagnostic Testing*

Pathogen(s) of interest:

Additional History: *Vaccinations, signs, stress factors, treatments, post mortem findings, pertinent feed or feed additives, clinical lab results, previous WADDL Case Numbers. (Attach additional sheets as necessary.)*

15 gravid females freshly collected from the wild population, 10 females from younger/recently collected (2018-2019), and 10 females from the historic (older, sickly) population in refugia are included in submission to compare disease, analyze trace minerals, conduct vitamin A tests and histopathology, and to look for differences between populations and possible explanations for females rupturing eggs and/or a lack of reproduction in captivity.

Also included is one sick male (live, freshly collected) exhibiting swelling similar to what we have seen in refugia. This animal was collected in this condition from the wild.

SAMPLE COLLECTOR:

Print Collector's Name _____ Collector's Signature _____

Veterinarian's or Clinician's Signature:

Condition(s) Suspected:

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/983 September 30, 2020
Memorandum

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

From: Huseyin Kucuktas, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fountain darters (*Etheostoma fonticola*) collected from the San Marcos River, (Case Number 20-70) and Comal River (Case Number 20-71), Texas.

On August 18, 2020, Southwestern Fish Health Unit (SFHU) staff received a total of 60 fountain darters from the San Marcos River (GNIS ID: 1375972), and 10 fountain darters from the Comal River, Texas. These fish were collected by staff at the San Marcos ARC and shipped overnight live to the SFHU for fish health testing. San Marcos ARC staff recorded collection of fountain darters from a total of 5 locations on the San Marcos River as follows: Twenty-five fish from the Upper San Marcos (Spring Lake) at latitude 29.7129° and longitude -98.1375°; 15 fish from the Middle San Marcos (below Spring Lake Dam) at latitude 29.8901° and longitude -97.9340°; 10 fish from the Middle San Marcos (Sewell Park Dock) at latitude 29.8875° and longitude -97.9341°; 20 fish from the Lower San Marcos River (Ramon Lucio Park) at latitude 29.8754° and longitude -97.9319°, and 10 fish from the Lower Comal River (Schlitterbahn Employee Parking Lot) at latitude 29.7101° and longitude -98.1276°, all in Hays County, Texas.

A total of 50 fish from San Marcos River were tested for virology, and 10 fish each from both San Marcos and Comal River were screened for parasites. Fish were euthanized upon arrival and samples (whole fish) from San Marcos River were collected for virology. Screening for *Centrocestus formosanus* was conducted by examination of the left side set of gills for 10 fish from both Rivers. Viruses screened for included those listed as NWFHS targeted pathogens as well as any other viruses that may be detected in cell culture. 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. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2016 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 4 out of 10, and 3 out of 10 fish examined in San Marcos and Comal River fountain darters, respectively. No viruses were detected from the fountain darters

collected from 4 different sites on the San Marcos River. 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 numbers 20-70, and 20-71 for all follow up correspondence.

cc: Trista Welsh-Becker, Southwestern Fish Health Unit/ Southwestern Native Aquatic Resources and Recovery Center

Lindsay Campbell, San Marcos Aquatic Resource Center

Southwestern Fish Health Unit
Diagnostic Case History and Lot Information

Please fill out as much history as possible and submit via email to the SNARRC Fish Health Unit Lead (trista_welsh-becker@fws.gov) at the time that samples are mailed out. NO samples should be mailed without prior authorization from SNARRC staff to ensure that timing and staffing is appropriate to evaluate the samples received. **FedEx is the only option** to use as a commercial carrier as others have business hours incompatible with our protocols.

Location submitting samples:_____ Staff member submitting samples:_____
Tracking number of shipment*:_____(*ALWAYS select the "**Hold at FedEx location**" option. This ensures that SNARRC staff can receive samples as quickly as possible for best results)
Date that signs/symptoms first noticed:_____Date samples taken: _____
Species:_____Eurycea nana_____Cultured ☐ Wild ☐ Age _____
Lot ID: _____ Tank#/system#_____
Type of samples submitted: _____ No. of fish/samples submitted: _____
System size and stocking density: _____Filtration method: _____
Water source: _____
Number of fish in affected system:
List ANY recent movements of animals into the facility or abnormal occurrences (within last 60 days):

_____

_____

Brief history of routine system maintenance including water changes/water flow and how often water quality checks performed.

_____

_____

Please explain the reason for the submission of samples including the signs noted, length of time, number of mortalities over time, appetite, behavior, lesions noted, etc.

_____

_____

Please list any treatments including vaccinations, date, dosage, agent used, and effects on animals post-treatment. Include any other available lot history.

_____

_____

_____

Most recent water quality analysis date: _____
Results (include units): DO _____ Temp _____ pH_____Ammonia :TAN_____UIA _____
Nitrite_____Salinity _____ Alkalinity _____ Nitrate _____

Signature of staff member

Southwestern Native Aquatic Resources & Recovery Center
Fish Health Unit
Wild Fish Health Survey Submission Form

Please fill out this form as completely as possible whenever submitting fish for a wild fish health survey. Starred items are required.

***Submitter name:** _____
***Submitter address:** _____
***Submitter phone and FAX:** _____
***Submitter email:** _____

***Collection date(s):** _____
***County and state of collection site(s):** _____
***GPS coordinates (in decimal degrees) of sample site(s):** _____
***Name of water body:** _____
Site description (i.e. lake, river, fork etc): _____
Capture method: _____
***Water temperature:** _____ 21C _____
Ambient temperature: _____
Flow (cfs): _____
Turbidity (ntu): _____
pH: _____
DO (mg/L): _____

Reason for the wild fish sampling at this site:

Southwestern Fish Health Unit
Diagnostic Case History and Lot Information

Please fill out as much history as possible and submit via email to the SNARRC Fish Health Unit Lead (trista_welsh-becker@fws.gov) at the time that samples are mailed out. NO samples should be mailed without prior authorization from SNARRC staff to ensure that timing and staffing is appropriate to evaluate the samples received. **FedEx is the only option** to use as a commercial carrier as others have business hours incompatible with our protocols.

Location submitting samples:_____ Staff member submitting samples:_____
Tracking number of shipment*:_____(*ALWAYS select the "**Hold at FedEx location**" option. This ensures that SNARRC staff can receive samples as quickly as possible for best results)
Date that signs/symptoms first noticed:_____Date samples taken: _____
Species:_____Eurycea nana_____Cultured ☐ Wild ☐ Age _____
Lot ID: _____ Tank#/system#_____
Type of samples submitted: _____ No. of fish/samples submitted: _____
System size and stocking density: _____Filtration method: _____
Water source: _____
Number of fish in affected system:
List ANY recent movements of animals into the facility or abnormal occurrences (within last 60 days):

_____

_____

Brief history of routine system maintenance including water changes/water flow and how often water quality checks performed.

_____

_____

Please explain the reason for the submission of samples including the signs noted, length of time, number of mortalities over time, appetite, behavior, lesions noted, etc.

_____

_____

Please list any treatments including vaccinations, date, dosage, agent used, and effects on animals post-treatment. Include any other available lot history.

_____

_____

_____

Most recent water quality analysis date: _____
Results (include units): DO _____ Temp _____ pH_____Ammonia :TAN_____UIA _____
Nitrite_____Salinity _____ Alkalinity _____ Nitrate _____

Signature of staff member

January 2020 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Lindsay Campbell, Ph.D.

And

Mark Yost (UNFH)

San Marcos Aquatic Resources Center
500 East McCarty Lane
San Marcos Texas, 78666
Phone: 512-353-0011

February 10, 2020

Task 1 Refugia Operations

Species Collection

On January 15, San Marcos Aquatic Resources Center (SMARC) and Uvalde National Fish Hatchery (UNFH) technicians collected Peck's cave amphipods (PCA) by hand around Spring Island in the Comal River, New Braunfels, Texas. The technicians collected 97 PCA for the survival study starting at UNFH. A new collection technique for PCAs was implemented utilizing divers to collect from deeper upwellings located around Amelia's Island. During this collection event, 12 Comal Springs salamanders were captured while collecting amphipods and returned to SMARC. Staff collected lures by hand at Spring Run 3 on the same day; 198 Comal Springs riffle beetles (CSRB) and four PCA were obtained. One-hundred CSRB were returned to UNFH and 98 CSRB plus the four PCA were retained by SMARC for our refugia.

Traps were deployed in Rattlesnake Cave and Well in January. Compared to the previous trapping expedition in October, water conditions in Rattlesnake Well had improved. The water levels in Rattlesnake Cave continued to drop, however many blind cave shrimp were observed and water quality was excellent. Three salamanders were collected for Refugia, two from the cave and one from the well. An additional three salamanders were observed but not collected. Two noteworthy events occurred during this trapping period. Firstly, after setting traps on January 6th, staff returned and discovered a recently deceased ring-tailed cat in Rattlesnake Well. The animal, which was missing its front paw, was removed from the site before it could foul the water. It is unclear how the animal accessed the site, as the opening was covered as best as possible. Secondly, on the last day of sampling in Rattlesnake Cave, staff discovered a recently deceased Texas blind salamander in a trap with a large, live crayfish. The tail of the salamander in the trap had been severed at its base and the animal would not have survived the injury. The cause of the injury was most likely the crayfish found in the same trap. Staff returned both to station and preserved the dead salamander and crayfish. Crayfish became introduced (by unknown means) and established to the cave many years ago. We actively remove this invasive from the cave when possible to decrease potential negative encounters such as these. As a note, salamanders have the ability to swim or climb out of the traps we use: they are not artificially restricted to stay in a trap with potential predators.

Refugia Population Propagation and Husbandry (Task 3 funding included in Task 1)

SMARC

Cal Fraser joined the Refugia staff during the month of January on a work detail from a USFWS station in Bozeman, MT. He provided much needed help during the month while staff needed to allocate more time to writing and setting up 2020 research projects.

Amelia Hunter inventoried the wild stock, F1, and F2 generations of the CSRB, wild stock Comal Springs dryopid beetles (CSDB), and wild stock PCA on station. This year we are changing the schedule for invertebrate inventory at SMARC for CSRB adults and larvae and for CSBD. To reduce disturbance, inventories will be every three months, with careful spot siphoning of containers once a month into a sieve over a bucket, with the sieve then checked for CSRB, CSDB, and larvae. Each container and species will be siphoned and checked separately. Food items will be refreshed as needed.

Amelia Hunter started constructing new invertebrate holding containers out of more durable and fully opaque (black) plastic totes for all species in the SMARC refugia. These containers are expected to reduce light-related stressors and resist cracking and leaking. The PCA containers also utilize a new serpentine inflow that produces flow from the bottom of the container, giving access to fresh water input throughout the bottom of the container, where they primarily inhabit. This contrasts previous designs with inflows coming from the surface of container or just two bars along opposite sides of the bottom of the container.

Amelia Hunter reviewed a recent Texas State University dissertation by Dr. Parvathi Nair, part of which covered PCA diet. The study showed that PCA in the wild consumed other smaller amphipod species and were known as a top predator in their habitat. Ms. Hunter had suspected in 2019 that the current food offerings were insufficient for PCA and therefore proposed to incorporate this new food source as soon as possible to reduce possible death, cannibalism, and starvation due to lack of palatable food source. Small amphipods are already cultured for food purposes for salamanders and fish; thus with consultation of Dr. Campbell, Ms. Hunter plans to utilize some of these as a PCA food source in captivity on a tests basis. Research plans for 2020 include trials to test different foods for PCA (live animal and manufactured diets) and observe behavior and consumption.

Ten Texas wild rice (TWR) plants collected in December 2019 completed their 30 day quarantine during January. SMARC staff thoroughly cleaned tillers, potted, and placed the plants in refugia raceway tanks. The quarantine tank was prepared for incoming tillers in the upcoming February collection.

Fountain darters in Refugia were moved to different tanks after an older tank was moved out, and two new tanks moved to the space. This was done to consolidate fountain darters into one area. In Quarantine, we swapped spaces for fountain darter tanks with salamander grow out systems to group each species in the same areas and to increase usable space in the building. Staff and volunteers hand made more habitat items for enrichment and cover for the fountain darters.

Salamander standing stock tanks were de-scaled and cleaned using 20-30% vinegar. New water flow bars were constructed. Cal Fraiser, a biologist detailing from Bozeman, MT, plumbed several systems to partial recirculation with UV treatment, including two systems in the Quarantine area. *Eurycea* grow-out systems in Quarantine were de-scaled, cleaned, and consolidated in the space previously used for fountain darter tanks, so that all salamander quarantine and holding would be in one area.

Texas blind salamanders F1 offspring were removed from the large blue insulated tank, in Refugia (originally from the Pad). Staff placed with two green raceways in a portion of the space and shifted the research work station. We moved fountain darters into these two green race ways. The Texas blind F1 offspring were moved into the former holding raceways of the fountain darters (after tanks were cleaned and disinfected). Linda Moon tagged 54 juvenile Texas blind salamanders collected in 2019. These were moved out of *Eurycea* grow-out systems in Quarantine into previously empty tanks in the Refugia.

Eggs were produced by Comal and San Marcos salamanders, and all were fertile. An F_X San Marcos female produced a clutch of 24 eggs and a wild stock San Marcos salamander produced a clutch of 25 eggs. Wild stock Comal salamanders produced two small clutches; one with seven eggs and one with ten eggs. Clutches produced in December hatched.

Refugia staff extracted DNA from salamander swabs and ran qPCR on the extracted DNA.

Twenty-five F_X San Marcos salamander offspring were donated to the University of Tennessee for a study investigating the impacts of *Batrachochytrium salamandrivorans* (Bsal) introduction and infection. Though BSal is not currently present in the Americas, the disease has devastated amphibian populations in Europe. San Marcos salamanders were selected as one of three representative salamanders in Texas. This study will proactively define the susceptibility of American amphibians and potentially lead to treatments, should the disease eventually spread to the Americas.

UNFH

Staff constructed new air supply manifolds for several of the remaining residence tanks in the Refugia Building, including those that currently house salamanders. This was done so that each of the seventeen systems would have air stones should the need arise to put fountain darters in these systems. Two new bulk utility racks were purchased and installed to organize the refugia spaces and store extra gear. One 2'x 8' rack was installed in the Invert Room to hold extra culture gear. One 4'x8' rack was also installed outside the Tank House by the Texas Wild Rice tanks to hold pumps, plumbing manifolds, cleaning implements, and grooming tools.

Texas wild rice repotting efforts continued this month. Plants repotted in December were in lower flow systems to allow for firmer rooting. Therefore, instead of caring for rice in just the four large tanks outside the Tank House, staff also had four additional tanks of plants at the quarantine rice area to maintain. At the end of the month, the plants collected in December were incorporated in the Refugia population and moved to Tank R12 from their separate quarantine tanks. Staff pressured washed a large TWR tank outside of the Tank. After the new TWR plants and the repotted TWR plants were moved out of the rice quarantine area, these tanks were also pressure washed.

San Marcos fountain darter (SMFD) health continued to be a concern in the Refugia Building, with increasing mortality occurring throughout the month in one tank of fish from the middle section of the San Marcos River. Thirty-five fish were collected from this tank as moribund or dead; the only other five SMFD mortalities for the month were from another tank that also

contained middle section fish. On January 8, samples of five live fish from the affected tank were sent to the Southwest Fish Health Unit (SFHU) to assess for external parasites, bacteriology, and virology. Tissue samples were also prepared and sent to WADDL for histopathology assessment. To date, virology and histopathology work at WADDL is ongoing. SFHU reported that no external parasites were present. This suggested to us that the 0.5% salt and 15 ppm formalin treatments given in December effectively removed the monogeneans found on fish in December. SFHU did not isolate any bacterial infection. However, SFHU histopathology showed gill inflammation, histiocytic dermatitis, coelomitis, and a mild infection of myxosporidia in the brain cavity. They concluded that the evidence points to either previous parasite infection or reduced water quality in the form of particulate matter being the leading cause of death. The former is likely the case, since we did note monogenean infections in November and December. The latter is unlikely since water quality measurements have been good in these tanks throughout this time period. Suggested SFHU corrective actions taken to date include, increasing water flows to this tank to double the fresh water turnover rate from 3 times daily to 6 times daily and performing 0.5% static salt bath 24-hour treatments every Monday, Wednesday, and Friday. Mortality has not slowed. Staff implemented extra precaution by siphoning this tank daily and isolating water and gear from this tank from the other systems in the Refugia Building going into February. Final reports from SFHU and WADDL are expected mid-February. Please note that middle-section San Marcos fountain darters at SMARC have also experienced higher mortality rates that fish from the other two sections, with inconclusive causes.

Task 2 Research

Comal Springs riffle beetle nutrition:

Amelia Hunter finished setting up six round experimental tanks to start food preference experiments in February. Four pellet diets were extruded at Bozeman Fish Technology Center and sent to SMARC this month. We are currently observing the pellets within the experimental tanks to determine optimal replacement schedules as pellets degrade. Cages are also being constructed to group pellets together for easy removal of diets from tanks with minimal disturbance to the beetles inhabiting other areas within the tank. Ms. Hunter will thoroughly check removed food bundles for CSRB.

Amelia Hunter completed sample preparation of the initial food offerings and one sample of Refugia CSRB tissue for dual ^{13}C:^{15}N stable isotope analysis to be conducted at University of California-Davis. This initial run will be used to make sure there is distinction among each food type offered during the experiments and if CSRB food preferences are discernable on a ^{13}C:^{15}N biplot. The reference CSRB tissue sample will be used to compare current Refugia diet signatures with those of the pellets and CSRB tissues after the preference test. This method has been used before in other research studies done by Texas State University (TXST), analyzing diet signatures. TXST allowed Ms. Hunter the use of their deep freezer, freeze drier, and microbalance necessary to prep samples sent for analysis.

Long-term salamander tagging experiment:

Salamander tagging research continued with Linda Moon and Kelsey Anderson completing the 10 month tag checks and beginning the 11 month tag checks. Linda Moon presented early findings of this study at the Texas Conservation Society meeting.

San Marcos salamander reproduction research:

Cal Fraser plumbed two separate raceway tanks for partial recirculation and with UV sterilizers in Quarantine in preparation to house San Marcos salamanders for the upcoming reproduction trial. After the study, these tanks can go back to being utilized to hold organisms during their quarantine period or if salvage occurs. Two racks of empty glass tanks and two empty raceway tanks remain open in Quarantine if any needs arise. The remaining male and female salamanders from the heritage San Marcos salamander population as well as 26 individuals (13 males, 13 females) from the younger Standing Stock population were separated by sex (and population type) and placed in completely isolated aquaria from each other and other salamanders. The hormone that will be used for the study was ordered and delivered.

At UNFH Rachel Wirick set up two research systems for pilot testing habitat modifications to increase San Marcos salamander reproduction. Each system is capable of holding 20 5.5-gallon aquaria per shelf. Staff purchased equipment for water conditioning: including coarse filtration through 100 and 50 micron pleated filters, UV sterilization of 40 ms/cm/sec, a sedimentation collection box and biofilter. Staff prepared 25 5.5-gallon aquaria by cutting holes and installing new bulkhead fittings, filtration, sterilization, delivery, supply plumbing, and 25 additional 5.5-gallon aquaria.

Peck's cave amphipod survival:

Makayla Blake continued setting up the rack system for the PCA experimental holding containers and began implementing the PCA study. After collections on January 16, UNFH returned 97 PCA for the first replicate of each of the three container designs. Ninety PCA were randomly distributed into three different treatment groups, 30 to each culture box. The excess PCA collected were quarantined separately and will join the UNFH PCA Standing Stock numbers. The first treatment solely the black, low density, Matala (Treatment A), the second solely green, medium density, Matala (Treatment B), and the third a combination of the black and the green Matala (Treatment C). These replicates will remain in the UNFH Quarantine for 30 days, then inventoried, and moved to the Invert Room in the UNFH Refugia. After that, inventories will occur every 45 days to reduce disturbance while still charting survival at regular intervals. The second replicate will start in February and the third in March. We staggered each replicate so as not to remove so many individuals from the wild population at one time.

BIO-WEST, Inc:

Comal Springs riffle beetle pupation enhancement

Performed general maintenance of flow-through mesocosms.

- Monitored water quality parameters.
- Checked larval development of individuals from relaunch treatment 15, and 16.
- Researched designs for new aquarium constructions for 2020.
- Recorded fecundity of treatments 7, 8, 15, and 16 adults.

Texas State University CSRB Pupation Research: Dr. Weston Nowlin presented his 2019 findings and 2020 research plans at EAA headquarters to Dr. Chad Furl and Dr. Lindsay Campbell. Students on the project continued to check on their experimental replicates

Task 4 Species Reintroduction

No work on species reintroduction was conducted this month.

Task 5 Reporting

All Refugia staff complied information for the annual report to give Dr. Campbell, who composed the full document. Mark Yost provided comments on the document. Dr. Britton provided review and suggested edits to the finished document. Dr. Campbell submitted the Edwards Aquifer Refugia Program 2019 Annual Report to the EAA on January 31st for their review.

All Refugia staff members worked on the materials for the monthly report.

Marta Estrada submitted the monthly Refugia invoicing package.

Task 6 Meetings and Presentations

On January 10 Linda Moon attended and presented at the Texas Conservation Symposium in Georgetown, Texas. She presented our work on the long-term salamander tagging experiment showcasing the results of the first 6 months of the three different tagging methods in the Texas blind, Comal Springs, and San Marcos salamanders.

On January 31, 2020, Kristy Kollaus, Chad Furl, and Damon Childs from the EAA made a site visit at UNFH. Refugia staff put a luncheon together for EAA staff prior to touring them around the Refugia facilities. EAA staff visited to observe refugia operations and discuss planned renovations to the Quarantine Building infrastructure.

Table 1. New collections and total census in January 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. Inventory for invertebrates is not conducted every month, if this is not a month that inventory is conducted census will be NA. Further details of these numbers can be found in supporting documents.

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	0	0	34	40*	588	489
Fountain darter: Comal	NT	NT	0	0	0	0	1	1	212	35
Comal Springs riffle beetle	98	100	0	198	30	0	NA	NA	87	NA
Comal Springs dryopid beetle	0	0	0	0	0	0	4	NA	8	NA
Peck's cave amphipod	4	97	23	124	0	0	NA	NA	241	NA
Edwards Aquifer diving beetle	0	NT	0	0	0	0	-	-	-	-
Texas troglobitic water slater	NT	NT	0	0	0	0	-	-	-	-
Texas blind salamander	3	NT	3	6	0	0	4	0	261	31
San Marcos salamander	NT	NT	0	0	0	0	11	2	343	303
Comal Springs salamander	12	NT	2	14	0	0	1	0	87	55
Texas wild rice plants	NT	NT	0	0	10	14	0	0	217	171

*Does not include the five fish sacrificed for fish health.

Summary of January Activities

January – Cal Fraser on detail with the Refugia Program from Bozeman, MT

Jan 6th through 21st – Trapping for Texas blind salamanders in Rattlesnake Cave and Well

Jan 6th – Dr. Nowlin CSRB pupation presentation at EAA

Jan 10th – Linda Moon presents at the Texas Conservation Society meeting

Jan 15th – Collect PCA from around Spring Island for research project

Jan 15th – Collect CSRB lures from Spring Run 3 for Refugia Standing Stock

Jan 29th – DNA extractions from salamander swabs

Jan 30th – qPCR on extracted DNA

Jan 31st – Edwards Aquifer Refugia Program 2019 Annual Report turned into the EAA

Pictures

Figure 1 Setting up for San Marcos salamander habitat manipulation at UNFH.

Figure 2 New storage racks by the TWR tanks (L) and in the Invert Room (R) at UNFH.

Figure 3 Makayla Blake (left) and Amelia Hunter (right) sort through lure samples and teach Cal Fraser (center).

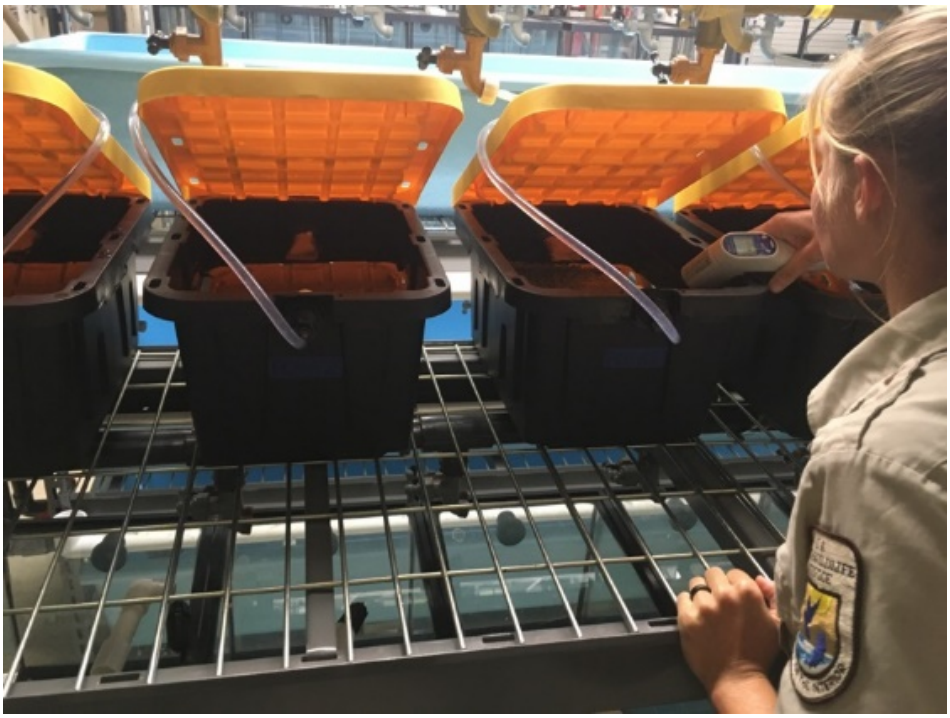


Figure 4 Makayla Blake checking the PCA survival experiment chambers.

Figure 5 Developing salamander egg from one of the several clutches deposited this month. The line of first cleavage can be seen down the center.

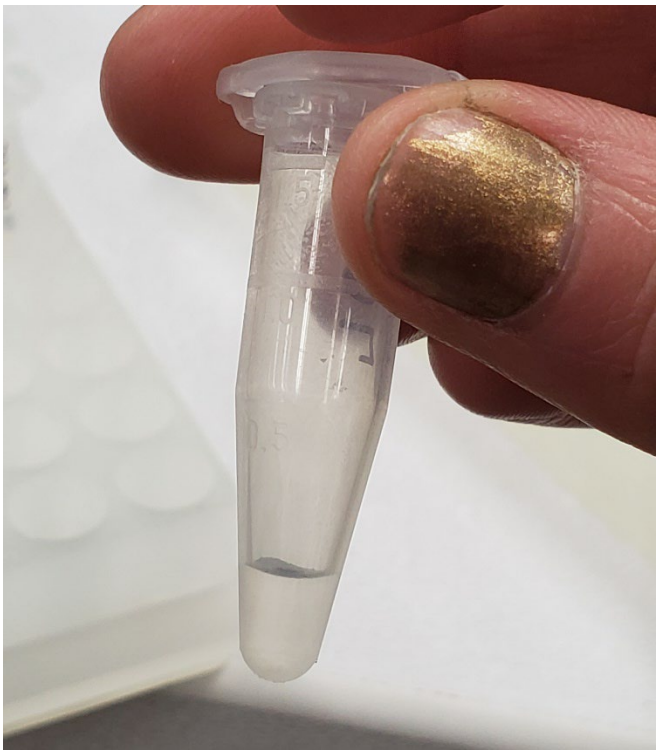


Figure 6 DNA extracted from salamander swabs.

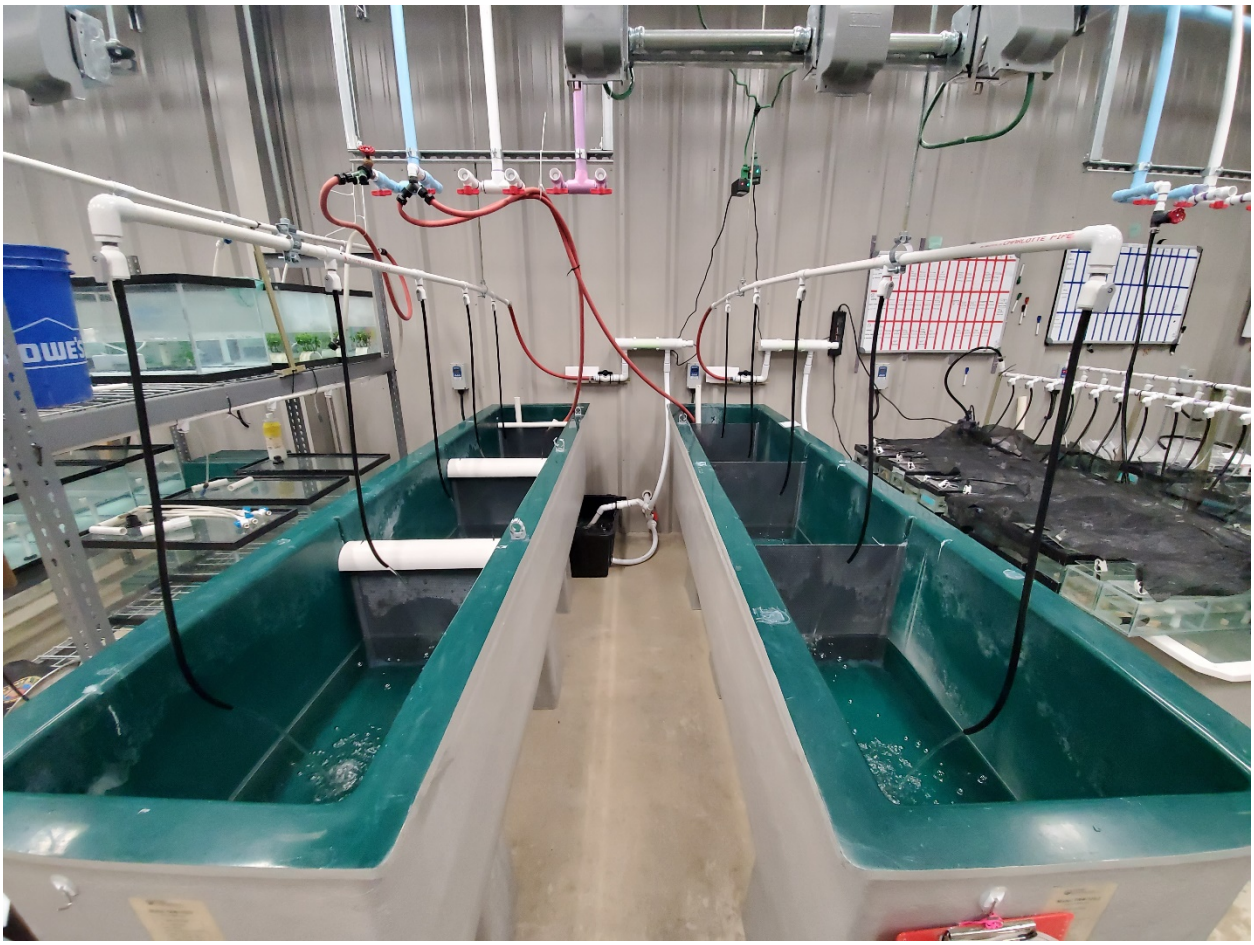


Figure 7 Quarantine raceway tanks plumbed for recirculation with UV sterilization.

Figure 8 Potting wild rice plants that completed their quarantine period.

Figure 9 Dr. Campbell puts her forklift training (and new forklift) to use moving around tanks during reorganization of our buildings.

February 2020 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Lindsay Campbell, Ph.D.

And

Mark Yost (UNFH)

San Marcos Aquatic Resources Center
500 East McCarty Lane
San Marcos Texas, 78666
Phone: 512-353-0011

March 10, 2020

Task 1 Refugia Operations

Construction

No work on construction projects by sub-contractors occurred in February.

Species Collection

On February 4, Benjamin Whiting and Mark Yost collected 14 Texas wild rice (TWR) plants from the San Marcos River and transported the tillers back to Uvalde National Fish Hatchery (UNFH).

On February 13, 2020, technicians from San Marcos Aquatic Resources Center (SMARC) collected Comal Springs riffle beetles (CSRB) by cotton lures set last month within Comal Springs (Spring Run 3), part of the Comal River, New Braunfels, Texas. The technicians collected 275 beetles and retained 274 for SMARC refugia wild stock, including 72 for nutrition experiments.

Staff collected ten fountain darters each from the Comal River and the San Marcos River, on February 21, 2020 for routine parasitology testing. Fish were sent alive to the Southwestern Fish Health Unit for analysis. These collections happened twice a year for on-going monitoring of an Asian trematode parasite called *Centrocestus* sp. in the rivers.

Refugia Population Propagation and Husbandry (Task 3 funding included in Task 1)

Our F1 larvae count of CSRB dwindled in the beginning of the year, following our donation of larvae to sub-contractors for experiments and low inventory of adult beetles that could produce more larvae to replace those donated. While staff collected more adult beetles in January 2020, they were not large enough for use in the experiments. Both sub-contractors, BIO-WEST, Inc. and Texas State University, needed larvae (5^{th} instar and above) in February 2020. So, Mark Yost transported 308 F1 larvae from UNFH refugia to supplement inventory at the SMARC refugia.

Kelsey Anderson conducted every-other-month inventory on all salamander holding systems at the SMARC. A fourth system for San Marcos salamanders was started and, after inventory, animals were dispersed in lower densities into all four tanks. These changes to the environment, including water flow patterns, new tank mates, and different habitat items in new locations were followed by our observations of increased exploration of their new tanks for salamanders of this species. Salamander clutches produced in January and December hatched and were counted; offspring were not included in the final count because they had not yet reached two months post-hatch.

Two 0.75-HP chillers arrived at UNFH during the last week of February and were lifted onto the mezzanine level above the Refugia area. In addition, the dysfunctional chiller, set up for the Invertebrate Room sump, was transported to the SMARC for troubleshooting by our

maintenance staff. A replacement, a new, unused 1.0-HP chiller, was transferred to UNFH for installation. This new unit was immediately put in place and installed.

Certified electricians replaced three faulty GFCI plugs at UNFH by the large TWR tanks, and all of the circuit breakers by the quarantine rice platform. These breakers were tripping, making the areas unable to run pumps continuously.

UNFH staff re-potting efforts for TWR continued this month, requiring the purchase of additional potting materials. In addition, they pressure washed Tank R11 in preparation of moving TWR plants back to this tank during the first week of March.

We continued to have concern for San Marcos River fountain darter (SMRFD) health in the Refugia Building at UNFH, with higher-than-normal mortality continuing throughout the month in Tank RE11. Twenty SMRFDs were collected from RE11 as moribund or dead fish and the only other SMRFD mortality for the month was from Tank RE8, all of which were "Middle" San Marcos River fish. We have continued to apply the corrective actions suggested by our Fish Health Unit, which include increasing water flows to this tank to double the exchange rate from 3 times daily to 6 times daily, and performing 0.5% 24-hour static bath salt treatments every Monday, Wednesday, and Friday. We have also implemented other basic biosecurity precautions that includes siphoning this tank daily, working in this tank last, and isolating gear from this tank from the other systems in the Refugia Building. The final Fish Health report (20-13) was issued on February 11, 2020, and noted that no pathogenic viruses nor bacteria were detected. Histopathology noted proliferative bronchitis due to the previous parasite load of monogeneans and a mild myxozoan infection of spores observed in the brain cavity. Fish mortality in this tank appears to be slowing down and will continue to be monitored and treated as noted above.

Makayla Blake inventoried the CSRBs, Comal Springs dryopid beetles (CSDB), and Pecks cave amphipods (PCA). All the CSRB and CSDB were moved from older, clear boxes to new black culture boxes after they were inventoried. A thin layer of the high-density, blue Matala material was added to each of these boxes as a base layer to keep leaves and cotton media off the bottom and to prevent anoxia. Ms. Blake collected nine F1 PCA from the boxes of gravid females used in the refugia, which has the new green, medium-density, Matala material.

Task 2 Research

Comal Springs riffle beetle nutrition: Amelia Hunter set up a system for diet preference experiments as a partial recirculation system. She then added food pellets to determine optimal replacement schedules, necessary as the pellets degrade. On February 11, 2020, Ms. Hunter noted water temperatures in the system were lower than optimal for CSRB and a small heating element was added to the sump to increase water temperature. Collection of riffle beetles occurred on February 13, 2020, which were sexed on station at SMARC using an Olympus microscope. Seventy-two adults in total were used for the experiment and divided evenly between the six tanks. Over the first weekend, a biologist reported that all the pellet types were fungused and the drains were clogged. Ms. Hunter quickly removed all fungused material and any floating debris and cleared the drains. Ms. Hunter observed that some beetles were trying to

climb out of the tank, a sign of stress. With the approval of Dr. Campbell and Dr. Britton, Ms. Hunter decided to cease the experiment and remove what beetles were still alive. Out of the 72 adults at the start, only 34 adults were alive. By this point, many adults were covered in fungus and could not be used for stable isotope analysis. Water quality parameters were also recorded upon ending of the experiment; no unusual points were read. Dr. Campbell and Ms. Hunter surmised that elevated temperature, 21 degrees Celsius and above, might have been an issue, and that they had used too many pellets of each type. Dr. Campbell and Ms. Hunter installed a UV sterilizer in the recirculating system and decreased the water temperature by 1-2 °C, still within a safe range for CSRB. Ms. Hunter started the pellet test again with fewer number of pellets. This setup ran for 48 hours and showed minimal signs of fungal growth. Ms. Hunter re-started the experiments with beetles from the same collection, excluding the ones already used, on February 25, 2020. She monitored water quality and checked for signs of fungus. Pellets were replaced every 48 hours. The experiment will run for two months.

Long-term salamander tagging experiment: Half of the final, 12 month, tag checks of the long-term tagging study occurred in February. A novel reader, Rachel Wirick, read the tags and weight, length, and photographs were taken of each salamander. Linda Moon and Kelsey Anderson completed 11 month tag checks.

San Marcos salamander reproduction research: Dr. Ruth Marcec-Greaves of the Detroit Zoo's National Amphibian Conservation Center visited SMARC to administer a hormone treatment to a group of San Marcos salamanders and consult on propagation. Dr. Marcec-Greaves is an amphibian reproductive specialist, who travels globally to consult on reproduction of imperiled species. In preparation for the visit, male and female salamanders, held in separate systems for one month, were combined into the same system, but kept from having access to each other mid-February. In January, Kelsey Anderson with the help of Dr. Campbell selected mature male and female salamanders from standing stock and separated them into independent systems by sex. In addition, another 24 animals (12 males, 12 females) from non-segregated systems were also selected for this experiment. This allows for comparisons in courtship occurrence in animals that are not segregated versus those held in mixed sex tanks. Dr. Marcec calculated the dose required of Luteinizing hormone-releasing hormone (LHRH) hormone for topical application to these San Marcos salamanders. She then trained our staff to mix the dosage and apply it to the salamanders. The hormone was applied topically to the head and nose, where the salamanders have glands that produce their own hormones. LHRH acts on the endocrine system to induce other reproductive hormones and behavior. Salamanders naturally produce LHRH during courtship and breeding. In some experimental groups, only males were dosed. And, in others, both sexes received doses. Groups were filmed for the first three days after full combination to monitor the salamanders after hormone application and to observe courtship behavior. Along with this, Dr. Marcec discussed with our staff several recommendations and strategies for general refugia tanks, including plans to cycle segregating and combining males and females

throughout the year, with a period of sharing water to prime pheromones before combining salamanders.

Rachel Wirick began setting up two systems for San Marcos salamander habitat manipulation at UNFH. This included cutting holes in glass aquariums, installing bulkhead fittings, making and installing tank lids, installing a water filtration system and UV sterilization system, installing a valve to create vacuum degassing, plumbing pumps, preparing system habitat, and preparing a biofiltration box for recirculating return water to flow through prior to reentering the sump. These chillers will tentatively be installed the first week of March so that we can begin conditioning one system for the San Marcos Salamanders that will be used for the habitat study.

Peck′s cave amphipod survivability: On February 20, Makayla Blake, Rachel Wirick, Mark Yost, Amelia Hunter, Kelsey Anderson, and Dr. Campbell collected Peck's cave amphipods (PCA) around Spring Island in the Comal River. In total, 97 PCA were collected for the second replicate of three for the study assessing how three different culture box habitat configurations affects survival. Ninety of these PCA were randomly distributed into three different treatment groups, 30 to each culture box, with the first being solely the black, low-density, Matala (Treatment A), the second being the green, medium-density, Matala (Treatment B), and the third being an equal combination of the black and the green Matala (Treatment AB).

Ms. Blake inventoried the January replicate this month, after their 30 day quarantine period. Seventy-six of the 90 survived, with treatment group survival percentages of 70.0% Treatment A, 90.0% Treatment B, and 93.3% Treatment C. It may be too soon to say conclusively, but since these were randomly placed in boxes, not graded by size, this may suggest that having the denser media provides refuge from cannibalism. The denser Matala material in these new brooding boxes may effectively provide refuge from cannibalism from the mothers. If we consistently see F1 production from these new brooding boxes, we could possibly improve producing PCA by upscaling a larger "garden" tank in the future using Matala to provide a nursery area for smaller PCA.

As of Monday, 9, 2020, neither BIO_WEST, Inc. nor Texas State University had submitted monthly summaries.*

BIO-WEST, Inc:

Comal Springs riffle beetle pupation enhancement
Comal Springs dryopid beetle

Texas State University CSRB Pupation Research:

Task 4 Species Reintroduction

No work on species reintroduction was conducted this month.

Task 5 Reporting

All Refugia staff members worked on the materials for the monthly report.

Marta Estrada submitted the monthly Refugia invoicing package.

Marta Estrada, Dr. Campbell, and Damon Childs (EAA) worked on finalizing 2019 accounting.

Dr. Campbell incorporated revisions from EAA staff to the Edwards Aquifer Refugia Program 2019 Annual Report. The report was reviewed by Dr. and Britton before final submission.

Task 6 Meetings and Presentations

Dr. Campbell attended a Species Status Assessment Workshop help by USFWS Ecological Services February 4-6, 2020.

Linda Moon presented a talk, "*The Role of Aquaculture in Refugia Conservation of Threatened and Endangered Species*," at the Aquaculture America 2020 Conference, February 11, 2020.

Dr. Campbell and Kelsey Anderson had a virtual meeting with the McCusker Lab (at the University of Massachusetts-Boston) about the ongoing regeneration research on February 18, 2020. They discussed the progress of the project and planed a manuscript for an upcoming special issue of a peer reviewed journal.

Dr. Ruth Marcec-Greaves visited SMARC and met with Refugia staff. Mark Yost and Rachel Wirick traveled to SMARC for the visit.

Table 1. New collections and total census in February 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. Inventory for invertebrates is not conducted every month, if this is not a month that inventory is conducted census will be NA. Further details of these numbers can be found in supporting documents. Numbers in (#) indicate those organisms that are in experiments.

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	0	0	0	0	0	0	171	21	396	472
Fountain darter: Comal	0	0	0	0	0	0	4	0	208	35
Comal Springs riffle beetle	274	0	1	275	98	96	NA	12	NA	116
Comal Springs dryopid beetle	0	0	0	0	0	6 (76)	NA	0	NA	1
Peck's Cave amphipod	0	97	16	113	4	0	NA	21	NA	142
Edwards Aquifer diving beetle	0	0	0	0	0	0	NA	0	NA	0
Texas troglobitic water slater	0	0	0	0	0	0	NA	0	NA	0
Texas blind salamander	3	0	7	10	3	0	0	0	264	31
San Marcos salamander	0	0	0	0	0	0	10	2	254(68)	301
Comal Springs salamander	0	0	0	0	12	0	1	0	98	55
Texas wild rice plants	0	14	0	14	0	0	0	0	217	171

Summary of February Activities

February 3 - 18 – Trapping for Texas blind salamanders at Primer's fissure and Johnson's well

February 4 - Texas wild rice tiller collection

February 13 - Comal Spring riffle beetle lure retrieval

February 19 - EAA consultant tour of SMARC Refugia buildings

February 20 - Peck's cave amphipod collection

February 21 - Fountain darter collection for parisiotology

February 24 - 27 - Dr. Ruth Marcec-Greaves visit to SMARC

Pictures

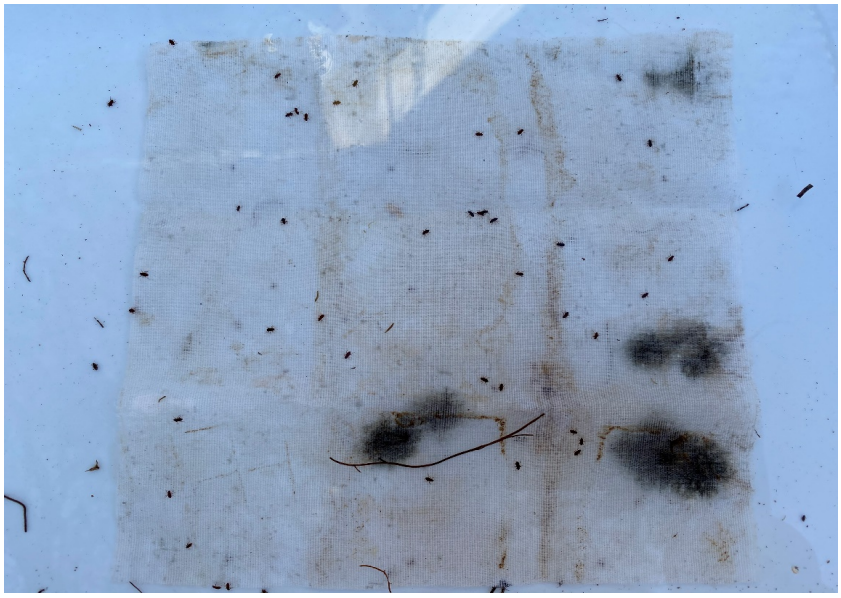


Figure 1 Comal Spring riffle beetles on a cloth lure.

Figure 2 Cal Fraser, Linda Moon, and Kelsey Anderson checking traps at Primer's fissure.

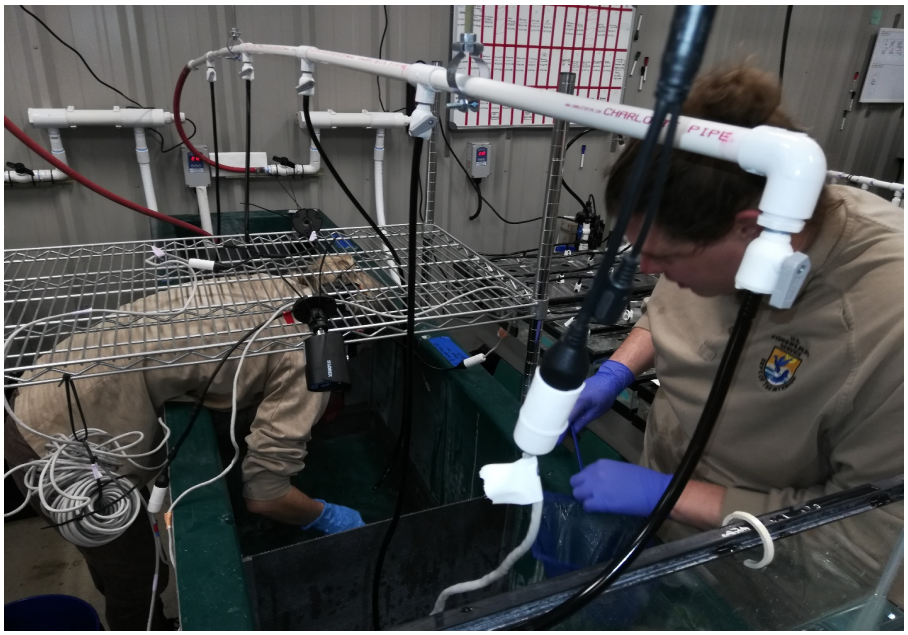


Figure 3 Dr. Campbell and Rachel Wirick gathering San Marcos salamanders out of the experiment tanks with camera set up for LHRH application.

Figure 4 Topical application of LHRH on a San Marcos salamander.

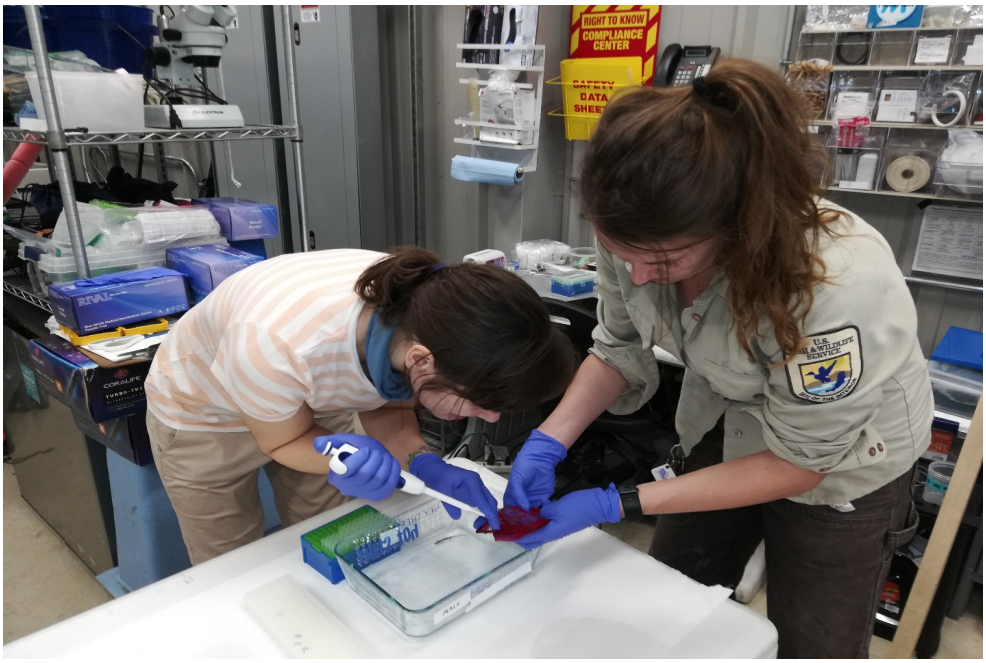


Figure 5 Dr. Ruth Marcec-Greaves demonstrates how to apply the hormone to Kelsey Anderson and watching staff.

Figure 6 Dr. Marcec-Greaves supervises staff in the application of LHRH.

March 2020 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Lindsay Campbell, Ph.D.

And

Mark Yost (UNFH)

San Marcos Aquatic Resources Center
500 East McCarty Lane
San Marcos Texas, 78666
Phone: 512-353-0011

April 10, 2020

Task 1 Refugia Operations

Construction

The contract for the 2019 EAA Quarantine Building HVAC Remediation project at Uvalde National Fish Hatchery (UNFH) was awarded to Firstop, LLC. Points of contact for the company throughout the project are Project Manager Oscar Maltos and Project Superintendent Raymond Bueno. A preconstruction conference call was held on March 11, 2020, attended by Rachel Wirick, as Acting EA Refugia Lead, along with Patricia Duncan, Project Leader, Valentine Cantu, Lead Biologist, and Mark Orton, Regional Mechanical Engineer. The meeting outlined the following roles during the project: Contracting Officer (CO) Ray Fletcher, Contracting Officer's Technical Representative (COR) Mark Orton, and the Field Inspector (CI) Patricia Duncan. Construction begins in April 2020.

Species Collection

US Fish and Wildlife Service divers Linda Moon and Justin Crow deep cleaned the net on Diversion Spring, in Spring Lake, San Marcos, TX, on March 3, 2020. Kelsey Anderson and Amelia Hunter provided surface support to the divers and assisted by scrubbing algae off the upper portions of the net. After letting the spring flush dislodged debris and algae from inside the net overnight, staff placed the collection cup on the cod end of the net. Additional buoys were deployed on the net at anchor points to help the net maintain its correct orientation. Staff checked the net on March 6, 10, 13, and 17, 2020. Biological technicians retained one live juvenile Texas blind salamander and 38 live San Marcos salamanders, 1 adult and 37 juveniles, for refugia purposes. The collection cup was removed on March 17, 2020, as all field work was suspended for staff health and well-being during the SARS-CoV-2 outbreak.

Refugia Population Propagation and Husbandry (Task 3 funding included in Task 1)

Amelia Hunter checked invertebrate culture containers and siphoned, as needed, to remove any accumulated debris at San Marcos Aquatic Resources Center (SMARC). Ms. Hunter siphoned the water-debris into a fine mesh sieve and examined for any invertebrates. Ms. Hunter built new invertebrate culture containers out of opaque containers to replace older models of clear containers. Additionally, Ms. Hunter collected water samples at the Refugia and at Spring Island, Comal, Texas for on-going microbiome work with Zack Mays of Texas State University.

Texas blind salamander females at SMARC deposited three clutches of egg at the end of March, one of 25 eggs, the second of 52 eggs, and the third of 11 eggs. All clutches were fertilized and are developing. A San Marcos salamander female deposited a clutch of eggs at UNFH on March 27, 2020. All 25 of the eggs are developing.

Kelsey Anderson weighed, measured, and checked for gonadal development in Texas blind juveniles that had reached one-year post collection. These data collected track individuals from the large group of juveniles that were collected in March-May 2019.

On March 13, 2020, staff and volunteers repotted 28 refugia Texas wild rice (TWR) plants into new pots and soil at the SMARC. TWR plants are repotted every 1 to 2 years or as needed if nutrients in the soil are depleted. Further scheduled large re-potting days at the SMARC were canceled due to quarantine procedures around SARS-CoV-2. At UNFH, annual TWR repotting efforts were completed this month. Staff also pressure washed Tanks R12 and R14 in preparation for TWR plants to move back into after they have firmly rooted in their new pots. All 14 plants collected in February were incorporated into the UNFH refugia.

The daphnia culture continued to grow, and Ms. Anderson moved them into a larger culture system. Both salamander and fountain darters actively consumed this new food item. Currently only a limited amount of daphnia are fed at a time until the culture grows large enough to sustain greater take for feeding.

Staff at SMARC purchased frozen freshwater *Mysis* shrimp (*Mysis diluviana*) and frozen *Calanus* copepods (*Calanus finmarchicus*) to test as another potential food source for our organisms. Biological technicians mixed the frozen *Mysis* with regular feeds to facilitate acceptance of this new food item. After initial hesitation all species of salamanders were observed to ingest the *Mysis*. Fountain darters were also observed to ingest *Mysis*. After successful intake of the food, we added it into the rotation of food items offered to the organisms. The size of the *Calanus* copepods made them compatible to feed to small juvenile organisms and both fish and salamander juveniles consumed them. Biological technicians added the frozen copepods as another food source for juveniles beside *Artemia* nauplii. Diversifying the food sources offered to our organisms can increase nutrition as each source can offer

different nutrients. A variety of food items, also, allows us to have back up food sources if one item becomes limited. At UNFH, staff set up a new amphipod tank in the Refugia area and seeded it with several batches of amphipods from the amphipod tank in the Tank House. Eventually, this system will feed all organisms in Refugia and Quarantine at UNFH.

Staff at UNFH moved invertebrate holding containers to a different system in order to perform annual cleaning and maintenance on their previous system. The system was acid washed and the drain system was configured to match the other drain systems in the room. The system is ready for future use.

The Refugia Program staff at UNFH made preparations to reinforce the TWR shade structures that were installed in December 2019. Patricia Duncan and Jeff Johns, Regional Structural Engineer, deemed these structures unsuitable for holding shade cloth, as they lack an engineering stamp that rates them as permanent structures that can withstand strong winds. These structures were not originally intended to be permanent structures. Jeff Johns' suggested solution was to weld cross braces to each structure in three separate overhead areas, as well as on both gabled ends. Since this will prevent the structures from being taken apart, we will also weld each socket joint held in place by eyebolts to make one solid structure. These structures would then be mounted upon 30" mobile home anchors driven into the ground. All plans were approved in March and supplies were ordered. EA staff will begin work in April.

Staff fabricated water inputs and drainpipes for progeny tanks over residence tanks in the UNFH Refugia building. Each aquarium will receive both incoming well water and conditioned water inputs. The modification design proved useful in the Quarantine building as Makayla Blake discovered nine F_1 fountain darters during inventory of the Comal fountain darters. The offspring were moved to a progeny tank set up over the adult tank.

Task 2 Research

Comal Springs riffle beetle nutrition: The experiment testing four types of manufactured diets continued this month and will conclude in April. Amelia Hunter traveled to Bozeman Fish Technology Center last year to make the four pellet types to test as alternative or supplemental food sources for Comal Springs riffle beetles. Gibson Gaylord and Wendy Sealey helped formulate the diets. The four pellet types are animal-based, plant-based, single cell-based, and

fiber log. No mortality events occurred in March, potentially due to the implementation of the following recirculation system modifications: UV sterilization, chiller set to 20 °C, monthly replacement of Vincon® tubing, and sump siphoning to remove organic matter and pond snails. Ms. Hunter exchanged pellets every Monday, Wednesday, and Friday to reduce fungal blooms, which can be lethal if the beetles become entrapped in the fungus.

Long-term salamander tagging experiment: The long-term salamander tagging experiment concluded this month with the final 12-month tag checks, photo documentation, and recording of length and weight. This study evaluated the retention and readability of visible implant elastomer (VIE), visible implant alphanumeric (VIA), and passive integrated transponder (PIT) tags in Texas blind, San Marcos, and Comal salamanders. Justin Crow served as the novel reader for this last set of tag checks. Individually tagging salamanders increases efficiencies in refugia operations and species management by allowing data, such as age, health, sex, capture location, and growth rate, to be tracked over the life span of an individual. Beyond refugia operations, long-term tags in individuals reintroduced to the wild facilitate mark-recapture evaluations.

San Marcos salamander reproduction research: Last month we dosed a subset of San Marcos salamanders with luteinizing hormone-releasing hormone (LHRH) before combining groups of females and males in sections of two large tanks. Two clutches were produced in March from this study. Both clutches came from San Marcos salamanders where males and females had been fully isolated from each other for one month before being combined after hormone application and both clutches were deposited within 14-days of dosage. We will continue to monitor the salamanders for egg deposition for three months total.

Peck's cave amphipod survivability: The third collection of Peck's cave amphipods (PCA) for the survivability research project was canceled due to precautions over the spread of SARS-CoV-2. We will determine a time to reschedule when social distancing procedures are over. Makayla Blake inventoried the PCA from the February collection after their 30-day quarantine period. Survival through the initial quarantine period was 93.3% overall. The PCA from the second collection, from February, was inventoried this month and 84 of the 90 survived through quarantine period for an overall survival of 93.3%.

BIO-WEST, Inc:

Comal Springs riffle beetle pupation enhancement

- Performed general maintenance of flow-through mesocosms.
- Monitored water quality parameters.
- Continued check of larval development of individuals from relaunch treatment 15, and 16.
- Performed additional literature review.
- Recorded fecundity of treatments 7, 15, and 16 adults.

Texas State University CSRB Pupation Research:

•Texas State conducted project management and invoicing
•Texas State coordinated with SMARC refugia director and staff
•Maintained growth/pupation chambers for experimentation at SMARC
•Conducted experiments
•Coordinated with refugia staff on the number of available larvae and the timing of their availability for experiments
•Sized larvae and used them in experiments; maintained own larval stock to supplement SMARC larvae used in experiments
•Maintained adult beetle populations at SMARC
•Coordination with molecular microbial ecology lab (headed by Dr. Camila Carlos-Shanley, Texas State University)
•Analyzed OM for protein, lipid, and carbohydrate extraction from biofilms and animals
•Retrieved and re-deployed OM sources at Comal Springs and at SMARC for use in experiments.

Task 4 Species Reintroduction

No work on species reintroduction was conducted this month.

Task 5 Reporting

All Refugia staff members worked on the materials for the monthly report.

Marta Estrada worked with Lisa Greigo-Lyon to submit the monthly Refugia invoicing package.

Staff worked on updating protocols for their facilities.

Task 6 Meetings and Presentations

The preconstruction meeting for the for the UNFH Quarantine Building HVAC Remediation project occurred March 11, 2020.

Damon Childs, Marta Estrada, and Dr. Kenneth Ostrand met on March 12, 2020 to go over the Refugia Program accounting.

Table 1. New collections and total census in March 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. Inventory for invertebrates is not conducted every month, if this is not a month that inventory is conducted census will be NA. Further details of these numbers can be found in supporting documents.

Species	SMARC March Kept	UNFH March Kept	Released	Total Collected	SMARC March Incorporated	UNFH March Incorporated	SMARC March Mortalities	UNFH March Mortalities	SMARC March Census	UNFH March Census
Fountain darter: San Marcos	NT	NT	--	0	0	0	14	18	382	454
Fountain darter: Comal	NT	NT	--	0	0	0	2	0	206	37
Comal Springs riffle beetle	NT	NT	--	0	202	0	NA	NA	NA	NA
Comal Springs dryopid beetle	NT	NT	--	0	0	0	NA	NA	NA	NA
Peck's Cave amphipod	NT	NT	--	0	0	0	NA	NA	NA	NA
Edwards Aquifer diving beetle	NT	NT	--	0	0	0	--	--	--	--
Texas troglobitic water slater	NT	NT	--	0	0	0	--	--	--	--
Texas blind salamander	1	NT	0	1	0	0	1	0	266	31
San Marcos salamander	39	NT	0	39	3	0	4(9)	0	241 (59)*	301
Comal Springs salamander	NT	NT	--	0	0	0	0	0	98	55
Texas wild rice plants	NT	NT	--	0	0	14	4	0	213	185

*Numbers in parenthesis are those San Marcos salamanders that are part of the population but in the reproduction experiment.

Summary of March Activities

March 2 – Diversion net cleaned

March 4 – Amelia Hunter collected water samples for microbiome project

March 6 – Diversion net checked

March 10 – Diversion net checked

March 11 – Preconstruction meeting for UNFH HVAC Remediation

March 11 - Amelia Hunter collected water samples for microbiome project

March 12 – Refugia Program accounting meeting with Damon Childs

March 13 – Diversion net checked

March 17 – Diversion net checked and removed

Pictures

Figure 1 Makayla Blake pressure washing Texas wild rice tanks at UNFH.

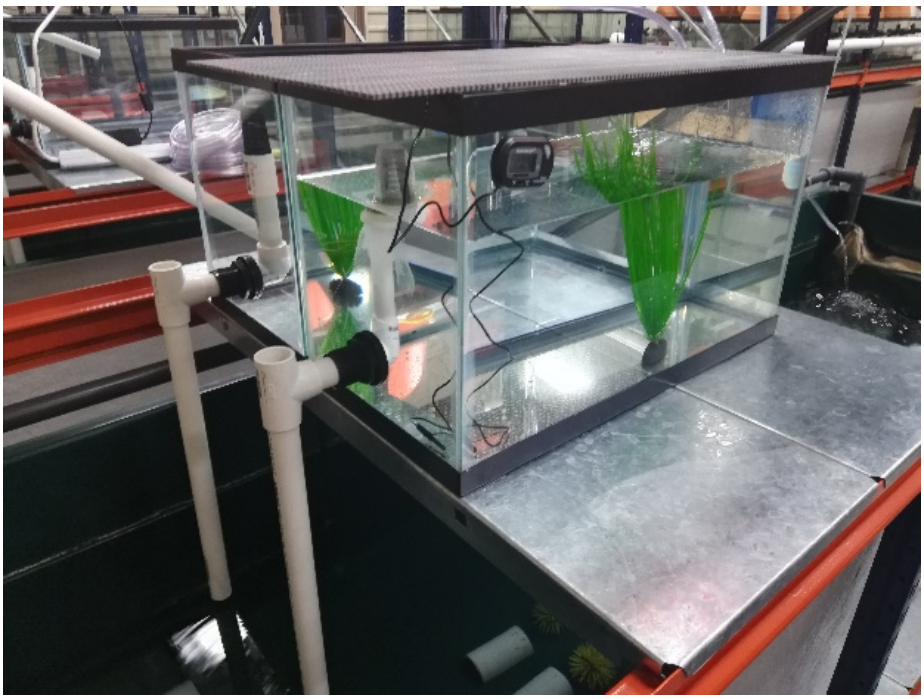


Figure 2 A new progeny aquaria holding a clutch of San Marcos salamander eggs at UNFH.

Figure 3: Larval San Marcos salamander. Yolk sac, front limb buds, gills, and eyes are visible.

Figure 4: Female San Marcos salamander depositing eggs at SMARC.

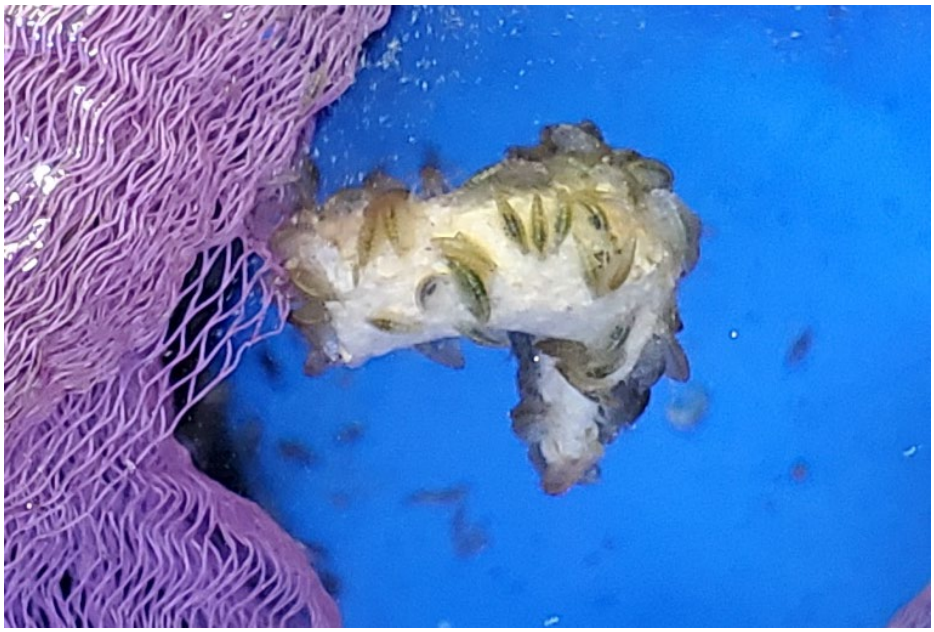


Figure 6: Amphipods congregate and feed on an extruded log food pellet designed as a supplemental food source for invertebrates.

Figure 7: Three one-year old Texas blind salamanders congregate on mesh in their aquarium near the water input. Palatal teeth are visible on the lower jaw.

Figure 8: A male fountain darter hiding in the SMARC display tank.

Figure 9: Texas blind salamander feeding on Mysis shrimp.

Figure 10: Feeding frenzy of San Marcos salamanders.

April 2020 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Lindsay Campbell, Ph.D.

And

Mark Yost (UNFH)

San Marcos Aquatic Resources Center
500 East McCarty Lane
San Marcos Texas, 78666
Phone: 512-353-0011

May 10, 2020

Task 1 Refugia Operations

Construction

Work began on April 1, 2020 for the 2019 EA Quarantine Building HVAC Remediation project (Contract Number 140F0220C0017) at Uvalde National Fish Hatchery (UNFH) (funds for project do not come from EAA). The project entails installing walls and a door to seal off the mezzanine level of the Quarantine building at UNFH. Additionally, contractors will install a thermostat, an exhaust fan, and automatically dampening louvers to remove heat waste from the chillers on the mezzanine, thus keeping it from warming the systems below in the Quarantine Building. They will also modify HVAC duct and grills so that the cooling system has more air flow in the building. The contractors started by installing plastic sheeting around the mezzanine level to prevent dust or debris from falling into the tanks below. Then they installed metal track on the floor and roof and installed metal studs for the walls, installing extra supports around the plumbing and HVAC duct penetrations through the new wall. Electricians moved some of the existing lights and conduit where the new doors would be and ran new electrical services and conduit to power the exhaust fan, the dampening louvers, and a new light switch inside the new room. Then, the contractors installed metal panels for the exterior wall, insulation within the walls, and metal sheeting for the interior wall. As the month concluded, the contractors started painting the new door frame and doors to match the color of the metal wall paneling. To date, none of the electrical equipment, such as the exhaust fan and dampening louvers have been received or installed. The contractors began work nearly one month after being awarded the contract, putting them nearly one month behind schedule. The contract is scheduled for completion by May 18th, and at present it does not appear that they will have everything complete by then. They also need to perform a complete HVAC replacement at the UNFH Shop Building, which was bundled into this contract, but work on this has not yet commenced. Further updates regarding the Quarantine Building renovations will be included in the next monthly report.

Species Collection

US Fish and Wildlife Service collaborated with BIO-WEST, Inc to receive fountain darters from the San Marcos River. BIO-WEST, Inc staff conducted annual spring biomonitoring activities for the EAA following proper protective measures against the spread of SARC-CoV-2. San Marcos Aquatic Resources Center (SMARC) staff did not come into contact with BIO-WEST, Inc staff. A SMARC staff member placed coolers for the fish at a designated drop off location and disinfected the outsides of the coolers after placement (insides of coolers had already been cleaned and disinfected for fish transport). BIO-WEST staff picked up the coolers from the location, transferred the collected fountain darters in the coolers, and then placed the coolers at the specified pick-up location. A SMARC staff member (wearing PPE) disinfected the outsides of the coolers and transported them to SMARC Quarantine. Only one staff member at a time picked up and acclimated the fish. Smaller transport coolers were used so one person could safely pick them up.

All other field work was suspended for staff health and wellbeing during the SARS-CoV-2 outbreak.

Refugia Population Propagation and Husbandry (Task 3 funding included in Task 1)

Amelia Hunter conducted inventory on all invertebrate species within the refugia.

Ms. Hunter cleaned, sterilized, and disassembled the research set-up for Comal Springs riffle beetle (CSRB) food preference experiment (see research section for more details). She and Dr. Campbell, in alternating shifts, reconfigured the system to hold Peck's cave amphipod (PCA) containers. During inventory, Ms. Hunter found *Hyalella* amphipods used as food for PCA in other invertebrate containers. We do not think the *Hyalella* harm the other species, but out of caution we moved the PCA to their own partial recirculation system. *Hyalella* are given as a food source to the PCA based on studies that indicate they are the food source for PCA in the wild. Mesh on outflow of the PCA holding containers did not allow adult *Hyalella* to escape, but these findings indicated they reproduced, and their young escaped.

Kelsey Anderson weighed, measured, and checked for gonadal development in Texas blind juveniles that reached one-year post collection. These data collected track individuals from the large group of Texas blind salamander juveniles that were collected in March-May 2019.

At the start of April, Ms. Anderson examined all sub- and mature-adult Texas blind salamanders for gonadal development. Several animals of previously unknown sex were identified as female for the first time. Young females present with very small ova that are difficult to find even while candled. As they age, the skin becomes thicker and candling becomes more challenging, so females are not usually confirmed until eggs are visible to the naked eye without candling. Some males presented with swollen, enlarged testes, and these males were selected for the pilot reproduction trial. Ms. Anderson and Dr. Campbell administered topical luteinizing hormone-releasing hormone (LHRH) to a subset of gravid Texas blind salamander females and a corresponding number of males. Ms. Anderson handled the salamanders, taking weights and relaying the information to Dr. Campbell (who was six feet separated). Dr. Campbell recorded the data, calculated the dose of LHRH, and drew the LHRH solution into the pipette. Both, wearing masks and gloves, came within arm's length of each other so Dr. Campbell could dispense the hormone onto the salamander Ms. Anderson held. The salamanders were placed in a partial recirculating system and videoed for courtship behavior. The cameras captured video of a female Texas blind salamander ovipositing eggs on mesh in the tank.

After moving 30 individuals into the reproduction trial, the remaining Texas blind wild stock were reorganized in the SMARC refugia. Animals were sorted into 15-mm total length ranges into four tanks. The rearrangement allowed Ms. Anderson to move three groups in glass aquaria – each with nine juvenile salamanders – into the three sections of a vacated raceway. Ms.

Anderson conducted a full inventory and the identification of each animal was recorded by tank by Linda Moon (appropriately social distanced and wearing PPE).

Ms. Anderson also conducted full inventories on San Marcos and Comal Springs salamanders. Following this, Visible Implant Elastomer tags, based on year and sex, were given to 51 San Marcos and 14 Comal Springs salamanders from previous collections. After tagging, animals were combined into general housing and dispersed evenly. All habitat was removed and replaced as per routine cleaning schedule.

Ms. Moon conducted a full inventory on all refugia darters. She removed all habitat items, cleaned and sterilized raceways, and replaced habitat with new items before returning fish to tanks in all fountain darter systems. She transferred the last group of fountain darters to their new tank in the same area as all other refugia fountain darters.

Ms. Moon prepared empty tanks in Quarantine for receiving fountain darters by adding appropriate habitat items for the species, adding standpipes and drain mesh covers, and flushing with water for 24 hours prior to the anticipated arrival of the fish.

Ms. Moon continued to re-pot Texas wild rice (TWR) plants.

All staff alternated days working on station with day teleworking. While teleworking, staff concentrated on revising Standard Operating Procedure documents and writing-up research results. Without collections and some research projects postponed, staff caught up on system maintenance. Dr. Campbell continued with color coding equipment by species to improve biosecurity so that nets, buckets, siphon tubes, etc. are not accidentally moved between systems.

EA staff at UNFH made plans to reinforce the TWR shade structures that were installed in December 2019, which included taking temporary portable structures, anchoring them to the ground, and welding structural cross braces to them to make them permanent structures. Materials for this project arrived at the end of the third week of April and preparations were made to commence work during the following week. However, Project Leader Patricia Duncan, under the direction of safety director Steven McEvoy, postponed the welding could until it can be conducted by a certified welder. The project will move forward as soon as a welder is identified. In the meantime, Patricia Duncan decided that the structures will be disassembled and stored once she determines a suitable place for the materials to be stored.

UNFH Refugia staff performed inventories in all the residence tanks in the Refugia and Quarantine Buildings, to include the fountain darters from the San Marcos and Comal Rivers, the San Marcos salamanders, the Texas blind salamanders, and the Comal Springs salamanders. Makayla Blake also performed inventory for the wild stock CSRB, CSBD, and refugia PCA, as well as for the January-Collection of the research PCA.

Another clutch of San Marcos salamander eggs was laid from April 15-17, 2020. Staff moved 32 eggs to a separate progeny tank for development.

Task 2 Research

Comal Springs riffle beetle nutrition: The experiment testing four types of manufactured diets finished on April 13, 2020. Amelia Hunter traveled to Bozeman Fish Technology Center in July 2019 to make the four pellet types to test as alternative or supplemental food sources for Comal Springs riffle beetles. Gibson Gaylord and Wendy Sealey helped formulate the diets. The four pellet types are animal-based, plant-based, single cell-based, and fiber log. Ms. Hunter humanly euthanized the beetles in the experiment and prepared the samples for isotopic analysis. She stored the samples in a -80 ºC freezer on the Texas State University campus. Samples will not be sent for analysis until the Stable Isotope Facility at University of California-Davis has reopened.

San Marcos salamander reproduction research: In February, we dosed a subset of San Marcos salamanders with LHRH before combining groups of females and males in sections of two large tank systems. An additional clutch of eggs was produced in April. We will continue to monitor the salamanders for egg deposition for three months total.

Rachel Wirick sorted and sexed all the San Marcos salamanders at UNFH to separate all males and females into two different systems in preparation for the habitat manipulation experiment. Once the salamanders have been separated by sex for four weeks, then males and females will be combined in the same tanks, but separated by divider screens

Peck's cave amphipod survivability: Dr. Campbell constructed two prototype brooding chambers for gravid PCA. In the past females have cannibalized their young after they are released from the brooding pouch. A flow-through tube design with a mesh divider traditionally used does not achieve high survivability for young. Ms. Hunter placed gravid PCA females in the two prototypes. Females were found during routine inventory of PCA holding containers. We will monitor the brooding chambers for young. Dr. Campbell and Kelsey Anderson began planning the logistics of videoing PCA food choice experiments factoring in changes due to social distancing.

Makayla Blake continued with the PCA habitat media study. Mrs. Blake inventoried the January collection PCA groups; seventy PCA still survived across all treatments. The February collection groups will be inventoried in early May.

BIO-WEST, Inc:

Comal Springs riffle beetle pupation enhancement

- Performed general maintenance of flow-through mesocosms.
- Monitored water quality parameters.
- Continued check of larval development of individuals from relaunch treatment 15, and 16.

• Performed additional literature review.
• Recorded fecundity of treatments 7, 15, and 16 adults.
• Launched 3 new treatments (TMS.1, TMS.2, and TMS.3) for medium to small sized F1 larvae

Texas State University CSRB Pupation Research:

· Texas State conducted project management and invoicing
· Texas State coordinated with SMARC refugia director and staff
· Maintained growth/pupation chambers for experimentation at SMARC
· Conducted experiments
· Coordinated with refugia staff on the number of available larvae and the timing of their availability for experiments
· Sized larvae and used them in experiments; maintained own larval stock to supplement SMARC larvae used in experiments
· Maintained adult beetle populations at SMARC
· Coordination with molecular microbial ecology lab (headed by Dr. Camila Carlos-Shanley, Texas State University). Extracted DNA and prepped samples for sequencing.
· Analyzed OM for protein, lipid, and carbohydrate extraction from biofilms and animals
· Retrieved and re-deployed OM sources at Comal Springs and at SMARC for use in experiments.

Task 4 Species Reintroduction

No work on species reintroduction was conducted this month.

Task 5 Reporting

All Refugia staff members worked on the materials for the monthly report.

Lisa Griego-Lyon with supervision by Marta Estrada submitted the monthly Refugia invoicing package.

Staff at both stations continued work on SOPs on telework days.

Task 6 Meetings and Presentations

Any meetings or presentations of note

Table 1. New collections and total census in April 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. Inventory for invertebrates is not conducted every month, if this is not a month that inventory is conducted census will be NA. Further details of these numbers can be found in supporting documents.

Species	SMARC April Kept	UNFH April Kept	Released	Total Collected	SMARC April Incorporated	UNFH April Incorporated	SMARC April Mortalities	UNFH April Mortalities	SMARC April Census	UNFH April Census
Fountain darter: San Marcos	91(49 FH)	NT	0	140	0	0	10	30	371	424
Fountain darter: Comal	NT	NT	NT	NT	0	0	2	0	204	37
Comal Springs riffle beetle	NT	NT	NT	NT	0	0	45	59	251	57
Comal Springs dryopid beetle	NT	NT	NT	NT	0	0	4	0	4	1
Peck's Cave amphipod	NT	NT	NT	NT	0	0	136	11	116(4)	132
Edwards Aquifer diving beetle	NT	NT	NT	NT	0	0	--	--	--	--
Texas troglobitic water slater	NT	NT	NT	NT	0	0	--	--	--	--
Texas blind salamander	NT	NT	NT	NT	0	0	4	0	236(30)	31
San Marcos salamander	NT	NT	NT	NT	11	0	7	1	248(55)	300
Comal Springs salamander	NT	NT	NT	NT	0	0	2	2	96	53
Texas wild rice plants	NT	NT	NT	NT	0	0	2	0	211	185

Numbers in ( ) indicate organisms that are part of the refugia population, but currently in experiments.

Summary of April Activities

April 13 – End of CSRB feeding experiment

April 21-23 – Staff received fountain darters from BIO-WEST, Inc as part of biomonitoring

Pictures

Figure 1 Construction to enclose the Quarantine Building mezzanine level to remove waste heat from the chiller units.

Figure 2 New San Marcos Salamander eggs collected in April (left) and developing eggs collected in March (right).

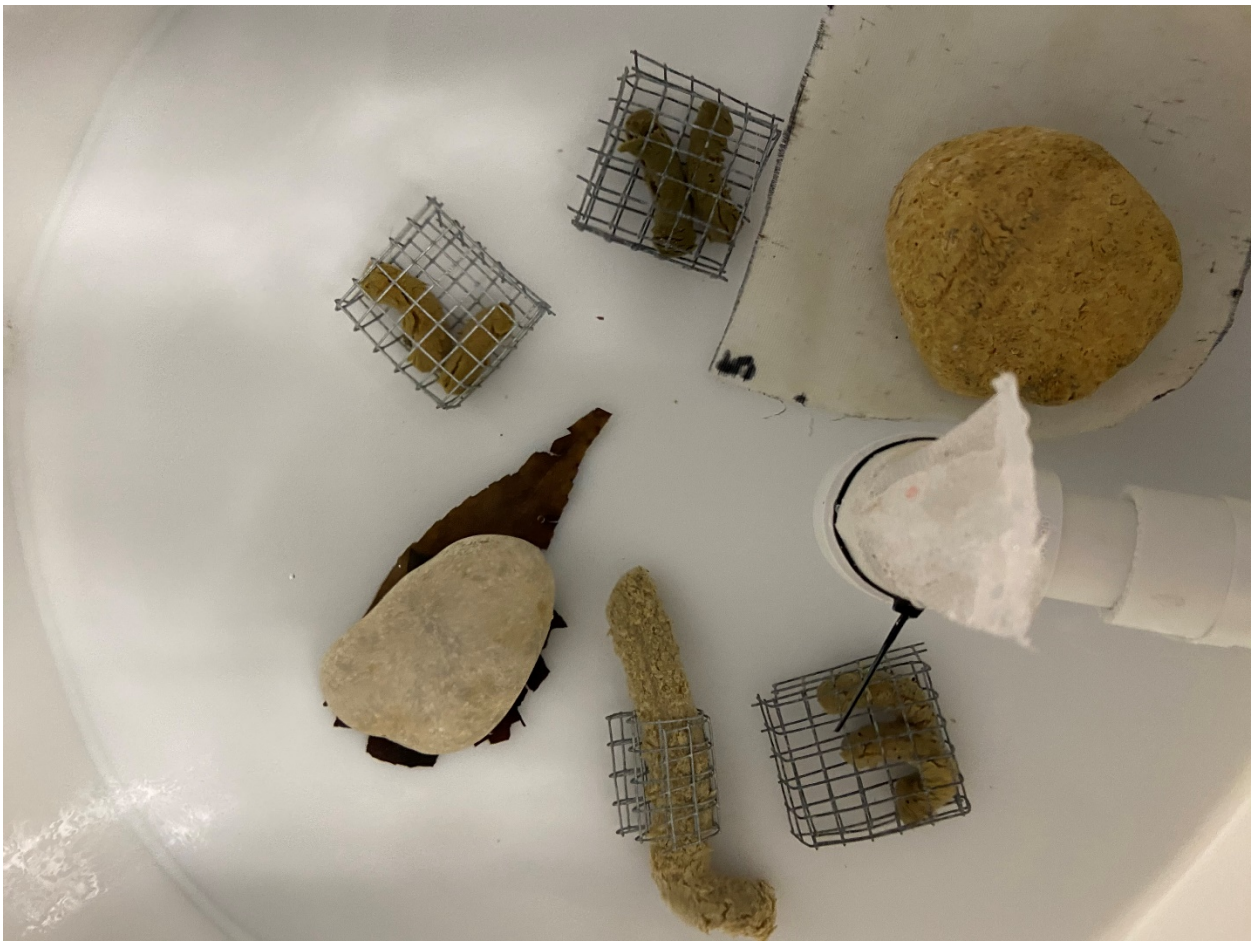


Figure 3 Arial view of CSRB food choice experiment. Clockwise from top: are animal-based pellet, cloth with developing biofilm held down by a rock, plant-based pellet, fiber log, decomposing leaf held down by a rock, and single cell-based pellet. Riffle beetle were introduced into the center of the grouping.

May 2020 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Lindsay Campbell, Ph.D.

And

Mark Yost (UNFH)

San Marcos Aquatic Resources Center
500 East McCarty Lane
San Marcos Texas, 78666
Phone: 512-353-0011

June 10, 2020

Task 1 Refugia Operations

Construction

Work continued on the 2019 EAA Quarantine Building HVAC Remediation project at Uvalde National Fish Hatchery (UNFH). Contractors received an extension for the completion date from May 18 to June 12, 2020. To date, they have enclosed the building walls on the exterior, installed batting insulation, put up the interior walls, installed the double man doors, painted all trim work to match the wall color, installed the automatically dampening louvers, and prepared a curb support for the exhaust fan that will be installed on the roof. The work that still remains includes blocking off the five upper louvers on the exterior wall of the mezzanine, installing the exhaust fan on the roof, changing the small duct supply grills with larger ones on the duct near the ceiling, modifying the main supply grill for the duct supplying air to the ground level, changing the return grill with a larger one to increase air flow, and installing power to a new light switch on the inside of the new mezzanine room. Since the room has been sealed, it has already made a difference by keeping waste heat from the chillers from reaching the ground level. However, the mezzanine room is much warmer and this has required us to augment the chiller set points higher to avoid over cooling the water due to the temperature discrepancy of the tank water and the dry well chamber on the chillers. Once the HVAC is working optimally by increasing the duct grills to allow more airflow, and the exhaust fan effectively removes waste heat on the mezzanine level, we hope the overall temperatures of the Quarantine Building will be closer to optimum.

Species Collection

Fieldwork was suspended for staff health and wellbeing during the SARS-CoV-2 outbreak. Randy Gibson donated 52 Peck's cave amphipods (PCA) to the refugia from biomonitoring work he conducted at Comal Spring, New Bruanfels, TX.

Refugia Population Propagation and Husbandry (Task 3 funding included in Task 1)

Kelsey Anderson moved Texas blind salamanders into a tank previously occupied by San Marcos fountain darters (after tank was cleaned and disinfected) in the Refugia area at San Marcos Aquatic Resources Center (SMARC). Larval Texas blind salamanders began to hatch from clutches produced in late March and early May. Texas blind salamanders produced another clutch of 32 eggs, from the group that received the topical luteinizing hormone-releasing hormone (LHRH) last month.

Linda Moon began the fountain darter largemouth bass virus (LMBV) exposure trail with F1 darters in the Quarantine area of the refugia buildings. We are exposing groups of five F1 LMBV negative fountain darters to groups of five Comal River LMBV positive fountain darters for a period of three months. Two control tanks have F1 LMBV negative fountain darters and Comal River LMBV negative fountain darters. Any signs of disease will be documented and all

mortalities frozen in a -20 °C freezer. At the end of the trial, all F1 fountain darters will be tested for LMBV.

Ms. Moon continued to re-pot Texas wild rice plants.

During May, we implemented using Survey123, at SMARC, to collect daily tank/system check data on all of our systems, after testing in previous months on a few systems. Paper records of mortalities and other significant events were kept concurrently to ensure that no information was lost during the transition. Thus far, the data collection has gone well and looks to streamline work efforts of re-entering paper datasheets into electronic form. Additionally, Dr. Campbell is working on producing an ARC-GIS dashboard that updates with data input so summaries of data collected can be viewed in real time.

Ms. Anderson, Ms. Moon, and Dr. Campbell rotated duties, caring for the invertebrates after the departure of Amelia Hunter. Ms. Hunter started a permanent position as a Listing and Recovery Biologist with Austin Ecological Services (see below for more staffing details). All staff alternated days working on station with days teleworking. While teleworking, staff concentrated on revising Standard Operating Procedure documents and writing-up research results.

Staff at UNFH performed inventories again during May in all the residence tanks in the Refugia and Quarantine Buildings before the departure of all the staff (see below for more staffing details). For the San Marcos Fountain Darters, there were 14 missing (seven from the Upper section and seven from the Middle section) from the inventory requiring an adjustment; these were counted as mortality losses but separated from observed mortality in records. For the Comal Fountain Darters, there was one missing from the inventory requiring an adjustment. For the San Marcos Salamanders, there was one missing requiring an adjustment. For the Comal Springs Salamanders, there were four missing from the inventory requiring an adjustment. The Texas Blind Salamander inventory was intact.

The two clutches of San Marcos Salamander eggs collected at the end of March and in mid-April hatched during May. A full inventory has not been performed to date, as they are very small and fragile. They seem to be growing well with the daily feeding of Artemia nauplii.

May was a month of many departures of the staff from the Refugia program. Amelia Hunter started a job with USFWS Austin Ecological services on May 11, 2020. All of the staff at UNFH departed in May (or on June 2) after accepting employment offers elsewhere. Makayla Blake is working for the USFWS at Tishomingo National Fish Hatchery in Tishomingo, Oklahoma, as a Fish Biologist. Rachel Wirick is working for the USFWS Southwest Native Aquatic Resources and Recovery Center in Dexter, New Mexico, as a Fish Biologist. Mark Yost will be working for the USFWS Ecological Services Office in Klamath Falls, Oregon, as a Hatchery Manager. Benjamin Whiting will be working for the Federal Emergency Management Agency throughout the country as an Environmental Specialist. All of these employees

contributed to the effectiveness of the Refugia program. In particular, Ms. Hunter, Ms. Blake, and Ms. Wirick have been with the program from the start and helped shape the success of the program, each having a unique contribution and showing great dedication to the species, program and conservation. Much of our advancement on the knowledge and care of the invertebrates comes from the work of Ms. Hunter and Ms. Blake. Mr. Yost was a great leader of the UNFH portion of the program, immensely contributing to advances made their and staff development. All will be missed, but we wish them well and know we will see great things from them as their careers advance. Most of the photos for this report will be of them.

Upper management is currently exploring options to advertise and back fill these vacancies. Dr. David Britton is transferring his duty station during June to UNFH to help cover for the vacant positions and train emergency hires coming in to cover a portion of the positions.

Task 2 Research

Comal Springs riffle beetle nutrition: Samples from the experiment testing four types of manufactured diets were sent to the Stable Isotope Facility at University of California-Davis at the end of May. We received confirmation of sample delivery to the facility. We await our samples being logged into the system and given an estimated date for analysis. The Stable Isotope Facility released a statement upon reopening that while they can receive samples that they give approval to be sent, analysis times are expected to be longer than usual due to the backlog of samples while the facility was closed due to COVID-19 quarantine.

San Marcos salamander reproduction research: May 25, 2020 marked the end of the three-month evaluation period of San Marcos salamanders dosed with LHRH. No additional clutches were produced in May. We will discuss our results with Dr. Ruth Marcec-Greaves and determine the next steps. At UNFH, San Marcos salamander reproduction research has been temporarily halted until more staff are hired to help care for the husbandry activities. All San Marcos Salamander refugia stocks have been separated by sex and habitat treatment items created.

Peck's cave amphipod survivability: Makayla Blake inventoried PCA from the second collection, the B-collection, from February, on May 7, 2020. Eighty-one of 84 survived from the previous inventory for an overall survival of 96.4%. Dr. David Britton inventoried PCA from the first collection, the A-collection, from January, on May 20-21, 2020. Sixty-five of the 70 survived from the previous inventory, for an overall survival of 92.9%.

BIO-WEST, Inc:

Comal Springs riffle beetle pupation enhancement

- Performed general maintenance of flow-through mesocosms.
- Monitored water quality parameters.

- Continued check of larval development of individuals from relaunch treatment 15, and 16.
- Performed additional literature review.
- Recorded fecundity of treatments 16 adults.
- Checked results of treatments 17 and 18 and relaunched them.

Texas State University, Nowlin Lab:

Matthew Stehle and Kirby Wright continued to check experimental replicates at SMARC. Materials with biofilm developed in Comal springs was retrieved and used for experimental replicates.

Task 4 Species Reintroduction

No work on species reintroduction was conducted this month.

Task 5 Reporting

All Refugia staff members worked on the materials for the monthly report.

Marta Estrada and Lisa Griego-Lyon submitted the monthly Refugia invoicing package.

Task 6 Meetings and Presentations

No large meetings or presentations were conducted this month.

Table 1. New collections and total census in May 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. Inventory for invertebrates is not conducted every month, if this is not a month that inventory is conducted census will be NA. Further details of these numbers can be found in supporting documents. Numbers in ( ) indicate organisms in research projects, but part of Refugia.

Species	SMARC May Kept	UNFH May Kept	Released	Total Collected	SMARC May Incorporated	UNFH May Incorporated	SMARC May Mortalities	UNFH May Mortalities	SMARC May Census	UNFH May Census
Fountain darter: San Marcos	NT	NT	NT	NT	0	0	17	37	347	388
Fountain darter: Comal	NT	NT	NT	NT	0	0	0	2	203	35
Comal Springs riffle beetle	NT	NT	NT	NT	0	0	NA	NA	NA	NA
Comal Springs dryopid beetle	NT	NT	NT	NT	0	0	NA	NA	NA	NA
Peck's Cave amphipod	52	NT	0	52	0	0	NA	NA (8)	NA	NA (146)
Edwards Aquifer diving beetle	NT	NT	NT	NT	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	NT	NT	0	0	0	0	0	0
Texas blind salamander	NT	NT	NT	NT	0	0	1 (1)	0	235 (29)	31
San Marcos salamander	NT	NT	NT	NT	0	0	8 (1)	5	240 (54)	295
Comal Springs salamander	NT	NT	NT	NT	0	0	3	4	96	49
Texas wild rice plants	NT	NT	NT	NT	0	0	3	0	208	185

Pictures

Figure 1 Construction to enclose the Quarantine Building mezzanine level to remove waste heat from the chiller units.

Figure 2 Amelia Hunter in front of a mural of a Comal Springs riffle beetle.

Figure 3 Rachel Wirick conducting DNA extraction from salamander skin swabs.

Figure 4 Makayla Blake finds a non-target species during fountain darter sampling.

Figure 5Mark Yost (right) topically applies hormone to a San Marcos salamander. Also pictured Rachel Wirick (left) and Dr. Ruth Marcec-Greaves (middle).

Figure 6 Ben Whiting helps conduct sampling for Comal Springs riffle beetles.

June 2020 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Lindsay Campbell, Ph.D.

And

David Britton, Ph.D. (for UNFH)

San Marcos Aquatic Resources Center
500 East McCarty Lane
San Marcos Texas, 78666
Phone: 512-353-0011

July 10, 2020

Task 1 Refugia Operations

Construction

Contractors completed work on the Quarantine Building HVAC Remediation project at Uvalde National Fish Hatchery (UNFH).

Species Collection

Staff from San Marcos Aquatic Resources Center (SMARC) collected Peck's cave amphipods (PCA) for an ongoing experiment conducted at UNFH on June 16, 2020. At the same time Dr. Lindsay Campbell and Randy Gibson placed lures (with masks and socially distanced) for Comal Spring riffle beetles (CSRB) on the Western shoreline of Landa Lake in the Comal River, New Braunfels, Texas. Randy Gibson placed and checked drift nets for food items for a PCA feeding experiment (see below), but also retained PCA and Comal Springs salamanders in the nets for refugia populations. Refugia staff also collected Comal Springs salamanders on the back-up field collection day for our captive assurance refuge population. All fieldwork activities must be approved at the regional level; supervisors must write out documentation as to the need for the fieldwork, description of the fieldwork and preventative measures against COVID-19, and fill out a risk assessment matrix to send up in a package.

Refugia Population Propagation and Husbandry (Task 3 funding included in Task 1)

Staffing

Joseph Barnett joined our team at SMARC as an Emergency Hire (Animal Caretaker WG-5) to help with basic husbandry duties.

Two emergency hire employees have accepted 30-day positions at UNFH; both have worked here in the past and will help cover the duties left by the departing staff. Dr. David Britton (Deputy Director of the San Marcos Aquatic Resources Center) has been temporarily re-assigned to UNFH to oversee the transition to new staff. Emergency hire Valerie Fischer began animal husbandry duties at UNFH on June 22, 2020. She will work for a maximum of 60 days in this temporary position. The other emergency hire is expected to start in early July.

The Service has recently changed the procedures for hiring new employees. Most new employees now must be hired under a national "batch hire" process, which will include only five types of positions per hiring round. The types of these positions are determined based on national priorities, determined at a regional and national management level. Through this process, we have been able to post job opportunities for four GS-5 Biological technicians (two at UNFH and two at SMARC). The open period for applications to the Biological Technician positions closed on June 9. These positions are 13-month terms that may be extended up to four years. We should be able to review candidates and conduct interviews in early July. We expect these new hires to start no sooner than late August.

We have also received approval to hire a permanent GS-11 Biologist for the Refugia program at UNFH, independently from the batch hire process. This position has not yet been posted. We hope to fill this Program Lead position by September. In the interim, Dr. David Britton will lead the Edwards Aquifer Refugia program at UNFH. Project Leader Dr. Pat Duncan and UNFH lead Biologist Valentine Cantu have agreed to assist Dr. Britton throughout this transition.

Husbandry

Linda Moon worked with Dr. Trista Welsh-Becker and local veterinarian Dr. Kim Doll on treatment of fountain darters after the finding of Cryptobia in the stomach and intestines of one fish sent for analysis at Washington Aquatic Disease and Diagnostic Laboratory (at SMARC).

Ms. Moon continued to re-pot Texas wild rice plants (TWR). Mr. Barnett began power-washing in-between and under TWR raceways, that will culminate in cleaning out the trench drains (all Refugia staff will need to participate in this job).

Dr. Campbell participated in a planning team video conference for the Texas Groundwater Invertebrate Forum.

Staff completed their annual Snorkel Swim Test.

A Texas blind salamander female deposited at clutch of 32 eggs on June 29, 2020. She is in the tank that was treated with luteninizing hormone-releasing hormone in April. This is the fifth clutch from this tank.

UNFH staff performed daily animal and plant care to include monitoring system operations and water quality, caring for and feeding refugia organisms, cleaning waste and algae from tanks, grooming plants, and monitoring animals for signs of stress and/or disease to put in isolation or treat as needed.

Dr. Britton inventoried two clutches of San Marcos salamanders that hatched in May and found 40 healthy individuals. They have grown rapidly with the daily feeding of Artemia nauplii or cut blackworms.

Task 2 Research

Long-term salamander tagging experiment: Dr. Campbell and Ms. Moon consulted with region biometrician Dr. Matthew Butler on the analysis of tagging data for the project, in particular the type of analysis and transformation of data for the different positions and breakage of Visible Implant Elastomer tags. They are working on a manuscript for the project, along with the final report to the Edwards Aquifer Authority due at the end of the year.

Peck's cave amphipod survivability: San Marcos Aquatic Resources Center (SMARC) Refugia staff collected PCA for the on-going holding container habitat survivability study. Dr. Britton is

over-seeing this project at UNFH due to the recent departure of the staff. This is the third replicate of the experiment. This replicate was originally scheduled to start in late-March, but due to COVID-19 it had to be suspended until now.

The PCA from the second collection, from February, were inventoried on June 22. PCA in the all-black Matala treatment had 100% survival. PCA in the all-green Matala treatment had 89.6% survival. PCA in the mixed (half black, half green Matala) had 92.3% survival. We did not find any juveniles in the boxes. The overall survival rate was approximately 94%.

Dr. Campbell conducted inventory on the PCA females in the experimental brooding chambers and found no neonates. All six females were accounted for, but two were no longer gravid. Of the four females originally put into the dual-chamber box design, three escaped into the larger holding container. All three were still gravid. Both females were found in the single chamber design; one gravid, one not. Dr. Campbell returned the four remaining gravid females to the single chamber design. The other two returned to refugia population containers. Dr. Campbell will inventory the brooding chamber again in July. When the refugia PCA population is conducted in July, any gravid females will again be placed in the two experimental designs for testing.

Randy Gibson placed driftnets on springs in the Comal River, New Braunfels, Texas, to collect *Lirceolus hardeni* (non-listed) for feeding trails of new food sources for our PCA refugia population. Kelsey Anderson tested the camera set-up and video clarity of the PCA and the different food items to be tested. Replicates started at the end of the month and will continue into July.

San Marcos salamander reproduction: The San Marcos salamander habitat manipulation pilot experiment at UNFH has been postponed until more staff are hired to help care for the husbandry activities.

BIO-WEST, Inc:

Ely Kosnicki continued to check the pupation chambers of the various experimental replicates.

Texas State University CSRB Pupation Research: Students continued to check the on-going experiments during June at SMARC. Students also places materials in the field to develop biofilm for the pupation experiments.

Task 4 Species Reintroduction

No work on species reintroduction was conducted this month.

Task 5 Reporting

All Refugia staff members worked on the materials for the monthly report.

Lisa Griego-Lyon and Marta Estrada submitted the monthly Refugia invoicing package.

Task 6 Meetings and Presentations

No special meetings were held regarding the EA Refugia Program besides daily and weekly staff meetings to communicate work assignments and accomplishments.

Table 1. New collections and total census in June 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. Inventory for invertebrates is not conducted every month, if this is not a month that inventory is conducted census will be NA. Further details of these numbers can be found in supporting documents.

Species	SMARC Jun Kept	UNFH Jun Kept	Released	Total Collected	SMARC Jun Incorporated	UNFH Jun Incorporated	SMARC Jun Mortalities	UNFH Jun Mortalities	SMARC Jun Census	UNFH Jun Census
Fountain darter: San Marcos	NT	NT	--	--	63	0	9	12	402	376
Fountain darter: Comal	NT	NT	--	--	0	0	1	0	202	35
Comal Springs riffle beetle	NT	NT	--	--	0	0	NA	NA	NA	NA
Comal Springs dryopid beetle	NT	NT	--	--	0	0	NA	NA	NA	NA
Peck's Cave amphipod	72	99	16	187	0	96	NA	NA (5)	NA	NA (231)
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	--	--	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	--	--	0	0
Texas blind salamander	NT	NT	--	--	0	0	1	0	234(29)	31
San Marcos salamander	NT	NT	--	--	0	0	5	13	290	282
Comal Springs salamander	32	NT	2	34	2	0	1	0	94	49
Texas wild rice plants	NT	NT	--	--	0	0	1	0	207	185

Summary of June Activities

June 16th – Collection of PCA and setting CSRB lures on Western shoreline

June 18th – Collection of Comal Springs salamanders

Pictures

Figure 1. Completed construction to enclose the Quarantine Building mezzanine level to remove waste heat from the chiller units.

Figure 2. Comal Springs salamanders collected from the Comal River, New Braunfels, Texas, in June.

Figure 3 Kelsey Anderson filling containers for the PCA feeding trials.

July 2020 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Lindsay Campbell, Ph.D.

And

David Britton, Ph.D. (for UNFH)

San Marcos Aquatic Resources Center
500 East McCarty Lane
San Marcos Texas, 78666
Phone: 512-353-0011

August 10, 2020

Task 1 Refugia Operations

Construction

The Quarantine Building HVAC Remediation project at Uvalde National Fish Hatchery (UNFH) has improved the cooling of the Quarantine building. Temperatures in the husbandry area remain around 75 °F with the two residence systems running (Comal fountain darters and Comal salamanders).

Species Collection

Staff collected Peck's cave amphipods (PCA) for Refugia purposes at San Marcos Aquatic Resources Center (SMARC). At the same time Dr. Lindsay Campbell and Randy Gibson collected lures (with masks and socially distanced) for Comal Spring riffle beetles (CSRB) on the Western shoreline of Landa Lake in the Comal River, New Braunfels, Texas. Amelia Everett, representing Austin Ecological Services, participated in CSRB lure pick-up and then she and Mr. Gibson continued on to evaluate Spring Run 4 and lures Mr. Gibson set there last month (not a part of Refugia collections). All fieldwork activities must be approved at the regional level; supervisors must write out documentation as to the need for the fieldwork, description of the field work and preventative measures against COVID-19, and fill out a risk assessment matrix to send up in a package.

Refugia Population Propagation and Husbandry (Task 3 funding included in Task 1)

Staffing

USFWS staff conducted interviews for the four GS-5 Biological Technician positions (two at UNFH and two at SMARC). These positions are 13-month terms that may be extended up to four years. Two new employees were processed for hiring at UNFH and are expected to begin work on August 17, 2020. Jennifer Whitt and Benjamin Thomas will serve as biological technicians. The interview and hiring process is still on-going at SMARC.

The lead Refugia biologist position GS-9/11 for UNFH was posted and has now closed. We are awaiting a certification list of candidates from our Human Resources Department. We hope to fill this Program Lead position by September. In the interim, Dr. David Britton will continue to lead the Edwards Aquifer Refugia program at UNFH. Project Leader Dr. Pat Duncan and UNFH Lead Biologist Valentine Cantu continued to assist Dr. Britton throughout this transition. Emergency hire employee Valerie Fischer completed her tour of duty on July 27, 2020. Emergency hire employee Juan Banda accepted a 30-day extension.

Husbandry

Ms. Moon continued to re-pot Texas wild rice plants (TWR) at SMARC. She also participated in online USFWS SCUBA diver continuing education courses.

Ms. Anderson and Dr. Campbell conducted inventory on all PCA in Refugia. Dr. Campbell started inventory on Refugia CSRB and will finish in August.

Staff made room in the Quarantine building at SMARC to accommodate BIO-WEST, Inc. study on CSRB lure efficacy.

Texas blind salamanders, that had previous been treated with Luteinizing Hormone Releasing Hormone (LHRH), deposited five clutches of eggs in July.

At UNFH, half of the San Marcos salamanders held in RE14 were moved to RE3 to reduce densities. Likewise, half of the San Marcos salamanders held in RE13 were moved to RE5 for the same reason.

A heater/chiller unit in the UNFH invertebrate room was replaced after the existing one failed due to calcium build-up. The failed unit is being repaired.

Dr. Britton installed a vacuum gauge on a variable-speed pump in the invertebrate room. This gauge will allow staff to precisely control the pump's vacuum used to de-gas supersaturated water coming from the well.

Maintenance staff began welding shade structures for placement over the Texas Wild Rice raceways at UNFH. Once welded, these structures will be anchored to the asphalt.

Task 2 Research

Peck's cave amphipod survivability:

Dr. Britton conducted inventories on the habitat-media survivability study. The PCA from the third collection, the C-collection, from June, were inventoried on July 16, 2020, after their 30-day quarantine period. PCA in the all-black Matala treatment had 50% (15 of 30) survival. PCA in the all-green Matala treatment had 80% (24 of 30) survival. PCA in the mixed (half black, half green Matala) had 83% (25 of 30) survival.

The PCA from the first collection, the A-collection, from January, were inventoried on July 8, 2020. PCA in the all-black Matala treatment had 100% (17 of 17) survival. PCA in the all-green Matala treatment had 88% (23 of 26) survival. PCA in the mixed (half black, half green Matala) had 72% (16 of 22) survival.

Dr. Campbell inventoried the test brooding boxes. All females were accounted for, however none were still gravid nor juveniles found. Females were returned to stock. Only one gravid female was added to the brooding boxes early in July, however by the end of July she was no longer gravid and returned to stock.

Kelsey Anderson conducted replicate trials of different food items for PCA and began reviewing video. Ms. Anderson has made a video clip of a PCA hunting, attacking, and consuming a

daphnia. She will continue to scan video for more interactions. Though not consistent across replicates, PCA ate daphnia, *Lirceolus hardenii*, *Hyalella*, and video showed them to be on the food pellets.

San Marcos salamander reproduction:

Dr. Campbell and Dr. Ruth Marcec-Greaves collaborated on designing the expanded experiment with topical application of LHRH to a larger number of salamanders. Our pilot study earlier this year proved the safety of using LHRH on the San Marcos salamanders and produced some success at increasing egg clutch deposition. San Marcos salamanders were separated by sexes in June. We believe that separation can spur interest in mating once salamanders are combined. When the salamanders have constant access to potential mates there might not be as much of a drive to mate, as that resource is always available. Separation for a period of time and then combination could create an instinctive behavior to act on a resource (access to mates) that has not previously been available. The few clutches we did get in the pilot study this year were from groups that had been separated before LHRH application and combination.

We will apply LHRH to the males as they initiate courtship in this species by producing pheromones in their mental glands and rubbing them on the females to induce receptivity. In our digital footage of these salamanders in the 2019 and 2020 pilot experiments, males were not as interested in initiating and pursuing courtship as expected, many times not participating in courtship behavior at all. Application of LHRH is anticipated to stimulate male hormone production and participation in courtship

Long-term salamander tagging research:

Dr. Campbell and Ms. Moon continued to analyze data on the tagging project with biometrician Dr. Matthew Butler. They focused on writing R code for the analysis and compiling results. They are working on a manuscript for the project, along with the final report to the Edwards Aquifer Authority due at the end of the year.

BIO-WEST, Inc:

Ely Kosnicki continued to check the pupation chambers of the various experimental replicates.

Texas State University CSRB Pupation Research:

Students continued to check the on-going experiments during July at SMARC. Students also retrieved materials from the field with developed biofilm for the pupation experiments.

Task 4 Species Reintroduction

No work on species reintroduction was conducted this month.

Task 5 Reporting

All Refugia staff members worked on the materials for the monthly report.

Lisa Griego-Lyon and Marta Estrada submitted the monthly Refugia invoicing package.

Task 6 Meetings and Presentations

Refugia Program staff conducted daily and weekly staff meetings to communicate work assignments and accomplishments.

Dr. Campbell and Kristy Kollaus had a video meeting discussing the mid-year progress of research projects on July 13, 2020.

Ms. Moon and Dr. Campbell had video meeting with Donelle Robinson, of Austin Ecological Services, to discuss fountain darters on July 20, 2020. The 5-year review of the fountain darter species assessment is due by the end of FY2020.

Table 1. New collections and total census in June 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. Inventory for invertebrates is not conducted every month, if this is not a month that inventory is conducted census will be NA. Further details of these numbers can be found in supporting documents. (#)These are refugia organisms involved in research studies.

Species	SMARC Jun Kept	UNFH Jun Kept	Released	Total Collected	SMARC Jun Incorporated	UNFH Jun Incorporated	SMARC Jun Mortalities	UNFH Jun Mortalities	SMARC Jun Census	UNFH Jun Census
Fountain darter: San Marcos	NT	NT	--	--	0	0	17	7	385	369
Fountain darter: Comal	NT	NT	--	--	0	0	4	0	198	35
Comal Springs riffle beetle	17	NT	0	17	0	0	NA	NA	NA	NA
Comal Springs dryopid beetle	0	NT	0	0	0	0	NA	NA	NA	NA
Peck's Cave amphipod	110	NT	61	171	0	0	9	NA (35)	202	NA (196)
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	--	--	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	--	--	0	0
Texas blind salamander	NT	NT	--	--	0	0	0	0	233(29)	31
San Marcos salamander	NT	NT	--	--	0	0	8	10	282	272
Comal Springs salamander	NT	NT	--	--	32	0	4	0	121	49
Texas wild rice plants	NT	NT	--	--	0	0	1	0	206	185

Summary of June Activities

July 6th – PCA group A inventory

July 13th – Mid-year research check-in

July 16th – PCA group C inventory after quarantine period

July 21st – Collect CSRB lures and collect PCA

July 22nd – Fountain darter discussion with Donelle Robinson

Pictures

Figure 1 Newly installed vacuum gauge on a variable-speed pump at UNFH. A vacuum is used to remove supersaturated gases from well water.

Figure 2 Peck's cave amphipods after a feeding trial, ready to move back to their Refugia holding containers.

Figure 3 Developing Texas blind salamander still in egg.

August 2020 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Lindsay Campbell, Ph.D.

Contributions by

Patricia Duncan, Ph.D. (for UNFH)

Edited by

Kenneth Ostrand, Ph.D.

San Marcos Aquatic Resources Center
500 East McCarty Lane
San Marcos Texas, 78666
Phone: 512-353-0011

September 10, 2020

Task 1 Refugia Operations

Species Collection

Staff from San Marcos Aquatic Resources Center (SMARC) collected fountain darters from the San Marcos River for Refugia purposes. This collection replaced the canceled collection event this past spring. Staff were able to safely socially distance from one another while collecting and river access was closed to the public, so they did not encounter others during collections. Staff also set traps for Texas blind salamanders in Primer's fissure and Johnson's well. Six adult salamanders were collected. We also lowered a GoPro camera into the two locations and video recorded Texas blind salamanders in both. All field work activities are approved at the regional level; supervisors must write out documentation as to the need for the field work, description of the field work and preventative measures against COVID-19, and fill out a risk assessment matrix to send up in a package.

Refugia Population Propagation and Husbandry (Task 3 funding included in Task 1)

Staffing

USFWS staff conducted interviews for the GS-9/11 at Uvalde National Fish Hatchery (UNFH); interview committee members were Dr. Ostrand, Dr. Britton, Dr. Duncan, and Trevor Luna (Willow Beach NFH).

Jennifer Whitt, a new four-year term biological science technician began work at UNFH on August 17, 2020. UNFH Emergency hire employee Juan Banda continued working through a 30-day extension of duty; however, his work hours have been reduced because he has returned to junior college. Dr. Britton continued to serve as the Refugia Program staff supervisor at UNFH. Dr. Patricia Duncan and Valentine Cantu assisted with monitoring and maintaining UNFH's Refugia Program populations during staff transition.

Joseph Barnett completed his SMARC Emergency Hire position in August. Mr. Barnett's assistance with Refugia daily operations was greatly appreciated. We were impressed with his work ethic and professional growth.

Husbandry

Linda Moon and Kelsey Anderson continued to re-pot Texas wild rice plants (TWR).

Dr. Campbell finished inventory on Refugia Comal Springs riffle beetles (CSRB) and Comal Springs dryopid beetles (CSDB). The SMARC Refugia invertebrate system became too calcified to operate properly. Dr. Campbell set up a new system in the Quarantine building following Standard Operating Procedures. Unfortunately, some CSRB losses were observed and recorded. While we thought that flow in the invertebrate system, though reduced, was adequate, we surmise that reduced flow contributed greater expected losses. Amelia Hunter (Austin ES

Field Office, formerly with Refugia program) was consulted about the CSRB losses and any changes we need to make.

Texas blind salamanders, that had previous been treated with Luteinizing Hormone Releasing Hormone (LHRH), deposited two additional clutches of eggs in August. This makes 13 clutches from the 15 females in the pilot study. The pilot study concluded at the end of August by removing the males from the tank and checking all the females for signs of egg regeneration.

At UNFH, higher than normal air temperatures resulted in a heater/chiller malfunction. Staff increased the internal temperature control tolerances and purchased large fans for both the Refugia and Quarantine area to improve air circulation around the units.

UNFH maintenance staff continued welding shade structures to go over TWR raceways.

Task 2 Research

Peck's cave amphipod survivability:

Dr. Britton conducted inventories on the habitat-media survivability study. The Peck's Cave Amphipods (PCA) from the February-collection were inventoried on August 6, 2020. PCA in the all-black Matala treatment had 92.3% (24 of 26) survival. PCA in the all-green Matala treatment had 96.7% (29 of 30) survival. PCA in the mixed (half black, half green Matala) had 96.7% (29 of 30) survival. No juvenile PCA were observed.

Dr. Britton trained Ms. Whitt on PCA inventory on August 21, 2020 with the January collection PCA. PCA in the all-black Matala treatment had 86.7% (13 of 15) survival. PCA in the all-green Matala treatment had 83.3% (20 of 24) survival. PCA in the mixed (half black, half green Matala) had 96% (24 of 25) survival. No juvenile PCA were observed.

San Marcos salamander reproduction:

Ms. Anderson and Dr. Campbell, aided by Ms. Moon and Mr. Barnett, initiated the full scale LHRH experiment with San Marcos salamanders. This experiment has 200 salamanders in it (100 females, 100 males), comparing number of egg clutches laid in groups where males are treated with topically applied LHRH and control groups where no hormone was applied.

Long-term salamander tagging research:

Dr. Campbell and Ms. Moon continued to analyze data on the tagging project with biometrician Dr. Matthew Butler. They edited R-code for analyses and composed tables and figures.

BIO-WEST, Inc:

Ely Kosnicki continued to check the pupation chambers of the various experimental replicates.

Texas State University CSRB Pupation Research:

Students continued to check the on-going experiments during July at SMARC. Students also retrieved materials from the field with developed biofilm for the pupation experiments.

Task 4 Species Reintroduction

No work on species reintroduction was conducted this month.

Task 5 Reporting

All Refugia staff members worked on the materials for the monthly report.

Lisa Griego-Lyon, Mark Dietrich, and Marta Estrada submitted the monthly Refugia invoicing package.

Task 6 Meetings and Presentations

Refugia Program staff conducted daily and weekly staff meetings to communicate work assignments and accomplishments.

EAA staff and SMARC staff conducted a virtual meeting August 12, 2020 discussing research projects in 2020 and for 2021.

Table 1. New collections and total census in August 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. Inventory for invertebrates is not conducted every month, if this is not a month that inventory is conducted census will be NA. Further details of these numbers can be found in supporting documents. (#)These are refugia organisms involved in research studies.

Species	SMARC Aug Kept	UNFH Aug Kept	Released	Total Collected	SMARC Aug Incorporated	UNFH Aug Incorporated	SMARC Aug Mortalities	UNFH Aug Mortalities	SMARC Aug Census	UNFH Aug Census
Fountain darter: San Marcos	229	NT	111	530	0	0	7	5	378	364
Fountain darter: Comal	NT	NT	--	--	0	0	2	2	196	33
Comal Springs riffle beetle	NT	NT	--	--	15	0	225	NA	26	NA
Comal Springs dryopid beetle	NT	NT	--	--	0	0	2	NA	0	NA
Peck's Cave amphipod	NT	NT	--	--	0	0	NA	NA (11)	NA	NA (185)
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	--	--	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	--	--	0	0
Texas blind salamander	6	NT	10	16	0	0	0	0	233(29)	31
San Marcos salamander	NT	NT	--	--	0	0	8	5	274	267
Comal Springs salamander	NT	NT	--	--	32	0	0	0	121	49
Texas wild rice plants	NT	NT	--	--	0	0	1	0	206	185

Summary of August Activities

August 3rd to 24th – Trap for Texas blind salamanders at Primer's fissure and Johnson's well

August 6th – PCA Habitat Experiment inventory

August 10th – Fountain darter collection at Spring Lake

August 11th – Fountain darter collection in the middle and lower sections of San Marcos River

August 12th – Research Meeting with EAA (virtual)

August 21th – PCA Habitat Experiment inventory

August 27th – Start of LHRH full trial with San Marcos salamanders

August 31st – PCA Habitat Experiment inventory

August 31st – Submit detailed outline of planed 2021 research to EAA

Pictures

Figure 1 Socially distanced and masked collection staff. L to R: Linda Moon, Kelsey Anderson, Dr. Lindsay Glass Campbell

Figure 2 Baby stinkpot turtle caught (and released) when collecting Fountain Darters, about the size of a silver dollar.

Figure 3 Welding of shade structures at UNFH.

Figure 4 Kelsey Anderson holds a male San Marcos salamander, while Dr. Campbell applies LHRH to its head. Salamanders then rested on wet paper towels for 3-minutes to allow the LHRH to soak into their skin. Mr. Barnett (background) and Ms. Moon assisted by moving salamanders to their appropriate destinations.

Figure 5 Joseph Barnett assists with trapping at Primer's fissure.

September 2020 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Lindsay Campbell, Ph.D.

Contributions by

Patricia Duncan, Ph.D. (for UNFH)

Edited by

David Britton, Ph.D.

San Marcos Aquatic Resources Center
500 East McCarty Lane
San Marcos Texas, 78666
Phone: 512-353-0011

October 9, 2020

Task 1 Refugia Operations

Species Collection

Staff from the San Marcos Aquatic Resources Center (SMARC) collected Peck's cave amphipods (PCA), Comal Springs dryopid beetles, and Comal Springs salamanders from the Spring Island area of the Comal River, New Braunfels, TX. Jennifer Whitt from the Refugia staff at Uvalde National Fish Hatchery (UNFH) assisted the SMARC staff and transported a portion of PCA to UNFH. Staff were able to safely socially distance from one another while collecting. All field work activities are approved at the regional level; supervisors must write out documentation as to the need for the field work, description of the field work and preventative measures against COVID-19, and fill out a risk assessment matrix to send up in a package.

Refugia Population Propagation and Husbandry (Task 3 funding included in Task 1)

Staffing

Thomas Funk and Braden West, two new four-year term biological science technicians began work at SMARC on September 14, 2020. The remaining refugia staff trained them throughout the month on husbandry tasks and fieldwork.

Ben Thomas, a new four-year term biological science technician began work at UNFH on September 28, 2020. Dr. Britton continued to serve as the Refugia Program staff supervisor at UNFH. Dr. Patricia Duncan and Valentine Cantu assisted with monitoring and maintaining UNFH's Refugia Program populations during staff transition.

Linda Moon ended her time working at the station to start her position as a permanent Biological Science Laboratory Technician at the Exotic & Emerging Avian Viral Diseases Unit at the USDA/ARS Southeast Poultry Research Center. Linda grew as a professional here going from a volunteer, to Biological Technician, and heading up our tagging study on three salamander species, all while completing her other work tasks. We thank her for her service, dedication, and valuable contributions to the Refugia program.

Husbandry

Linda Moon and Kelsey Anderson continued to re-pot Texas wild rice plants (TWR).

Several staff conducted inventories of PCA, all fountain darters, and Comal Springs salamanders. Dr. Campbell, assisted by Ms. Anderson, altered the PCA system to make room for additional holding boxes for the September collection and beyond.

All SMARC Refugia staff worked on training the new SMARC Refugia employees.

At UNFH, in order to collect the abundant amphipods found in filamentous algae in outside tanks, a modification was made to the amphipod collector. With the dense filamentous algae, the

existing amphipod collector failed to allow separation of the amphipods from the algae without causing amphipod mortality. After testing several substrates, the use of Matala Biomedia was found to work to reduce algae from escaping the anoxic chamber of the amphipod separator, while allowing amphipods to crawl through and accumulate in the isolation bucket. This modification has allowed collection of abundant amphipods to use for feed and to seed indoor amphipod cultures.

Maintenance staff continued welding shade structures for placement over the Texas wild rice raceways at UNFH.

The peer reviewed paper "Characterizing the regenerative capacity and growth patterns of the Texas blind salamander (*Eurycea rathbuni*)" in Developmental Dynamics was published on-line September 4, 2020. It covers research initiated by Dr. Glass Campbell, who reached out to Dr. McCusker, at the University of Massachusetts—Boston, about documenting a case of double limb regeneration in a Texas blind salamander donated to the station. From there Dr. Vierra flew to Texas to conduct a surgery on a Texas blind salamander that had been collected with one limb missing, in an attempt to stimulate limb regeneration. The surgery was successful and the salamander now has a fully functional limb where it previously only had a stump for at least one year (the time we observed it in Refugia). Kelsey Anderson diligently worked documenting the limb growth. Both Ms. Anderson and Dr. Glass Campbell are co-authors on the paper. Basic life history information such as growth, reproduction, collection, and care are documented in the paper. Two videos were included as supplement material for the paper. One video shows Texas blind salamander courting behavior, including deposition of a spermatophore packet. The second video shows the salamander using her regenerated limb. Citation:

Vieira, WA, Anderson, KA, Glass Campbell, L, McCusker, CD. Characterizing the regenerative capacity and growth patterns of the Texas blind salamander (*Eurycea rathbuni*). Developmental Dynamics. 2020; 1-16. **https://doi.org/10.1002/dvdy.245**

Task 2 Research

Peck's cave amphipod survivability:

Jennifer Whitt conducted inventories on the habitat-media survivability study. The Peck's Cave Amphipods (PCA) from the February-collection were inventoried on September 21, 2020. PCA in the all-black Matala treatment had 91.7% (22 of 24) survival. PCA in the all-green Matala treatment had 75.9% (22 of 29) survival. PCA in the mixed (half black, half green Matala) had 75.9% (22 of 29) survival. No juvenile PCA were observed. This was the last inventory of the February group for the study.

Four brooding females found during inventories at SMARC were moved to the test brooding chambers.

San Marcos salamander reproduction:

Six San Marcos salamanders were euthanized and sent to Washington Aquatic Disease and Diagnostic Laboratory for follow-up barium (and other metals) analysis. After finding high levels of barium in the salamanders last year, staff changed the food items fed to the salamanders to those that were not high in barium. The follow up analysis should indicate if this change had any effect on the barium levels.

BIO-WEST, Inc:

Ely Kosnicki continued to check the pupation chambers of the various experimental replicates.

Texas State University CSRB Pupation Research:

Students continued to check the on-going experiments at SMARC.

Task 4 Species Reintroduction

No work on species reintroduction was conducted this month.

Task 5 Reporting

All Refugia staff members worked on the materials for the monthly report.

Lisa Griego-Lyon, Mark Dietrich, and Marta Estrada submitted the monthly Refugia invoicing package.

Task 6 Meetings and Presentations

Refugia Program staff conducted daily and weekly staff meetings to communicate work assignments and accomplishments.

Table 1. New collections and total census in September 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. Inventory for invertebrates is not conducted every month, if this is not a month that inventory is conducted census will be NA. Further details of these numbers can be found in supporting documents. (#)These are refugia organisms involved in research studies.

Species	SMARC Sept Kept	UNFH Sept Kept	Released	Total Collected	SMARC Sept Incorporated	UNFH Sept Incorporated	SMARC Sept Mortalities	UNFH Sept Mortalities	SMARC Sept Census	UNFH Sept Census
Fountain darter: San Marcos	NT	NT	--	--	220	0	10	4	584	360
Fountain darter: Comal	NT	NT	--	--	2	0	5	1	191	32
Comal Springs riffle beetle	NT	NT	--	--	0	0	NA	NA	NA	NA
Comal Springs dryopid beetle	6	NT	0	6	0	0	NA	NA	NA	NA
Peck's Cave amphipod	111	50	14	175	74	0	32	NA (16)	244	NA (185)
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	--	--	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	--	--	0	0
Texas blind salamander	NT	NT	--	--	0	6	0	0	268	31
San Marcos salamander	NT	NT	--	--	0	0	8	4	263*	263
Comal Springs salamander	14	NT	3	17	0	0	10	0	111	49
Texas wild rice plants	NT	NT	--	--	0	0	3	0	203	185

*Six San Marcos salamander wildstock from SMARC euthanized and sent for follow-up barium test, not counted as mortalities

Summary of September Activities

September 14th – Thomas Funk and Braden West started work at SMARC

September 28th – Ben Thomas started work at UNFH

September 29th – Peck's cave amphipod and Comal Springs salamander collection

Pictures

Figure 1 Texas blind salamanders on habitat enrichment items.

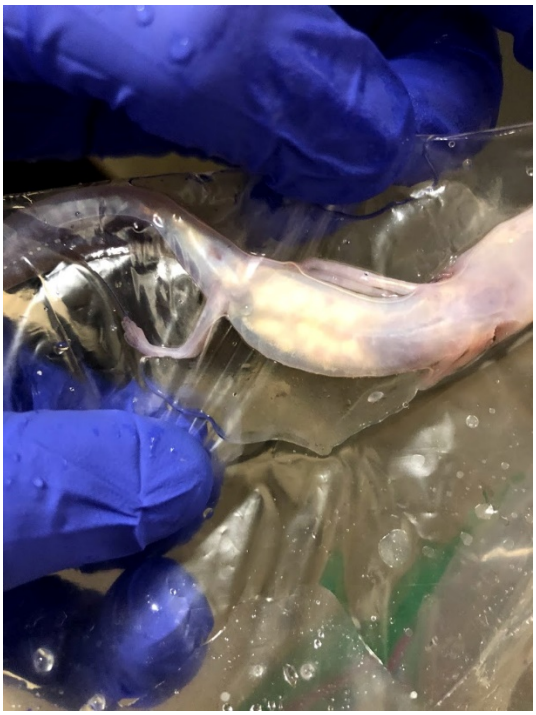


Figure 2 Texas blind salamander female very gravid.

Figure 3 Ben Thomas feeding Texas blind salamanders at UNFH.

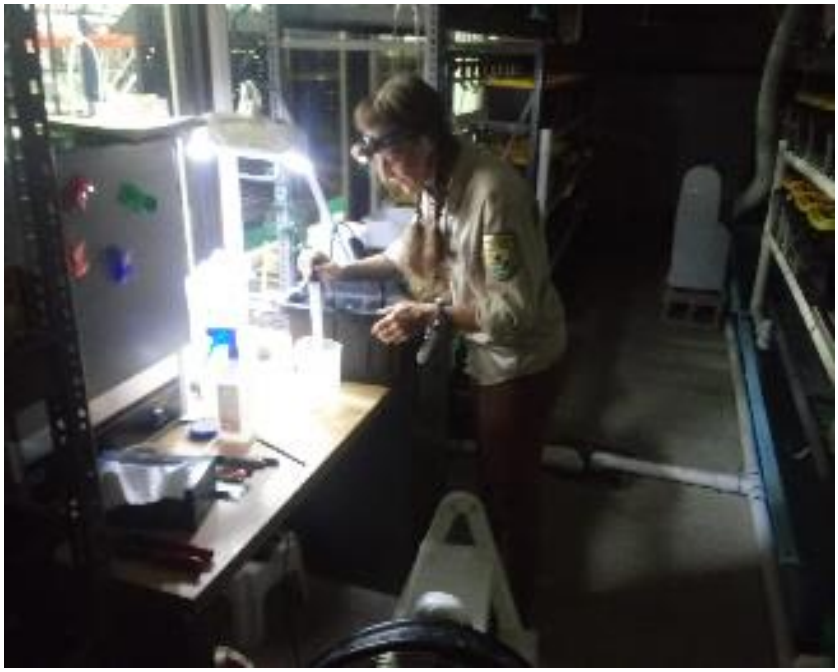


Figure 4 Jennifer Whitt conducts inventory on Peck's cave amphipods in the habitat experiment at UNFH.

October 2020 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Lindsay Campbell, Ph.D.

Contributions by

Patricia Duncan, Ph.D. (for UNFH)

Edited by

David Britton, Ph.D.

San Marcos Aquatic Resources Center
500 East McCarty Lane
San Marcos Texas, 78666
Phone: 512-353-0011

November 10, 2020

Task 1 Refugia Operations

Staffing

On October 8, 2020, Dr. Pat Duncan, Project Leader at Uvalde National Fish Hatchery (UNFH) took over leadership of the Edwards Aquifer Refugia Program at UNFH and supervision of the two new biological technicians. Dr. Duncan and Lead Fish Biologist, Val Cantu continued training the new hires and managing the EAA program on site.

Supervision of new biological technicians at San Marcos Aquatic Resources Center (SMARC) was transferred to Dr. Lindsay Campbell on October 8, 2020.

Species Collection

Staff collected Fountain Darters from the San Marcos River and received Fountain Darters from Bio-West collected during fall biomonitoring for Refugia purposes for SMARC, UNFH, and sample sent to USFWS Fish Health evaluation. Kelsey Anderson and Dr. Campbell trained the new staff at SMARC and UNFH on how to collect darters and the different collection locations in the San Marcos River. Ms. Anderson and Dr. Campbell also trained the new staff from both station on how to set lures for Comal Springs riffle beetles (CSRB) in the Spring Runs of the Comal River, New Braunfels, TX. We set traps for Texas blind salamanders in Rattlesnake well; however, staff had to remove a dead squirrel from the well on the first day of trapping. We hoped there would be enough water flow and turn over to improve the water quality during the trapping period, but ammonia levels did not drop into normal ranges. No salamanders were caught or seen. Ms. Anderson provided surface support for USFWS divers participating in a botany research project. The divers and Ms. Anderson took the opportunity of being in the same area as Texas wild rice stands to collect tillers for refugia. All fieldwork activities are approved at the regional level; supervisors must write out documentation as to the need for the fieldwork, description of the fieldwork and preventative measures against COVID-19, and fill out a risk assessment matrix to send up in a package.

Husbandry

Thomas Funk and Braden West continued to take over more of the husbandry duties as they became familiar with the systems. This month they learned about the incoming quarantine procedures for fountain darters and Texas wild rice.

Ms. Anderson and Dr. Campbell conducted inventories of Comal Springs riffle beetles and Comal Springs dryopid beetles, using this as an opportunity to teach Mr. Funk and Mr. West the process.

At UNFH, problems with pumps or plumbing were repaired in several systems to improve water quality and care of fountain darters and salamanders in the Refugia. Jennifer Whitt helped construct a new manifold with valves to improve well water flow rates into each tank section of

the system holding Texas blind salamanders. Ben Thomas assisted with repair of the pump leak on the outlet end of a fountain darter refugia tank.

At UNFH, the location of the heater/chiller units on the mezzanine outside and above the Refugia and Quarantine Rooms have caused problems with reading the temperature the units' probes for water regulation. The sensor in the heater/chiller units often read 3 to 4ºC above or below water temperature in the tanks systems in the rooms below. We have decided to bring the controllers down to the level of the tanks. On October 19, electricians were called to visit the UNFH to provide a quote to rewire the controllers located on the mezzanine of the Refugia and Quarantine Rooms into the rooms near the tank systems. We will also move the probes from the heater/chiller plumbing and submerge them directly into the water of the tank system. This will allow a direct measurement of temperature from the tanks and provide a more accurate reading.

Maintenance staff continued welding shade structures for placement over the Texas Wild Rice raceways. At the end of the month, welding on 90% of the structures was completed. A rented crane will move the structures over the tanks. This is scheduled for November 19. Once in place the final pieces will be welded on the structures and they will be anchored into the ground.

Task 2 Research

Staff continued to analyze data and writing up completed and on-going 2020 research projects. Dr. Campbell began to write full drafts of 2021 research proposals.

Peck's cave amphipod (PCA) survivability:

Jennifer Whitt conducted inventory on the June/C PCA group. PCA in the all-black Matala treatment had 100% (13 of 13) survival. PCA in the all-green Matala treatment had 100% (20 of 20) survival. PCA in the mixed (half black, half green Matala) had 88% (23 of 24) survival. No juvenile PCA were observed.

San Marcos salamander reproduction:

Three clutches of eggs were deposited from the scaled up Luteinizing Hormone Releasing Hormone (LHRH) experiment, two in tanks treated with LHRH and one in a non-treated tank.

BIO-WEST, Inc:

Ely Kosnicki continued to check the pupation chambers of the various experimental replicates.

Texas State University CSRB Pupation Research:

Students continued to check the on-going experiments at SMARC.

Adult CSRB Nutrition and Survival

Zachary Mays defended his master's thesis "The effect of captivity on the endangered Comal Springs riffle beetle, *Heterelmis comalensis*." Mr. Mays is a student of Dr. Camila Carlos-

Shanley, who is working with the Refugia program on CSRB gut microbiome research. Mr. Mays worked on a portion of the research comparing the microbiomes of CSRB collected from the wild and those that had been in the refugia population for over a year. Analysis revealed that the microbiomes of wild CSRB were significantly different from those of captive CSRB. The microbiomes were different in both size and composition.

Dr. Campbell video conferenced with Dr. Carlos-Shanley (TxSt) about CSRB microbiome research 2020.

Task 4 Species Reintroduction

No work on species reintroduction was conducted this month.

Task 5 Reporting

All Refugia staff members worked on the materials for the monthly report.

Lisa Griego-Lyon, Mark Dietrich, and Marta Estrada submitted the monthly Refugia invoicing package.

Task 6 Meetings and Presentations

Refugia Program staff conducted daily and weekly staff meetings to communicate work assignments and accomplishments.

Dr. Campbell met with Kristy Kollaus of EAA to exchange refugia SOPs and discus 2021 Research projects.

Dr. Campbell participated in a video conference with Kristina Tolman, Kristy Kollaus, and EAA interns to discuss refugia operations around the Fountain Darter and San Marcos salamander.

Table 1. New collections and total census in October 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. Inventory for invertebrates is not conducted every month, if this is not a month that inventory is conducted census will be NA. Further details of these numbers can be found in supporting documents. (#)These are refugia organisms involved in research studies.

Species	SMARC Oct Kept	UNFH Oct Kept	Released	Total Collected	SMARC Oct Incorporated	UNFH Oct Incorporated	SMARC Oct Mortalities	UNFH Oct Mortalities	SMARC Oct Census	UNFH Oct Census
Fountain darter: San Marcos	245	257*	133	635	0	0	23	7	543	353
Fountain darter: Comal	NT	NT	--	--	0	0	9	0	180	32
Comal Springs riffle beetle	NT	NT	--	--	0	0	7	51	19	17
Comal Springs dryopid beetle	2	NT	0	2	4	0	0	1	4	0
Peck's Cave amphipod	28	NT	0	28	0	0	NA	18	NA	270
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	--	--	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	--	--	0	0
Texas blind salamander	NT	NT	--	--	0	6	3	2	265	29
San Marcos salamander	NT	NT	--	--	0	0	8	2	255	261
Comal Springs salamander	NT	NT	--	--	14	0	3	2	122	47
Texas wild rice plants	10	NT	0	10	0	0	0	2	203	183

*An additional 18 Fountain Darters were transferred to UNFH from SMARC that had been collected in August and held in quarantine.

Summary of October Activities

Oct 5th- 23rd – Trap for Texas blind salamanders in Rattlesnake Well

Oct 7th – Dr. Campbell and Kristy Kollaus meet

Oct 9th – Dr. Campbell video conference with EAA interns

Oct 15th – SMARC staff collect fountain darters

Oct 22nd – SMARC and UNFH staff collect fountain darters

Oct 26th – All Refugia Program staff set lures for CSRB

Pictures

Figure 1 New Refugia Program biological technicians on their first fountain darter collection. L to R: Jennifer Whitt, Ben Thomas, Braden West, Thomas Funk.

Figure 2 Thomas Funk collects fountain darters below Spring Lake Dam.

Figure 3 Jennifer Whitt collects fountain darters at Spring Lake.

Figure 4 Kelsey Anderson demonstrates how to cover a lure with rocks and take water quality readings.

Figure 5 Ben Thomas works to fix a leaky pipe join.

Figure 6 Jennifer Whitt works on water lines.

November 2020 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Lindsay Campbell, Ph.D.

Edited by

David Britton, Ph.D.

San Marcos Aquatic Resources Center
500 East McCarty Lane
San Marcos Texas, 78666
Phone: 512-353-0011

December 10, 2020

Task 1 Refugia Operations

Staffing

On November 9, 2020, we welcomed Adam Daw, stationed at Uvalde National Fish Hatchery (UNFH), as our team lead for husbandry and collections. Mr. Daw is completing his Ph.D. from the University of Southern Mississippi where his dissertation is on environmental factors affecting life histories in two species of copepods. Mr. Daw has experience in aquaculture from the Thad Cochran Marine Aquaculture Center (University of Southern Mississippi), the Pacific Aquaculture and Coastal Resources Center (University of Hawaii at Hilo), the Wrigley Marine Science Center (University of Southern California), the National Resource Center for Cephalopods (University of Texas Medical Branch), and the Laboratory for Oceanographic and Environmental Research (Texas A&M University at Galveston).

Species Collection

The EAA Refugia team set traps for Texas blind salamanders in Primer's Fissure and Johnson's Well in November. We tail clipped and released six salamanders, visually identified ten more salamanders, and collected seven for our Refugia. UNFH staff (Val Cantu, Jennifer Whitt, and Ben Thomas) collected Comal Springs salamanders and Peck's cave amphipods (PCA) from the Comal River on November 12, 2020. Four salamanders were collected at Spring Run 1, three salamanders from Spring Run 3, and one salamander was collected near Spring Island. Additionally 38 PCA were collected at Spring Run 3. These animals were returned to UNFH and are being held in quarantine.

All fieldwork activities are approved at the regional level; supervisors must document the need for the fieldwork, describe the fieldwork and preventative measures against COVID-19, and fill out a risk assessment matrix to send up in a package.

Paige Najvar, Donelle Williams, and Aubry Buzek joined Ms. Anderson and Dr. Campbell checking traps for Texas blind salamanders on November 10, 2020. Afterward, they returned to the SMARC to tour the Edwards Aquifer Refugia buildings. Ms. Buzek documented the fieldwork and posted a Tweet about it from @USFWSSouthWest. FWS Director Aurelia Skipwith then re-Tweeted the post from @USFWS spotlighting our work at a national level. The U.S. Fish & Wildlife Service then shared the post on their primary Twitter page and on LinkedIn.

Husbandry

In quarantine, 14 *E. pterophila* (Comal Springs salamanders) and 5 *E. rathbuni* (Texas blind salamanders) were tagged and moved into refugia systems. One of the Texas blind salamanders collected in August had a heavily injured, fungused back leg, which we amputated under anesthesia. The animal responded well and remains in isolation as it regenerates the lost limb. Mr. West and Mr. Funk continue to build and install new water lines and drainage systems on a new two-level rack system to hold all *E. rathbuni* offspring produced in 2020. A new, non-

chilled water line was also run to the invertebrate sink. New maru moss balls were purchased for fountain darters and are being leached in clean aquariums before being added to darter systems.

In Texas wild rice tank 4 staff dropped the flow bar deeper into the tank and raised several pots containing struggling plants to encourage growth. Plants were also repositioned into sunnier spots and shade cloth was opened for this system. At the same time, algaecide was applied to tank 5 and staff increased cleaning efforts to control algae. Mr. West and Mr. Funk began clearing water line holes of calcium deposits weekly. Amphipod populations in rice tanks had returned to normal levels and collection of amphipods from rice tanks resumed in November after one month of rest. After receiving U-line channels, staff prepared to install the new grating system in the rice building, which will greatly improve cleaning efforts and build-up of debris in the main drainage system.

Ms. Anderson and Mr. West visited the EARDC lab and retrieved various driftnet pieces to sample the artesian well in December. Justin Crow and Ashley Seagroves were cleared to dive and returned to Diversion Spring in San Marcos to retrieve the net. The net and pieces were returned to station for repairs and thorough cleaning such that the site can be sampled again in December. All wild Texas blind juveniles collected en masse from Diversion in March – May 2019 have reached 1.5 years in captivity; data continues to be collected every 6 months on the cohort to generate growth and developmental data for the species.

Dr. Trista Welsh-Becker, USFWS veterinarian and Supervisory Microbiologist at the Fish Health unit in Dexter, NM visited in November. This was Dr. Becker's first visit to the facility, though she has been working with the EAA species since early 2020. Staff showed Dr. Becker the facility and animal housing. Dr. Becker performed both a salamander and fish necropsy and demonstrated how to perform basic slide preparation techniques that may improve disease diagnostic capabilities and turnaround time.

At UNFH shade structures for Texas wild rice (TWR) are now in place. The maintenance crew began work anchoring the shade structures around the in-ground raceways that hold the TWR. This project is scheduled for completion in December.

Task 2 Research

Dr. Campbell finished 2020 research reports on Long-term Tagging Methods in Aquatic Salamanders, Comal Springs Riffle Beetle Nutrition (with input from Amelia Hunter), and San Marcos Salamander Reproduction (with input from Kelsey Anderson). Dr. Britton completed the Peck's Cave Amphipod Survival research report (with input from Kelsey Anderson, Dr. Campbell, and Jennifer Witt). Dr. Campbell completed and turned in 2021 research proposals on Texas Wild Rice Genetic Assessment, Refugia Level F1 Comal Springs Riffle Beetle Production, and Experimental Research Stemming from Comal Springs Riffle Beetle Microbiome Analysis.

Jennifer Whitt conducted the last inventory for the PCA habitat experiment. Ten of 13 PCA (77%) survived in low density habitat. Seventeen of 20 PCA (85%) survived in medium density habitat. And, 21 of 23 PCA (91%) survived in mixed density habitat. This inventory event was the last for this experiment.

BIO-WEST, Inc:

Ely Kosnicki continued to check the pupation chambers of the various experimental replicates.

Texas State University CSRB Pupation Research:

Students continued to check the on-going experiments at SMARC.

Task 4 Species Reintroduction

No work on species reintroduction was conducted this month.

Task 5 Reporting

All Refugia staff members worked on the materials for the monthly report.

Lisa Griego-Lyon, Mark Dietrich, and Marta Estrada submitted the monthly Refugia invoicing package.

Task 6 Meetings and Presentations

Refugia Program staff conducted daily and weekly staff meetings to communicate work assignments and accomplishments.

Dr. Campbell and Dr. Britton provided a research update during a meeting with Dr. Furl on November 6, 2020.

Paige Najvar, Donelle Robins, and Aubry Buzek from the USFWS Austin Ecological Services branch accompanied Kelsey Anderson and Dr. Lindsay Campbell to check traps for Texas blind salamanders at Primer's fissure and Johnson's well.

Table 1. New collections and total census in November 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. Inventory for invertebrates is not conducted every month, if this is not a month that inventory is conducted census will be NA. Further details of these numbers can be found in supporting documents.

Species	SMARC Nov Kept	UNFH Nov Kept	Released	Total Collected	SMARC Nov Incorporated	UNFH Nov Incorporated	SMARC Nov Mortalities	UNFH Nov Mortalities	SMARC Nov Census	UNFH Nov Census
Fountain darter: San Marcos	NT	NT	--	--	188	0	73	15	664	338
Fountain darter: Comal	NT	NT	--	--	0	0	8	0	172	32
Comal Springs riffle beetle	NT	NT	--	--	0	0	NA	NA	NA	NA
Comal Springs dryopid beetle	NT	NT	--	--	0	0	NA	NA	NA	NA
Peck's Cave amphipod	NT	38	7	45	0	0	NA	23	NA	243
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	--	--	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	--	--	0	0
Texas blind salamander	7	NT	16	23	0	6	3	0	262	29
San Marcos salamander	NT	NT	--	--	0	0	9	4	246	257
Comal Springs salamander	NT	8	0	8	0	0	0	0	122	47
Texas wild rice plants	NT	NT	--	--	10	0	6	0	207	183

Summary of November Activities

Nov 2nd- 20th – Trap for Texas blind salamanders at Primer's fissure and Johnson's well

Nov 3 & 4th – Dr. Welsh-Becker (Southwest Regional Veterinarian) conducted a site visit at UNFH & SMARC

Nov 6th – Dr. Campbell and Dr. Britton had a research update meeting with Dr. Furl

Nov 10th – Paige Najvar, Donelle Robins, and Aubry Buzek accompany Kelsey Anderson and Dr. Lindsay Campbell to check traps for Texas blind salamanders

Nov 19th – SMARC Refugia Staff attended EAHCP appreciation event at Landa Park

Nov 30th – Turned in 2020 Research Reports and 2021 Research Proposals to EAA

Pictures

Figure 1 Social media (Twitter) post by USFWS Southwest (regional) and USFWS Director.

Figure 2 New tag (black, black, red) on a wild Texas blind salamander.

Figure 3 (L to R) Tommy Funk, Braden West, Dr. Lindsay Campbell, and Kristin Tolman at the EAHCP appreciation event.

Figure 4 Texas wild rice shade structures installed over tanks at UNFH.

December 2020 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

David Britton and Adam Daw

San Marcos Aquatic Resources Center
500 East McCarty Lane
San Marcos Texas, 78666
Phone: 512-353-0011

January 10, 2021

Task 1 Refugia Operations

Species Collection

On December 3, 2020, refugia staff from Uvalde and San Marcos collected invertebrate lures from Spring Run 3 in Landa Park (New Braunfels, Texas). In total, 104 Comal Springs riffle beetles were collected with 63 going to San Marcos and 41 to Uvalde. Additionally, 14 Peck's cave amphipods at Spring Run 3 on the same date.

On December 9, 2020 SMARC biotechnicians collected 70 CSRB and one Comal Springs dryopid beetle from downed wood at Spring Island.

On December 10, 2020 SMARC biotechnicians collected 33 San Marcos salamanders below the falls at Spring Lake.

On December 7, 11, 14, and 16, Kelsey Anderson, Thomas Funk, Braden West, and Lindsay Campbell from the San Marcos Aquatic Resources Center checked traps at the Artesian Well (Texas State University) for Texas blind salamanders. None were collected. Seven Texas blind salamanders in quarantine were incorporated into the refugia population.

On December 15, 2020, Refugia staff collected Texas wild rice (TWR) tillers from the San Marcos River (historical River Sections E and F) with the assistance of two SCUBA divers. An average of ten tillers were collected from 13 plants. The plants were collected from 0.4 m to 2.1 m depths, and were returned the greenhouse at the San Marcos Aquatic Resources Center.

Husbandry

Kelsey Anderson (SMARC) started an Access database to help individually track Texas blind salamander life history data.

Facilities personnel at the Uvalde National Fish Hatchery (UNFH), with assistance from the Refugia staff, cemented anchors into the ground for canopy structures over the Texas wild rice tanks.

At UNFH, refuge system maintenance was conducted including preparing systems to be brought back online to increase tank space for organisms and allow for the rotational disassembly and thorough cleaning of all systems.

In order to reduce the heat load in the UNFH invertebrate room, we have decided to move the chiller units to the outside of the building. Preparations are ongoing for relocation of the water chillers to the outside, including the dismantling and cleaning of one of the four rack systems.

Inventory was taken for all captive organisms at UNFH, with the Texas wild rice inventory ongoing. Eggs were observed in numerous San Marcos Fountain darter tanks in the captive population over the course of the month. Refugia staff at Uvalde assisted with the daily care of UNFH facilities and animals during the holidays.

Staff

A new research biologist, Desiree Moore, was hired at SMARC with a tentative start date of January 4, 2021.

UNFH's Administrative assistant Mark Dietrich passed away on December 27, 2020, following complications after surgery. Marta Estrada and Lisa Griego-Lyon assumed his duties until a new Administrative assistant is hired for UNFH.

Task 2 Research

Questions from the EAHCP team about the research proposals for 2021 were answered and submitted to the EAA. Proposals included research on 1) genetics of Texas wild rice, 2) increasing Comal Springs riffle beetles F1 adult production at refugia level, 3) altering the microbial environment for riffle beetles based on microbiome analysis, and 4) San Marcos Salamander reproduction.

BIO-WEST, Inc:

Dr. Ely Kosnicki completed a Comal Springs riffle beetle luring experiment. BIO-WEST wants to continue this research in 2021. They expressed gratitude for being allowed to utilize the space at the SMARC. Experimental tanks were moved out of the quarantine space and temporarily stored elsewhere on station. BIO-WEST reported that they learned several things this year that they expect we could improve upon next year.

Texas State University CSRB Pupation Research:

Students continued to check the on-going experiments at SMARC.

Task 4 Species Reintroduction

No work on species reintroduction was conducted this month.

Task 5 Reporting

All Refugia staff members worked on the materials for the monthly report.

Lisa Griego-Lyon and Marta Estrada submitted the monthly Refugia invoicing package.

Task 6 Meetings and Presentations

On December 8, 2020 Ken Ostrand and David Britton attended the virtual meeting of the EAHCP Refugia Research Work Group.

On December 9, 2020 Ken Ostrand attended the virtual meeting of the EAHCP Comal Springs Riffle Beetle Work Group.

On December 17, 2020 David Britton, Ken Ostrand, and Lindsay Campbell attended the virtual EAHCP Joint Committee Meeting. David Britton presented an overview of operations and research activities.

Table 1. New collections and total census in December 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. Inventory for invertebrates is not conducted every month, if this is not a month that inventory is conducted census will be NA. Further details of these numbers can be found in supporting documents.

Species	SMARC Dec Kept	UNFH Dec Kept	Released	Total Collected	SMARC Dec Incorporated	UNFH Dec Incorporated	SMARC Dec Mortalities	UNFH Dec Mortalities	SMARC Dec Census	UNFH Dec Census
Fountain darter: San Marcos	NT	NT	--	--	0	161	63	16	601	480
Fountain darter: Comal	NT	NT	--	--	0	0	0	0	172	27
Comal Springs riffle beetle	133	41	0	174	0	0	0	2	NA	14
Comal Springs dryopid beetle	0	1	0	1	0	0	0	0	1	0
Peck's Cave amphipod	14	0	0	14	101	171	0	19	265	322
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	--	--	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	--	--	0	0
Texas blind salamander	0	NT	0	0	7	0	0	0	269	29
San Marcos salamander	33	NT	7	33	0	0	13	0	226	246
Comal Springs salamander	NT	NT	0	8	0	0	0	1	122	49
Texas wild rice plants	13	NT	--	13	10	0	1	9	206	174

Summary of December Activities

December 3, 2020 - collected Comal Springs riffle beetles and Peck's cave amphipods from lures at Spring Run 3.

December 9, 2020 - collected Comal Springs riffle beetles and Comal Springs dryopid beetles at Spring Island.

December 10, 2020 - collected San Marcos salamanders below Spring Lake.

December 7, 11, 14, and 16, 2020 - checked traps at the Artesian Well (Texas State University) for Texas blind salamanders.

December 15, 2020 - collected Texas wild rice (TWR) tillers from the San Marcos River (historical River Sections E and F)

Pictures

Figure 1. Jennifer Whitt, Lindsay Campbell, Kelsey Anderson, and Ben Thomas sorting Comal springs riffle beetles during collection at Spring Run 3 in Landa Park, New Braunfels, Texas.

Figure 2. Ben Thomas and Jennifer Whitt collect San Marcos Fountain darter eggs from refuge tanks during inventory.