5.1.1 Refugia Program

Introduction

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

This annual work plan and associated cost estimate have been developed per the requirements of contract number 16-822-HCP for the Implementation of the Refugia Program under the Edwards Aquifer Habitat Conservation Plan (EAHCP). The tasks and subtasks that follow provide the details for the services to be performed in 2021, which provide for the maintenance of a refugia population of the Covered Species (Table 1), including salvage, propagation, and restocking of the species (if species-specific habitat triggers occur and species are extirpated), plus research conducted on the Covered Species.

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

Common Name	Scientific Name	ESA Status
Fountain darter	*Etheostoma fonticola*	Endangered
Comal Springs riffle beetle	*Heterelmis comalensis*	Endangered
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Comal Springs salamander	*Eurycea pterophila*	Petition Rescinded
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, prey, competitors, pathogens, parasites; or on their habitat, including food, water, and shelter.

2021 Assumptions

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

- Target numbers for the standing and refugia stocks to be housed at both the UNFH and SMARC are established by the USFWS-EAA Refugia Contract (Contract # 16-822-HCP).
- Species capture rates are expected to be similar to historic values.
- Mortality rates of specimens held in captivity are expected to be similar to historic values.
- Target species collection numbers from the 2021 work plan are expected to be reached.
- Staff members remain employed at the two Service facilities throughout the performance period.

Target for 2021 (Deliverables and Methods by Task):

Task 1. Refugia Operations

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

Table 2. Species target refugia numbers and census.

Species	Standing Stock	Refugia Stock	Salvage Stock	Anticipated SMARC census (Jan 2021)	Anticipated SMARC census (Dec 2021)	Anticipated UNFH census (Jan 2021)	Anticipated UNFH census (Dec 2021)
Fountain Darter (Comal)	1000	1000 including specimens within the standing stock	2000	#	#	#	#
Fountain Darter (San Marcos)	1000	1000 including specimens within the standing stock	2500	500	500	500	500
Texas Wild-Rice	430	430 including specimens within the standing stock	1500	215	215	215	215
Texas Blind Salamander	500	500 including specimens within the standing stock	500	250	250	40	60
San Marcos Salamander	500	500 including specimens within the standing stock	500	250	250	250	250
Comal Springs Salamander	500	500 including specimens within the standing stock	500	115	135	80	105
Peck's Cave Amphipod	500	500 including specimens within the standing stock	500	250	250	250	250
Comal Springs Riffle Beetle	500	500 including specimens within the standing stock	500	75	75	75	75
Comal Springs Dryopid Beetle	500	500 including specimens within the standing stock	500	*	*	*	*
Edwards Aquifer Diving Beetle	500	500 including specimens within the standing stock	500	*	*	*	*
Texas Troglobitic Water Slater	500	500 including specimens within the standing stock	500	*	*	*	*

We will not collect Comal fountain darters until we have a better understanding of their mortality rates

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

Collection: In 2021, we will collect Covered Species as required to reach and maintain target standing and refugia stock numbers as shown in Table 2. Species collections will be coordinated with other ongoing HCP activities (e.g. Biological Monitoring Program) so that collections for refugia do not adversely impact other efforts. Species specific collections will be carried out through a variety of passive and active collection methods. Prior to collections, Hazard Analysis Critical Control Point (see Appendix A, 2017 Work Plan) will be conducted to minimize aquatic invasive species transfer. Collection efforts will be documented and reported to the EAA. Captured specimens will be distributed 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 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. The following sections briefly describe planned 2021 collection, maintenance, and propagation efforts for each species.

Fountain Darters:

Collection: In 2021, Fountain darters from the San Marcos River will be collected primarily in coordination with the Spring and Fall Biomonitoring events to create efficiencies and reduce habitat disturbance. After fountain darters are collected via drop nets for biomonitoring, USFWS staff will retain them for refugia purposes. Specimens will be collected along a longitudinal gradient. 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 Cape's dam). 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. Collections will target additional fish so that, as individuals perish, the remainder within the captive population should not decrease below the target number.

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. Until we have a better understanding of the high mortality rates of incoming Comal fountain darters we will conduct limited collections from the wild, unless salvage is needed.

As part of quarantine procedures, 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, mixed with purchased frozen food sources. Ponds will be utilized to produce zooplankton and amphipods. 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 (Figure 1), with a break during summer months when wild rice does not fare well due to heat stress. In 2021, collections for SMARC and UNFH will target stands that are not already part of the refugia population or require supplementation. The refugia populations will reflect the wild populations in both their respective proportion, based on the most recent Texas wild-rice survey data, and historical genetic diversity (Wilson et al. 2016). During tiller collection, the geographic coordinates, area coverage, and depth of the stand or individual plant will be recorded so the collection location of the clone is known. Tiller collection will be done by wading and SCUBA diving. Georeferenced aerial imagery will be captured with a small drone over the San Marcos river to help identify distinct TWR stands used for tiller collection. Knowing which TWR stands tillers were collected from is important in maintaining accurate husbandry records.

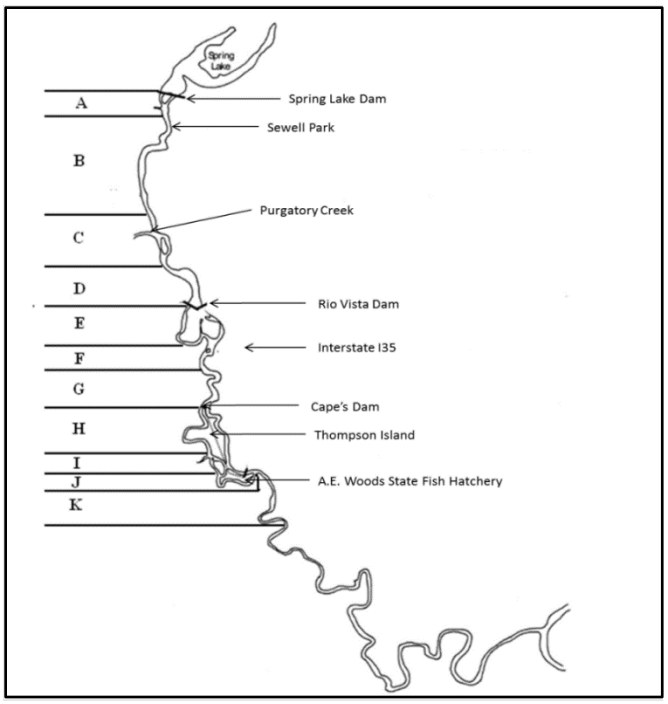

Figure 1. Letters define designated San Marcos River reaches where Texas wild rice is collected for refugia populations.

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 using nets and traps. Traps will be deployed quarterly for approximately 14 consecutive days with traps checked every 2-4 days to collect Texas blind salamander individuals from Primers Fissure, Johnson's well, Rattlesnake cave, and Rattlesnake well (Table 5). To avoid oversampling these habitats, 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.

As part of quarantine procedures, all Texas blind salamanders 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 or other facility to screen for *Batrachochytrium dendrobatidis* (Bd, commonly referred to as chytrid fungus) and *Batrachochytrium salamandrivorans* (Bsal) prior to specimen incorporation into the general refugia population. 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 regularly tested positive for Bd. Clinically, the salamanders appear normal and do not have any lesions or signs of disease. Positive testing for Bsal will be treated more cautiously as it has not yet been documented in this area (or anywhere in North America); these salamanders would remain in quarantine until further study and recommendations from FWS Fish Health.

Maintenance: Salamanders will be individually tagged to retain information on collection location, date, and other life history events. Water quality will be monitored and recorded weekly. Salamanders will be fed live foods reared or purchased, mixed with purchased frozen food sources. Ponds will be utilized to produce amphipods.

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

San Marcos salamanders:

Collection: San Marcos salamanders will be collected up to quarterly from below Spring Lake dam and with SCUBA teams in Spring Lake (Table 5). The drift net on Diversion Springs will be checked routinely and specimens will be kept from this location as space in quarantine and need allows. We will avoid collections close to the HCP Biological Monitoring Program assessment events. All specimens will be transported live and maintained in the SMARC and UNFH refugia.

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 or other facility to screen for *Batrachochytrium dendrobatidis* (Bd, commonly referred to as chytrid fungus) and *Batrachochytrium salamandrivorans* (Bsal) prior to specimen incorporation into the general refugia population. Duplicate 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.

Maintenance: Salamanders will be tagged to indicate year collected and gender. Water quality will be monitored and recorded weekly. Salamanders will be fed live foods reared or purchased, mixed with purchased frozen food sources. Ponds will be utilized to produce amphipods.

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 5) by hand with dipnets using snorkelers. Close coordination with the HCP biological monitoring program will take place to ensure that to the degree practicable, refugia collections do not overlap with specific 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.

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 or other facility to screen for *Batrachochytrium dendrobatidis* (Bd, commonly referred to as chytrid fungus) and *Batrachochytrium salamandrivorans* (Bsal) prior to specimen incorporation into the general refugia population. Duplicate 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.

Maintenance: Salamanders will be tagged to indicate year collected and gender. Water quality will be monitored and recorded weekly. Salamanders will be fed live foods reared or purchased, mixed with purchased frozen food sources. Ponds will be utilized to produce amphipods.

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 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 Run 1, Spring Run 3, Western Shore, and areas surrounding Spring Island (Table 5). Riffle beetles will be collected with cotton lures following EAHCP standard operating procedures (Hall 2016). New protocols established by the CSRB Work Group in 2019, include: 1) the same spring orifice will not be sampled two times in a row, 2) all riffle beetle adults and larvae will be collected from the lures, and 3) 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 be maintained by collection date. 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 5). Adult Peck's cave amphipods will be collected with drift nets and by hand collection at variety of locations (drift nets: Spring Run 3, N = 2; Spring Island and associated Spring Lake habitats: hand collection).

Maintenance: Specimens will be maintained by collection date. Peck's Cave amphipods will be maintained within custom-built aquatic holding units and fed commercial flake fish feeds.

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

Comal Springs dryopid beetle:

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

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

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

Edwards Aquifer diving beetle:

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

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

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

Texas troglobitic water slater:

Collection: Texas troglobitic water slater are primarily found in Artesian Well on Texas State Campus. Recent research by Will Coleman shows these are deep aquifer species that are rarely found at the surface. Mr. 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 5. A tentative schedule for all species sampling during 2021. Collections listed here are subject to change with extenuating circumstances such as weather and coordination with external partners. EAA and partners will be notified of sampling dates as they become known or changed.

Edward's Aquifer Species Collection Plan 2021			
Date (month)	**Interval**	**Location**	**Target Species**
January	14 Consecutive day with traps check 2-3 times a week	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
February	14 Consecutive day with traps check 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
February	1 day sampling event	San Marcos River	Texas wild-rice
March	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
March	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
March	1 day sampling event	Comal Springs	Comal Springs salamander
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

Edward's Aquifer Species Collection Plan 2021			
Date (month)	**Interval**	**Location**	**Target Species**
April	1-day sampling event	San Marcos River	Texas wild-rice
April	Throughout, coincide with bio-monitoring	San Marcos River	Fountain darters
April	Drift net, donated from bio-monitoring	Comal Springs	PCA
April	Set lures	Spring Runs, Landa Lake	CSRB, CSDB, PCA, TTWS
May	14 Consecutive day with traps check 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
May	1-day sampling event	San Marcos River	Texas wild-rice
May	Collect 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
July	14 Consecutive day with traps check 2-3 times a week	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander

Edward's Aquifer Species Collection Plan 2021			
Date (month)	**Interval**	**Location**	**Target Species**
July	Collect lures	Western Shore	CSRB, CSDB, PCA, TTWS
August	14 Consecutive day with traps check 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
August	1-2 day sampling event	Spring Lake and below dam	San Marcos salamander
September	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
September	1 day sampling event, hand pick	Landa Lake	Peck's Cave amphipod, SMARC
September	1 day sampling event	Comal Springs	Comal Springs salamander
October	14 Consecutive day with traps check 2-3 times a week	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
October	Throughout, coincide with bio-monitoring	San Marcos River	Fountain darters
October	Drift net, donated from bio-monitoring	Comal Springs	PCA
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 2021			
Date (month)	**Interval**	**Location**	**Target Species**
November	14 Consecutive day with traps check 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
November	1 day sampling event, hand pick	Landa Lake	PCA
November	1 day sampling event	Comal Springs	Comal Springs salamander
November	Collect 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	Set lures	Spring Runs, Landa Lake	CSRB, CSDB, PCA, TTWS

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 2021.

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:

Any maintenance to the program buildings beyond routine will be reported to the EAA as they occur.

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:

At the SMARC, we will employ two research biologists, one of which will serve as the Research Lead for Edwards Aquifer Refugia Program. A Biologist at UNFH will serve as the Husbandry and Collections Lead and will direct biological technicians at UNFH and the SMARC. The two program Leads mentoring, and training lower-graded employees, oversee facility maintenance and repair, develop and implement budgets, and organize activities that relate to all contract activities. The program leads will manage and coordinate research, propagation, culture, and field activities related to the refugia. The Leads are expected to provide proper and efficient use of facilities and staff resources. These leads will work with the Center Director and the Deputy Director to ensure that contractual obligations are met in a timely manner. In coordination with

the Center Director, they will prepare all the required written materials required for the reimbursable agreement reporting. Likewise, the Leads will also prepare oral presentations to be used as briefing statements, outreach presentations, internal reports, work summaries, and technical presentations at professional meetings. The two Leads will continue to work and communicate regularly with partners, Service personnel and other researchers to effectively meet Service and reimbursable agreement goals.

Under the direction of the Lead Biologist at UNFH, five Biological Science Technicians, two at SMARC and three at UNFH, will continue to assist with the collection, daily upkeep, maintenance, propagation, and research efforts for the ten species at the SMARC and UNFH. This includes maintaining experimental and culture production systems, keeping records along with entering, filing, and collating data. The technicians will also generate basic summary statistics and graphic analyses of data and document program accomplishments through the composition of Standard Operating Procedures (SOPs), reports, and manuscripts.

Permitting:

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

Biosecurity:

Both the UNFH and SMARC will practice biosecurity procedures in Refugia and Quarantine areas, and conduct appropriate biosecurity procedures on field equipment.

Task 2. Research

The Research Plan for 2021 will involve a series of activities ranging from increasing survival rates of various invertebrate species, salamander reproduction, and Texas wild-rice genetics. The following section describes the basic components of each of these proposed 2021 activities.

Project 1:

Title: Texas wild-rice genetic evaluation of both wild and refugia plants

Species: *Zizania texana*

Principal/Co-PI: FWS staff, sub-contractors (TBD) for genetic analysis

Overview: Staff with collect Texas wild-rice tissue samples from all Refugia Standing Stock and along the range of Texas wild-rice habitat within the San Marcos River. Samples will be analyzed for genetics to (1) characterize the genetic diversity of plants in the river, (2) see if the Refugia Standing Stock plants represent wild populations, (3) compare current wild genetic diversity to historical genetic diversity.

Budget: $71,940.26

Benefit to the Refugia: Inform collection strategies from river populations. Determine if refugia populations represent wild populations.

Expected Results: The results of the study will be presented as a report to the EAA and potentially a peer reviewed journal article. Development of a genetic management plan for Texas wild-rice or the building block for the genetic management plan.

Project 2:

Title: Continuation of San Marcos salamander reproduction

Species: *Eurycea nana*

Principal: FWS staff and/or sub-contractor(s) TBD

Overview: This study will assess the effects of habitat manipulation on reproductive success of San Marcos salamanders. We will also produce a status assessment report detailing all of the activities we have tried to improve captive reproduction for this species.

Budget: $29,764.24

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. We will also submit a status assessment report covering the last four years of work, attempting to improve captive propagation of this species.

Project 3:

Title: Increasing Comal Springs riffle beetle (*Heterelmis comalensis*) F1 adult production at the Refugia level

Species: *Heterelmis comalensis*

Principal: BIO-WEST with FWS staff

Overview: We will test different densities of CSRB larvae and a modified tank design, and adding wild cultivated biofilm to increase CSRB propagation success.

Budget: $124,402.51

Benefit to the Refugia: Increased survival rates of Comal Springs riffle beetles and increased F_1 production.

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

Project 4:

Title: Comal Springs dryopid beetle captive culture and propagation

Species: *Stygoparnus comalensis*

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

Overview: Investigate improved captive breeding practices for *Stygoparnus comalensis*, the Comal Spring dryopid beetle (CSDB), and gain a better understanding this species natural habitats.

Budget: $78,000.00

Benefit to the Refugia: Increase wild stock Comal Springs dryopid beetles in captivity and increase survival.

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 5:

Title: Altering the microbial environment based on microbiome analysis

Species: *Heterelmis comalensis*

Principal/Co-PI: Dr. Carlos-Shanely (Texas State University) and FWS staff

Overview: We will test exposure of *Staphylococcus* spp. found in SMARC CSRB on the survival of CSRB larvae. If larval supply and time allow, we will use the same design to test *Chromobacterium* spp. found in SMARC CSRB and well water. We will finalize the research into the identification of the specific microbiome from Comal Springs riffle beetle gut content analysis to compare wild microbiomes to those of captive microbiomes.

Budget: $54,692.69 for new research plus we request roll-over of the remaining $41,431.43 of the original $100,000 budget approved in 2020.

Benefit to the Refugia: A better understanding of factors influencing survival of CSRB in captivity.

Expected Results: We will submit an interim report to the EAA at the end of 2021. A final report of the study will be given in 2022 to allow for the long life cycle of the CSRB and accommodate the various sequential steps. A peer-reviewed journal article may be submitted of the study in 2022.

Project 6: (Not a new project – Will not use 2021 budget)

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: This research commenced in 2020 and will be completed in 2021. 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: We request roll-over of the remaining $31,521.08 of the $40,000 approved for this project in 2020.

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.

Task 3. Species Propagation and Husbandry

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

Task 4. Species Reintroduction

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

Reintroduction Plan for 2021: None

Any anticipated triggers being prepared for: Given current weather predictions, spring flows, and the Edwards Aquafer water level, none are anticipated during the 2021 performance period.

Task 5. Reporting

5.1 Species specific Propagation plans (SOPs): Refine throughout year as needed
5.2 Species specific Genetic Management plans: Texas wild-rice, contingent on when genetic study results are finished
5.3 Species specific Reintroduction plans: Refine as needed
5.4 2021 EAHCP Annual Program reporting– A year-end report of 2021 activities will be provided to the EAA no later than 1/31/2022.
5.5 Program reporting as required by ITP and TPWD. TPWD Scientific Research Permit Report will be filed July 31, 2021.
5.6 Descriptions and photographs of procedures from collections to restocking – Photographs and documentation of collection and restocking will be included in the monthly report to the EAA CSO along with the year-end report.
5.7 Summaries of any data analyses, research, or genetic analyses – Research projects and results of collection efforts will be provided to the EAA in the monthly reports, year-end documentation, and stand-alone documents (agreed upon by Center director and HCP CSO).
5.8 Description of terms and conditions of any permits received – As permits are received, their contents will be conveyed to the EAA.
5.9 Monthly electronic reports to HCP CSO: A monthly report of all activities will be provided

to the HCP CSO. We anticipate providing the report by the 10th of each month for the previous month's activities.

Task 6. Meetings and Presentations

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

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

Cost estimate:
See table to follow.

U.S. Fish and Wildlife Service 2021		Task Budget Amount	Total Task Budget Amount
TASK 1	**Refugia Operations**		**$ 608,214.74**
	SMARC Refugia & Quarantine Bldgs.		
	Equipment & Building Maintenance	$ 10,000.00	
	Utilities	$ 40,000.00	
	UNFH Refugia & Quarantine Bldgs.		
	Equipment & Building Maintenance	$ 30,000.00	
	Utilities	$ 40,000.00	
	Construction	-	
	SMARC Species Husbandry and Collection Salaries	$ 94,646.84	
	UNFH Species Husbandry and Collection Salaries	$ 178,004.83	
	Water Quality System Maintenance & Additions	$ 15,000.00	
	Divers	$ 2,885.00	
	Fish Health	$ 8,000.00	
	SMARC Reimbursibles	$ 40,000.00	
	UNFH Reimbursibles	$ 40,000.00	
	Subtotal	*$498,536.67*	
	Admin Cost Subtotal	*$109,678.07*	

TASK 2	**Research**		**$ 540,743.29**
	Dryopid Propagation	$ 78,000.00	
	Cooperative Agreement with BIO-WEST ($78,000)		
	Roll-over of $31,521.08 from 2020 budget	$ 31,521.08	
	CSRB Pupation	$ 124,402.51	
	Cooperative Agreement with BIO-WEST ($95,000)		
	USFWS Staff ($26,402.51)		
	Materials ($3,000.00)		
	USFWS Salamander Reproduction	$ 29,764.24	
	USFWS Staff ($28,764.24)		
	Materials ($1,000.00)		
	USFWS/TXT Microbes CSRB	$ 54,692.69	
	SMARC Staff ($28,682.69)		
	Cooperative Agreement with Texas State Univ. ($25,000)		
	Materials ($1,000.00)		
	Roll-over from 2020 budget $41,431.43	$ 41,431.43	
	TWR Genetics Study	$ 71,940.26	
	USFWS Staff ($33,968.60)		
	SNARRC Admin ($1,000.00		
	Materials ($15,000.00)		
	Research Oversight	$ 11,480.00	
	Subtotal	*$ 443,232.21*	
	Admin costs for Task 2	*$ 97,511.09*	
TASK 3	**Species Propagation and Husbandry**	-	-
	Subtotal	-	
TASK 4	**Species Reintroduction**	-	-
	Subtotal	-	
TASK 5	**Reporting**		**$ 59,814.64**
	SMARC Staff	$ 33,178.78	
	UNFH Staff	$ 15,849.61	
	Subtotal	*$ 49,028.39*	
	Admin costs for Task 5	*$ 10,786.25*	

TASK 6	**Meetings and Presentations**		**$ 13,333.85**
	SMARC Staff	$ 7,114.64	
	UNFH Staff	$ 3,814.74	
	Subtotal	*$ 10,929.38*	
	Admin costs for Task 6	*$ 2,404.46*	
	TOTAL	**$1,222,106.51**	

Projected (2021) Budget Summarized by Task:

Task 1: $ 608,214.74
Task 2: $ 451,741.23 (this does not include any funds not spent by contractors in 2020 that will also asked to rollover into 2021)
Task 3: $0
Task 4: $0
Task 5: $ 59,814.64
Task 6: $ 13,333.85

Projected (2021) Subcontractor Expenses Summarized by Task

Task 1: Southwest Regional Fish Health Unit, Dexter NM $8,000 (Health Diagnostics)
Task 2: BIO-WEST $173,000; Texas State University $25,000
Task 3: $0
Task 4: $0
Task 5: $0
Task 6: $0

Timeline of 2021 Milestones

(List major deliverables)

January	Continue with species collection
	Subcontract research awards executed
	2022 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.

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

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

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

Continuation of San Marcos Salamander (Eurycea nana) Reproduction: Refugia habitat and captive propagation

2021 Interim Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Desiree Moore and Dr. Katie Bockrath

San Marcos Aquatic Resources Center

U.S. Fish and Wildlife Service

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Background

Producing offspring reliably is important for the management of captive assurance populations. If a catastrophic event were to occur, the Refugia population must produce offspring to be able to effectively conserve and reintroduce that species to the wild. San Marcos salamanders (*Eurycea nana*) are a federally threatened aquatic species endemic to San Marcos, TX and to the Edwards Aquifer. Although a Refugia population of San Marcos salamanders has been held at the San Marcos Aquatic Resources Center (SMARC) for years, we cannot reliably produce enough offspring in the event that a future reintroduction becomes necessary. The Refugia program needs the ability to produce many offspring in a short amount of time.

Dr. Ruth Marcec-Greaves, an amphibian reproductive specialist with the Detroit Zoological Society, consulted with theRefugia program on San Marcos salamander reproduction. She theorized manipulating habitat within salamander tanks might stimulate reproduction. Therefore, this project was designed to examine two habitat characteristics hypothesized to relate to salamander reproduction, darkened tanks and a textured tank floor. Uvalde National Fish Hatchery (UNFH) staff designed the project, purchased materials, and partially set up the system atUNFH before Rachel Wirick moved to another job. We began this experiment in 2021.

Objectives

The objective for 2021 was to examine the effects of habitat modifications on San Marcos salamander reproduction.

Methods

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. The research system consisted of 44 5.5-gallon aquaria with perforated polyvinyl chloride (PVC) lids. Staff prepared aquaria by cutting holes and installing new bulkhead fittings, filtration, sterilization, delivery, and supply plumbing.

Each aquarium contained artificial habitat like the type used at the SMARC and UNFH (i.e., rocks and artificial plants). Half ofthe aquaria were covered on all sides to decrease light penetration (hereafter dark) and the other half were left uncovered (hereafter light). It is hypothesized that a dark environment may reduce stress and allow mating behaviors to go uninterrupted. Half of each of the dark and light aquaria had pond liner affixed to the bottom of the inside (hereafter rough) and the other half were without (hereafter smooth). Anecdotal evidence suggests salamanders may have difficulty conducting mating behaviors on smooth surfaces due to slipping. Therefore, there were 11 aquaria in each treatment group (i.e., dark/textured, dark/smooth, light/textured, and light/smooth). Once aquaria were prepared,they were filled and set to a flow-through system with partial recirculation.

One male and one female adult of similar size were randomly assigned to each aquariumto monitor reproduction for 90 days. Salamanders were starved 24hrs before and after moving to aquaria but were otherwise treated the same as refugia San Marcos salamanders that were not in the experiment (e.g., feeding, cleaning). Aquaria were checked for eggs and mortalities daily for 90 days. Eggs were transferred to a separate glass aquarium within 72 hours of oviposition. We recorded the number of eggs in each clutch and the date(s) the eggs were found and removed. Mortalities were replaced with a similar individual (i.e., same sex, similar size) upon discovery. After 90 days, salamanders were placed back in their refugia tanks, and the system was cleaned and prepared for the next trial. We completed two trials, began a third trial, and plan to complete the third trial in 2022 to properly cover the duration of the typical San Marcos salamander breeding season at the SMARC (September-February).

Results

The first 90-day trial began May 5, 2021. During the first trial, two male salamanders died from unknown causes. The first was observed in the light/textured treatment on day 41 of the trial. The second was observed in the dark/textured treatment on day 49 of the trial. Both salamanders were replaced the day they were observed as a mortality. No oviposition occurred in any salamander tank at the UNFH during the first trial.

The second 90-day trial began August 18, 2021. During the second trial, five female salamanders died or were replaced due to health concerns. The first was observed as lethargic and retaining water abnormally and was replaced in the light/smooth treatment on day 58 of the trial. The second was a mortality observed with burst capillaries in the head and tail in the light/textured treatment on day 70 of the trial. The third was observed with burst capillaries in the tail and bleeding near the cloaca and was replaced in the dark/smooth treatment on day 76 of the trial. The fourth was a mortality observed with burst capillaries with water retention in the light/smooth treatment on day 82 of the trial. The last lost her tail but had no other visible signs of distress. She was placed in a hospital tank for recovery and was replaced in the dark/smooth treatment on day 84 of the trial. No oviposition occurred in any salamander tank at the UNFH during the second trial. In order to keep all trials comparable across the entire breeding season and to not introduce additional variation that could confound potential results, no changes will be made to the third trial set up.

Life-History Aspects of *Stygoparnus comalensis* Barr and Spangler, 1992 (Coleoptera: Dryopidae): Comal Springs Dryopid Beetle Research 2021

PREPARED FOR:

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San Marcos Aquatic Resources Center

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Executive summary

The focus of this study was to build upon research of *Stygoparnus comalensis* that had been conducted in 2017-2019. Due to the longevity of this species and growth rate, it was important to build on the existing data collected and reported by BIO-WEST in 2019. The main objectives were to 1) continue monitoring adults, eggs, and larvae, 2) develop new aquaria for rearing all life stages, and 3) investigate natural habitats for reliable collections sites of this species.

A total of 288 eggs were recorded as being produced among 16 female subjects. The longest surviving female lived for ca. 452 days and produced 66 eggs during that time. There was a strong relationship between the number of eggs produced and the length of time females were bred, producing an egg ca. every 7-8 days. Extrapolating out to 630 days of captive breeding indicated the female reproductive potential of ca. 86 eggs.

A total of 10 pupae were produced. The shortest duration from oviposition to pupation was 323 days while the longest duration was recorded over 513 days (387.7 ± 62.5 days; n = 10). Four adults (two of each sex) were produced, but only two of these were observed as pupae before eclosion and were noted to pupate for 14 and 19 days, respectively. The four adults were observed to take 422.5 ± 6.0 days to reach adulthood from oviposition (n = 4). Unfortunately, the adults produced from this study did not reproduce.

A total of 52 larvae were produced, representing ca. 18% hatching rate. There was a considerable amount of variability in the measurements taken from the photos. Principal components analysis of dorsal-lengths measured showed that axis 1 (PC1) explained 96.8% of the variation. There were insufficient data to make practical instar estimations. Graphs based on PC1 suggested only four instars, while the graph of body length suggested 5 instars. Length of final instars were consistent with published data.

The new aquaria were referred to as BlackBoxes and were fashioned from 2.5 gal tanks. Each contained conditioned leaf and woody material as well as a sapling from the genus *Platanus*. The idea behind the BlackBox design was that females could oviposit anywhere in the aquarium and larvae would have a means of surviving. Only two eggs were produced at the time of this report, but the experiment is ongoing.

Four surveys to find reliable collecting locations were generally unsuccessful. Inspection of natural habitat from a known reliable collecting location revealed two late-stage larvae burrowed in a small scrape of submerged and degraded wood. There is a considerable amount of evidence to indicate that females do not make any special migration to oviposit. Eggs clearly developed even though completely submerged. This is not the first species of dryopid to have a submerged larval habitat and it is likely that other species reside in such habitats but are difficult to study and therefore have gone unnoticed.

Introduction

The Comal Springs dryopid beetle (*Stygoparnus comalensis*) is an endangered species (USFWS 1997) known from subterranean habitats of the Edwards Aquifer (EA). *Stygoparnus comalensis* is known from a few locations in Comal and Hays counties, Texas, where 39.4 and 139 ha of surficial and subsurface critical habitat, respectively, have been designated for it (USFWS 2013). Threats to *S. comalensis* and other species of the EA include pollution, competition from exotic species, and over pumping of water

(Bowles and Arsuffi 1993). A goal of the Edwards Aquifer Habitat Conservation Plan (EAHCP) is to have functional refuges that contain self-propagating captive populations. A better understanding of the basic life-histories information, especially with regard to habitat and growth of various life stages, is fundamental towards meeting that goal.

The adult phase of *S. comalensis* displays a thin cuticle and vestigial eyes and wings (Barr and Spangler 1992). The larval phase of this species is distinguished by having vestigial eyes and the spiracle of abdominal segment 8 located on the upper third of the segment, unlike the more lateral location of spiracles on other segments. Conservation of this species is important; however, studies are difficult due to its rarity and the fact that there are no surrogate species for comparison. Wild-caught adults have been maintained in captivity for 11-21 months (Barr and Spangler 1992, Fries et al. 2004); however, it is unknown how long the adults live. The first pupation events of this species were recorded in 2019 after 11-15 months (BIO-WEST 2019).

The larvae of many dryopid species are considered terrestrial, occurring in soils and damp decaying wood along stream banks and shallow floodplain depressions (Brown 1987, Ulrich 1986). Barr and Spangler (1992) projected that larvae may live in air pockets at the ceilings of subterranean spaces, since they were sampled from near-surface habitats. Larvae float and appear to have a hydrophobic integument, suggesting that they may reside in terrestrial or semiaquatic habitats.

The focus of this study was to build upon research conducted from 2017 to 2019. Due to the longevity of this species and growth rate, it was important to build on the existing data collected and reported by BIO-WEST (2019). A brief overview of the research report for cooperative agreement F18AC00065 is given in the background section below.

Background

Life-histories study 2018 and 2019

The first comprehensive study of life-histories aspects of *S. comalensis* was reported in January 2019 (BIO-WEST 2019) as part of cooperative agreement F18AC00065 with the USFWS. A brief description of the findings of that project are given in this background section. Please refer to that report for more details.

During the process of collecting study subjects, it was noted that hand collecting adults from coarse woody materials was an effective means of obtaining specimens. It was also noted from field collections and discussion with Randy Gibson (US Fish and Wildlife Service, San Marcos Aquatic Resources Center) at the time that collections of this species tended to be associated with tree roots. In particular, though not exclusive, roots of *Platanus* appeared to be more often associated with reliable collecting sites.

A behavioral study with regard to varying levels of flow was implemented for *S. comalensis* as well as *Heterelmis comalensis*. In general, *Stygoparnus* moved against the flow towards a food resource, regardless to the intensity of flow. However, individuals tended to stay in a food resource if placed in them at the beginning of the trials.

A reliable method for separating the sexes, based on internal features that could be seen through the translucent cuticle, was developed during this study (Kosnicki 2019) (**Fig. 1**). Attempts were made to determine if females oviposited above or below the water line; however, it remained indeterminant due

to the fact that eggs were not cemented loosely oviposited and were naturally subject to sinking. Containers used to examine oviposition sites inevitably had to be disturbed, resulting in eggs becoming submerged (if they were emergent) and essentially untraceable with regard to their original location.

Fig 1. A female and male representative of *Stygoparnus comalensis* (modified from BIO-WEST 2019).

A breeding program was developed to track the fecundity of females. Eggs were recovered from breeding chambers and transferred to rearing chambers; this was a transfer from a submerged aquatic habitat to a terrestrial habitat since larvae required air to respire (**Fig. 2A, 2B**). Tracking of larvae included identifying that they had a tendency to burrow in conditioned wood dowels that were provided (**Fig. 2C**). Photographs were taken of larvae at various times in an attempt to document growth, identify the number of instars that they would go through, and determine the length of time and conditions leading to pupation and eclosion to adult. The first pupation events were recorded in July 2019, with the first F1 reared adults recorded soon thereafter. By the end of 2019 six pupae had developed and an estimate of 12 larvae and ca. 20 unhatched eggs remained. The last mating pair of adults had died in December 2019.

An experiment was initiated to test if larvae could hatch and survive from submerged eggs. Eggs were placed at the bottom of a mating chamber with leaves placed at the water surface interface. The idea was that larvae hatching from submerged eggs could float to the surface and grab onto leaf material. Three of 14 eggs hatched and had viable larvae attached to leaf material at the water surface.

New insights regarding the life history of *S. comalensis* were revealed during the course of the 2018-2019 research; however, many questions remained. More time was needed to study the life cycle. Information regarding the optimum larval habitat, number of instars, length of time to pupation, pupation requirements, adult longevity, and fecundity were still being recorded and observed. Furthermore, it was of interest to gain a better understanding of this species in its natural habitat so that better husbandry practices could be implemented in the laboratory.

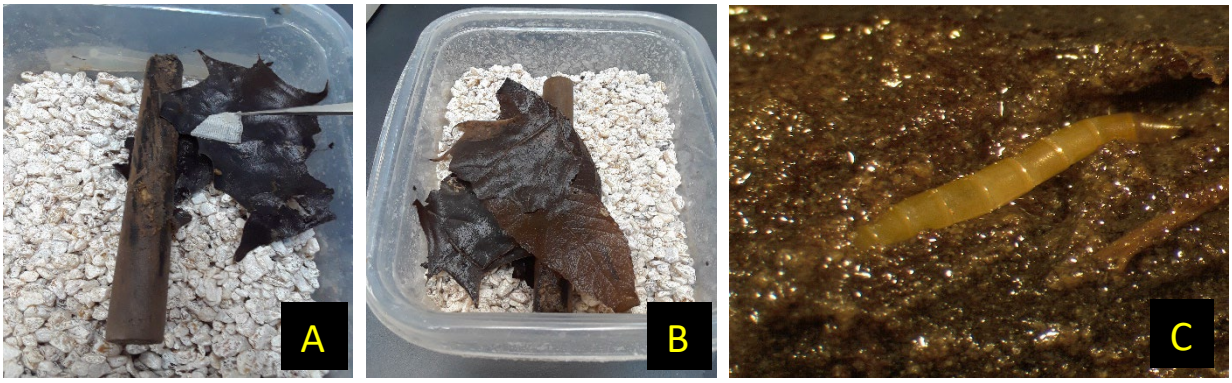

Fig. 2. Rearing chambers and *Stygoparnus comalensis* larvae. Panel A shows an egg being transferred on a strip of cotton (BIO-WEST 2019). Panel B shows how the eggs were incubated after the transfer (BIO-WEST 2019). Panel C shows a larva burrowing into the wooden dowel (2020).

Purpose and objectives

The purpose of this study was to continue tracking the growth and development of first generation (F1) larvae and other life stages already reared during cooperative agreement F18AC00065. It was also of interest to explore the natural habitat of this species in hopes to identify alternative locations where study subjects could be collected and habitat associations could be identified. Furthermore, the development of new aquaria that could house all life stages for more convenience of refuge production was also a goal of this study. Specific objectives are given below.

Continued monitoring of adults, eggs, and larvae

The last mating pair of adults died in December 2019; however, the complete results of the fecundity study were not available for the final report. Viable eggs and growing larvae were still being maintained at the SMARC into 2020. A main objective of this study was to continue tracking larvae for growth and instar estimation. And to continue to rear pupae and adults to get an indication of how long it takes captively reared individuals to reach these stages. Furthermore, it was an objective to produce F2 offspring.

New aquaria

The study of life-histories aspects of *S. comalensis* in captivity has been labor intensive due to the searching and transfer of eggs from one container to another which is not practical for long-term refugia operations. Because the current refugia aquaria have not been designed for production, it is possible that larvae are produced but are not able to reside in the aquaria with given habitat configurations. A new aquaria design to allow for the complete life cycle is of interest for establishing a self-propagating long-term refuge.

One of the strong observations regarding the habitat of *S. comalensis* is that at the time this study was initiated, reliable collecting sites were associated with *Platanus* root systems. Furthermore, it was noted that pupation and eclosion events in captivity took place within woody material. However, few pupae successfully eclosed to adult and adults did not appear healthy. One thought was that larvae were not provided with essential nutrients for completing their life cycle to healthy productive adults. The association to plant roots may have been an indication to the types of nutrients needed for proper *S.*

comalensis health. Therefore, new designs were sought to be constructed with a living plant in addition to providing habitat for all life stages.

Investigation of natural habitat and collecting locations

Stygoparnus comalensis has historically been collected in reliably from Comal Springs at Spring Run 2 (Barr and Spangler 1992) but intermittently at other locations (see Gibson et al. 2008). During cooperative agreement F18AC00065, only the Spring Island area of the Comal Springs was a reliable location to find subjects for experimentation and captive study. Considering that other entities would be utilizing these locations for refuge collection or competing studies, new locations to collect test subjects was strongly desirable. Additionally, it was of interest to investigate the distribution of this species within the Comal Springs since it has been elusive in the past. It is also of interest to investigate habitat characteristics that are different and common to existing collection locations. Furthermore, observing larvae in their natural habitat would be of great value in developing an appropriate holding system for refuge purposes. Number of eggs were counted per female and regressed over the time the female was alive in captivity.

Methods

Continued monitoring of adults, eggs, and larvae

Egg production

At the time the 2019 report was produced, there were still six females and seven males being utilized for egg production. Egg production was monitored ca. every month until the last mating couple was found dead. Due to space issues, some females had to be held in small groups of no more than three with a similar number of males. Because of this, overall egg production had to be split between the total number of females; however, production per female was estimated from females that were kept in a breeding chamber without other females present. In cases where there was more than one female in a breeding chamber, but one of the females died early and before more than 5 eggs were produced, the egg total was allocated to the surviving female and was tracked from there. The longevity of an individual was recorded up to the day it was found dead. See BIO-WEST(2019) for more details.

Pupation and eclosion to adult

Observations with regard to pupation and adult eclosion continued with larvae from the 2019 report (BIO-WEST 2019). The number of days to reach pupa and adult were estimated from the day oviposition was recorded for the egg clutch the individual originated.

Larval growth

Length measures that were initiated in 2018 were continued through 2019 and 2020 on laboratory-reared larvae. Hatched larvae were mapped for their position within a rearing chamber after they were first detected and were coded with this position in conjunction with the egg clutch from which they originated (see BIO-WEST 2019). Because larvae are rare and fragile, larvae were handled as little as possible and were photographed unrestrained. Over time multiple larvae within the same chamber were not able to be separated and thus were coded as each of the possible larvae within the chamber. Measurements were conducted with an Olympus cellSens Standard® or Digimizer from each photo and

a list of those measures are given in **Appendix A**. Photos from individuals were only used for consecutive photo dates where it was apparent that the larva grew; if the growth was not appreciable between one photo date to the next, earlier photo measurements were used to avoid pseudoreplication of using measurements of the same larva in the same instar more than once.

Number of days from the date eggs were transferred to rearing chambers to the photo date of the larval measurement were used to estimate the age of each larva and in turn the approximate age of the estimated instar. Where there was ambiguity of which larvae was measured, the average of the possible oviposition dates was used to estimate the larval age. In this way the age was days from estimated time of oviposition.

Because photos were not taken at each check, the number of days between checks was also recorded to keep track of the number of days from egg clutch detection to larval-hatch detection, pupation detection, and adult detection. From these data number of days to hatching and number of days of pupation and eclosion to adult were estimated.

Instars were estimated by finding inflection points based on the second derivative (*f''(x)*) of a smooth spline of the ranked natural logarithm of body lengths measured from the anterior of the pronotum to the posterior of the 9th tergite (PTBL). Principal component analysis was also used to find a linear component of the length measures of the pronotum, mesonotum, metanotum, abdominal tergite 1, and tergite 9 of each larva. The *f''(x)* of a smooth spline of principal component 1 (PC1) was also used to find inflection points as representative estimates of separate instars. The *f''(x)* represents the change in rate of a change in rate where inflections are indicated at zero; theoretically, abrupt changes from one "size class" to another should reflect a strong change in the *f''(x)* from positive to negative or negative to positive and therefore this could be interpreted as a demarcation between instars. Previous studies have used the inflection as the curve descends from a positive value to a negative value where the positive value is descending from a value greater than the average of all positive *f''(x)* values (BIO-WEST 2019). The combination of both these methods were compared to make decisions on instar number and the number of days from egg detection was used to estimate the length of time it took for a larva to reach that instar. Analyses were performed in R with the *features* package (R Development Core Team, 2017).

New aquaria

The construction and implementation of new aquaria for rearing all life stages were delayed due to the Covid-19 pandemic as access to the SMARC facility was limited until vaccinations were available. In addition, BIO-WEST had to move its entire flow-through operations on two occasions during the course of this study. Prototype aquaria were designed and built at the BIO-WEST office at San Marcos, Texas, and were tested for basic flow conditions with municipal water. However, actual aquaria housing test subjects were not implemented at the SMARC until 21 July 2021.

The aquaria, referred to as BlackBoxes (**Fig. 3**), consisted of a 2.5 gal tank with a 3/4 in intake-hole drilled at the bottom rear of the tank, fitted with couplings to seal the hole. A threaded 1/8 in barb was attached to the bottom of the couplings so an intake hose could be attached to supply the tank with water while emulating an upwelling. A 500 µm plastic mesh was secured between the couplings. A 1 3/4 in drain was created by drilling a hole in the front face of the tank and fitting it with a bulkhead. The inside surface of the bulkhead had a 500 µm plastic mesh glued to it with hot glue. High-density Matala biofilter was cut to fit the perimeter of the inside portion of the tank at the outflow drain. The

lengthwise center of the biofilter was drilled to provide a space for conditioned habitat and food resources (**Fig 3**).

Habitat and food resources included pebble sized limestone rocks, *Platanus* leaves conditioned in the laboratory for 4-6 weeks, *Platanus* twigs cut to ca. 2.5 cm diameter and 25 cm length conditioned in the laboratory for > 1 year, *Platanus* bark conditioned in the laboratory for > 1 year, high-density biofilter (as described above), and 1-2 *Platanus* saplings. Rocks, leaves, twigs, and bark were placed on the bottom of the tank around the inflow. The saplings were fit through the biofilter and situated so that the roots would be directly above the inflow coupling while the leafy portion of the sapling would emerge through the top of the biofilter. A twig was also placed within the center of the biofilter, lengthwise. The biofilter was fit into the tank at the level of the outlet drain so that it would be partially emergent and submerged at the same time (**Fig. 3A**). A second and third version of this aquarium were created with the addition of a 1/2 in standpipe in the corner by the main outflow (**Fig. 3B**). The standpipe was slightly higher than the main outflow drain and served as an emergency drain in case the main drain was clogged.

Saplings of the genus *Platanus* were retrieved from the banks of the Blanco River and Comal Springs, in Hays and Comal counties, respectively, during the spring of 2021. Saplings were kept outside in a bucket with water from the Blanco River and observed for conditions influencing growth. Fertilizer was applied to a subset of the saplings and another subset was planted in potting soil. Leaves were trimmed to maintain the height of the saplings and insects were removed from time to time. Live saplings were added to the new tank constructions before launching with adult subjects. The entire tank was wrapped in black plastic except for a small opening that allowed the sapling to emerge (**Fig. 3C**). Because of the black plastic, these aquaria were referred to as BlackBox 1 (without the standpipe drain) and BlackBox 2 (with the standpipe drain).

Adult test subjects were obtained from locations where the species was known to be reliably collected at Comal Springs. Specific locations are not given here because the rarity of this species necessitates its protection. On 21 July 2021, five females and two males were collected and launched within BlackBox 1. On 18 August 2021, five females and one male were collected and placed within BlackBox 2. On 17 September 2021, one female and one male were collected and placed within BlackBox 2.

The idea was that adults would have their pick of available habitats. If females did indeed prefer to migrate to a terrestrial resource, that was provided. Submerged woody material was provided for females if that was a resource they might oviposit within. If eggs hatched underwater outside of woody material, presumably, they would float to the surface where they would be able to grab onto the biofilter and hopefully make their way to the woody material provided. The *Platanus* sapling was provided as a potential food resource for both adults and larvae. Tanks were inspected after several months to determine where eggs were oviposited and where larvae may end up.

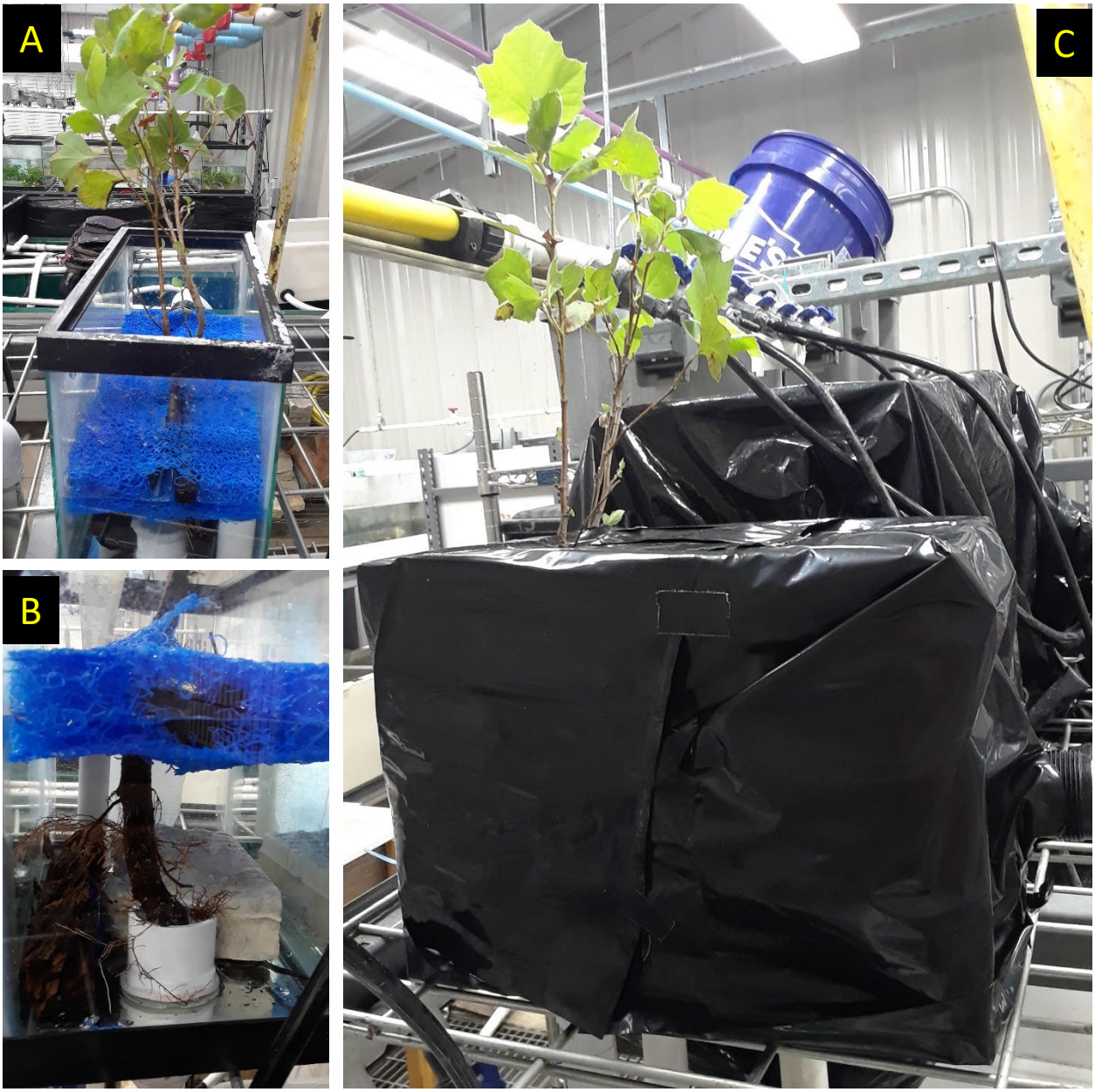

Fig. 3. BlackBox aquaria setup for *Stygoparnus comalensis* life stages. Panel A shows the position of the top back portion of BlackBox 1, showing the sapling's position within the biofilter, the location of the twig positioned in the center of the biofilter with the roots placed above the inflow, and the outflow drain at the far side. Panel B shows a closer view of the sapling position within the inflow coupling from BlackBox 2; the waterline is apparent, and the standpipe can be viewed in the back of the aquarium. Panel C shows a lateral view of BlackBox 1 fully covered by black plastic and fully operational.

Investigation of natural habitat and collecting locations

Surveys were conducted at multiple locations within the Comal Springs as an attempt to find more reliable collection sites for *S. comalensis*. Habitats that were targeted had to have visible spring flow and an association with tree roots. The first surveys were conducted by placing leaf and polycotton lures in springs, while later surveys were conducted by placing pieces of wood within the springs. Woody

materials were also inspected during times lures were set and retrieved. Set and retrieval times would often span several days, especially during retrievals; if lures or woody materials were still in good condition, they were visited repeatedly over a span of days or weeks.

Survey 1 lures were set in late June 2020, and retrieved in mid-August 2020, within 12 springs at Spring Runs 1, 2, 3, the western shoreline, and Spring Island areas; woody materials were also inspected during launch and retrieval. Survey 2 lures were set in late September 2020 and retrieved in late October 2020 within 10 springs at the western shoreline and Spring Runs 1 and 2. Survey 3 lures and woody material were set in late January 2021 within 15 springs at Spring Runs 1, 2, and 3; retrievals and wood inspections were made in mid-March through April 2021. Survey 4 was initiated in mid-August 2021 within 15 springs at the Spring Island area and Spring Run 3 and was monitored through mid-October. Woody materials were also dissected in the field on some occasions to better determine if larvae were burrowing within submerged woody material.

Results and discussion

Continued monitoring of adults, eggs, and larvae

Egg production

Egg production was monitored through December 2019, at which point a total of 288 eggs were recorded as being produced among 16 female subjects, though not all subjects produced eggs. By April only two females and two males were still alive and producing eggs. The last couple was found dead on 18 December 2019. This same couple was initiated in a breeding chamber on 4 October 2018, producing a total of 55 eggs during that time. However, the longest surviving female lived for ca. 452 days and produced 66 eggs during that time. There was a strong relationship between the number of eggs produced and the length of time females were bred (**Fig. 4**), producing an egg ca. every 7-8 days; however, during the most productive times, individual females were calculated to oviposit at a rate of one within three days (3.4 ± 0.8 days, n = 11). The maximum time an adult has been held in captivity was 21 months (Barr and Spangler 1992). It is unknown if production decreases longevity as energy is likely utilized; however, to extrapolate out to 630 days of captive breeding at these rates would indicate the female reproductive potential of ca. 86 eggs. It was noted from time to time that eggs retrieved from the breeding chambers contained visibly viable and developed larvae. Since these eggs were submerged and could have been submerged for 30 days or more, this was considered evidence that some larvae may hatch in a submerged condition.

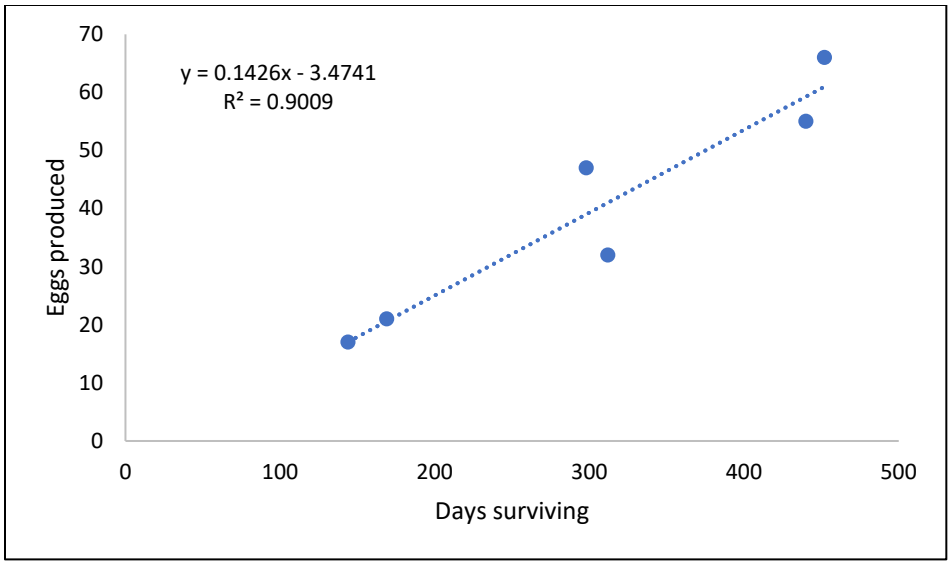

Fig. 4. Eggs produced per female over time breeding in captivity.

Pupation and eclosion to adult

A total of 10 pupae were produced from the original cooperative agreement F18AC00065 study. The shortest duration from oviposition to pupation was 323 days, while the longest duration was recorded over 513 days (387.7 ± 62.5 days; *n* = 10). Four adults (two of each sex) were produced, but only two of these were observed as pupae before eclosion and were noted to pupate for 14 and 19 days, respectively. The four adults were observed to take 422.5 ± 6.0 days to reach adulthood from oviposition (*n* = 4). One of the adults died pharate (immediately after eclosion). None of the females successfully produced eggs, and none of the adults survived for more than 3 months. On 21 August 2019, a larva > 407 days old was transferred to a rearing chamber with 3 pupae to try to invoke pupation, possibly as a response to the hormonal changes expressed by the existing pupae. This larva had been tracked in a late stage of development each month for about 4 months up to that point. Twenty-one days after the transfer, this individual was found transformed to a pupa.

Unfortunately, the adults produced from this study did not reproduce. It is surmised that they lacked essential nutrients or amino acids. As described above, this was partially the reasoning behind utilizing living *Platanus* as part of a food resource.

There is uncertainty or perhaps unreliable estimates of how long eggs may incubate before hatching. The longest record between observing a freshly oviposited egg and a newly hatched larva from the same clutch was 171 days. It is also uncertain whether larvae hatching after a longer incubation period are more developed, hardy, or faster growing compared to larvae that hatch within 60 days or less from oviposition. Therefore, the results given here should be used with caution; however, these data represent the most informative baseline understanding of time of growth in a captive setting.

Larval growth

A total of 52 larvae were produced from 288 eggs (produced during 2018-2019) that were transferred to rearing chambers, representing ca. 18% hatch rate. Most of the larvae were photographed when they were initially observed. With this there was a disproportionately higher number of early instars

measured than later instars being measured. Because of their burrowing behavior, it was also difficult to find later-instar larvae for photographing.

There was a considerable amount of variability in the measurements taken from the photos. Test subjects were live unrestrained specimens, freely moving and not oriented consistently in the same configuration for each photo. Body segments may have been overlapped or completely extended and some photos may have been taken at an angle rather than from directly overhead, and thus partially out of focus. Overall body length was difficult to take because of these reasons and even more because the head was nearly always retracted to some extent. In general, most of the measures were probably inconsistent and so the results are presented here with caution.

Principal components analysis of dorsal length measures showed that axis 1 (PC1) explained 96.8% of the variation and the loadings for each measure were relatively equivalent across this axis (**Table 1**). Ordination of dorsal-lengths PC1 space indicated a progression of larger larvae from left to right (**Fig. 5**).

Measure	PC1	PC2
PrNL	0.448818	-0.01351
MsNL	0.447221	-0.46629
MtNL	0.449230	-0.30939
Ab1L	0.449254	-0.02686
Ab9L	0.441497	0.82822

Table 1. Loadings for PCA axis 1 and 2 for dorsal lengths. PrNL = pronotal length; MsNL = mesonotal length; MtNL = metanotal length; Ab1L = abdominal segment 1 length, and Ab9L = abdominal segment 9 length. PC1 and PC2 explain 96.8% and 1.6% of the variation, respectively.

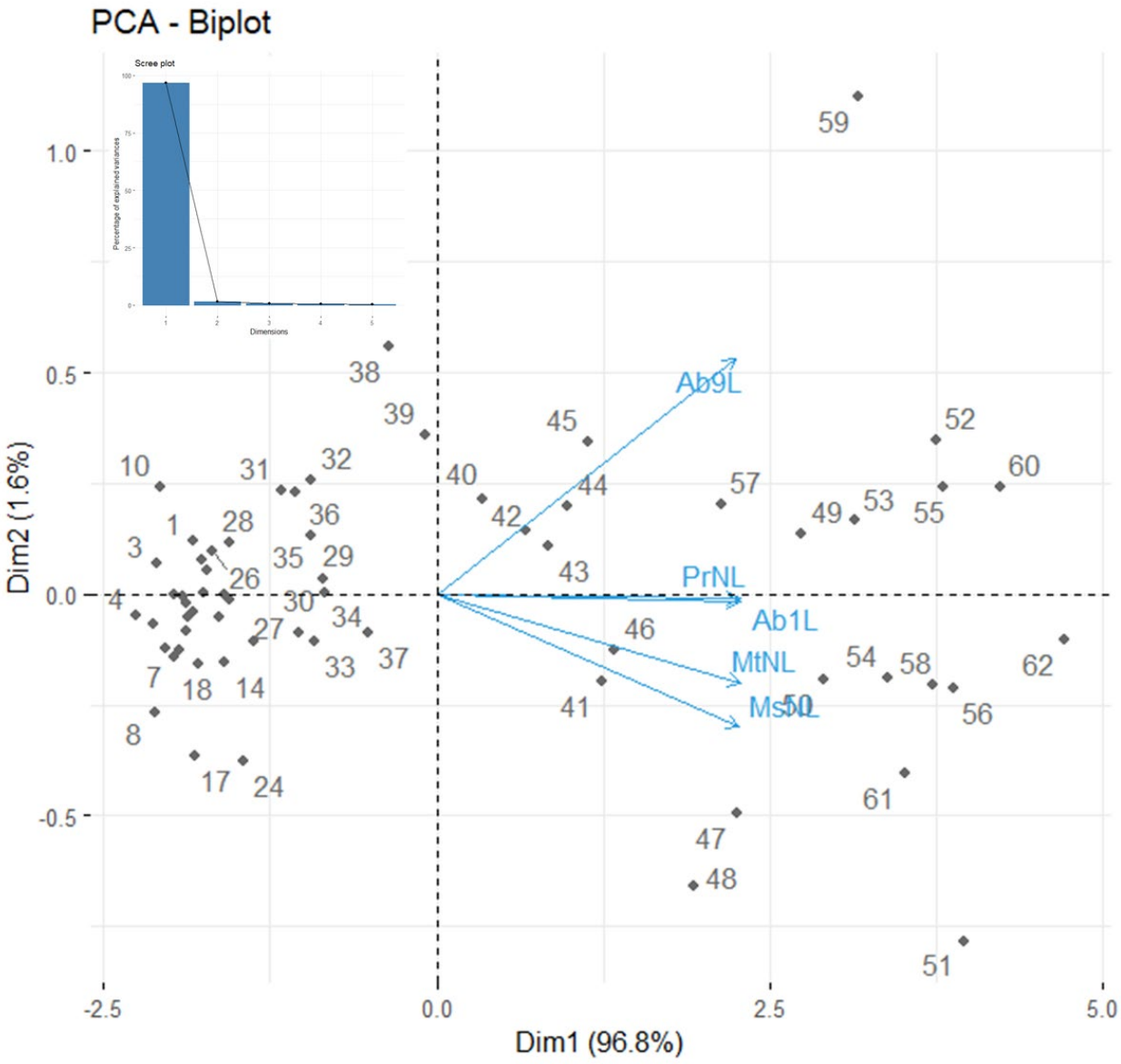

Fig. **5**. Scree plot and ordination of individual measures (see **Table 1** for descriptions) cover PC axis 1 (Dim1) and 2 (Dim2). Arrows indicate the loadings for each measure.

There were insufficient data to make practical instar estimations (**Fig. 6** and **7**). There were some inflections that appeared to denote instars from both graphs. Graphs based on PC1 of dorsal length measures appeared less useful compared to the PTBL measures with regard to identifying instars. Using the major inflections of the *f''(x)*, the PTBL graph suggests five instars with an estimate of 85.5, 94.4, 122.1, 153.6, and 328.5 days from hatching to reach each instar, respectively. The PC1 graph suggests only four with an estimate of 87.6, 90.5, 152.3, and 318.7 days from hatching to reach each instar, respectively. On the other hand, considering some of the weaker inflections, both graphs could indicate seven instars, though at different points. Both graphs appear to indicate strong inflections separating the first three or four instars with roughly the same individual measures. There is also a hint from both graphs that there may be a weak inflection, representing a demarcation between the first and second instar within the first 5-11 measures. Such a demarcation would indicate that the first molt happens

rather soon, say within 3 weeks of hatching and it is possible that the first instar was only photographed on a few occasions. Clearly more data are needed to make more discernable conclusions with regard to the number of instars and the length of time to reach each instar.

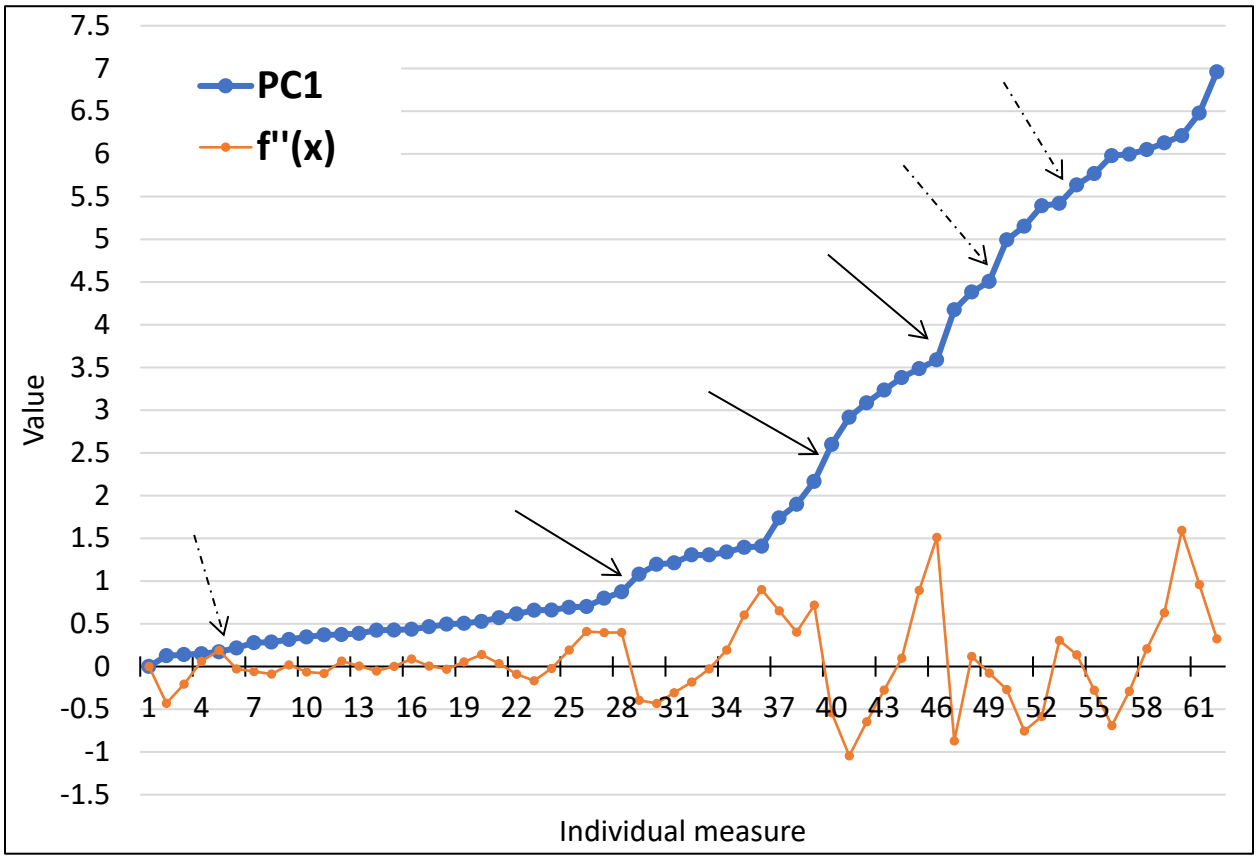

Fig. 6. Graph of the second derivative (*f''(x)*) of principal component 1 (PC1) of dorsal-length measures (see **Table 1** for description of measures). The x-axis represents PC1 ranks where more progressive development is indicated by higher ranks. Inflection points from *f''(x)* indicate potential separations of instars. The *f''(x)* was multiplied by 10 and 2.51 was added to PC1 to help visualize and fit the graph. Solid arrows indicate potential instar demarcations represented by stronger inflections. Dotted arrows indicate potential instar demarcations represented by weaker inflections.

It is also likely that the measurements taken from the photos were too variable or rather inconsistent with regard to the positioning of each larva. For instance, the surmised demarcation of the first and second instar noted above may be the result of inconsistent photography and measure. Therefore, more precision may be required for each photo in terms of positioning of larvae and the angle from which the photo is taken in addition to acquiring more data.

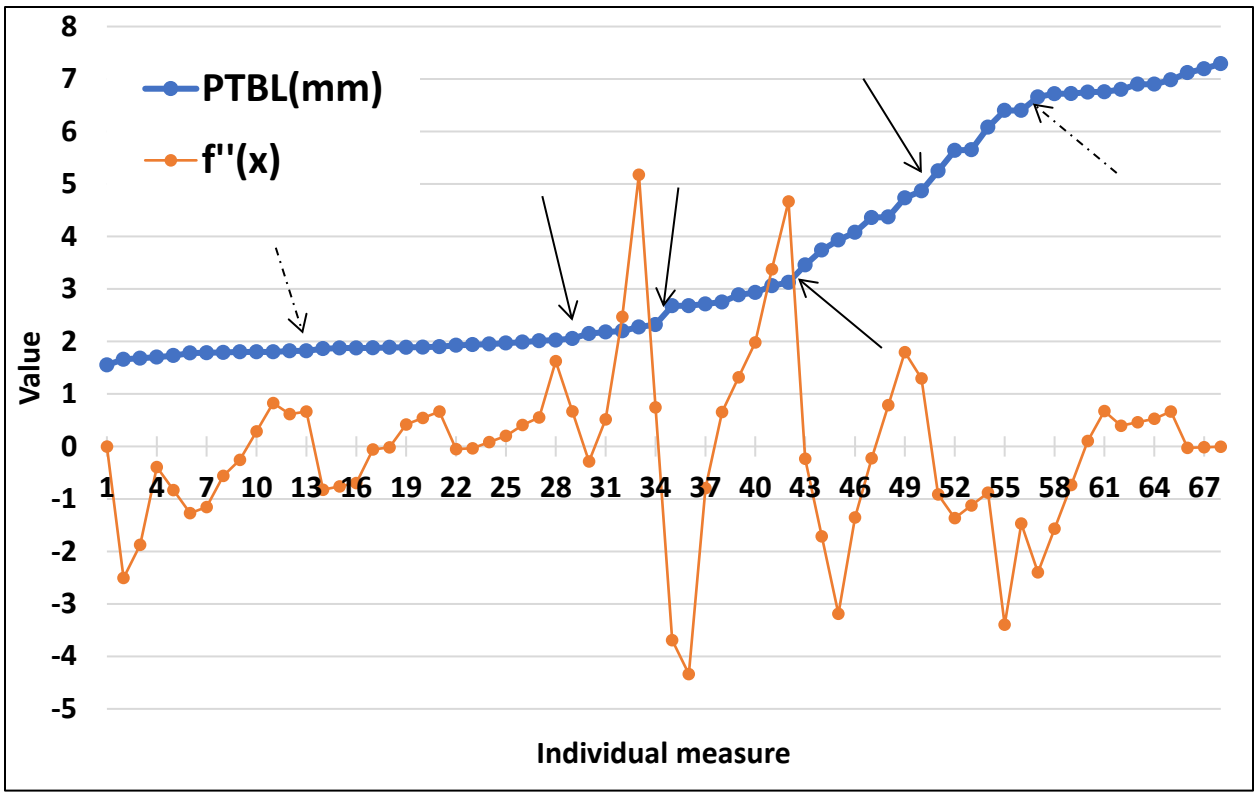

Fig. 7. Graph of the second derivative (*f''(x)*) of the natural logarithm of the body length measured in millimeters from the anterior of the pronotum to the distal end of the 9th abdominal tergite (PTBL). The x-axis represents rank of individual body lengths where more progressive development is indicated by higher ranks. Inflection points from *f''(x)* indicate potential separations of instars. The *f''(x)* was multiplied by 100 and the PTBL is represented by the actual measures (rather than the natural logarithm) to help visualize and fit the graph. Solid arrows indicate potential instar demarcations represented by stronger inflections. Dotted arrows indicate potential instar demarcations represented by weaker inflections.

The description of *S. comalensis* indicated that late instar larvae (by association) were 6-8 mm in length and 0.8-1.0 mm in width (Barr and Spangler 1992). The longest length from reared larvae during this project was 7.68 mm (including a partially retracted head) and the largest width was 0.78 mm measured across the mesonotum of a separate subject (**Appendix A**). Thus, it appears that the reared larvae were reaching a length expected; however, it is noted that the original description included few individuals for the diagnosis.

The estimated length of time to reach each instar is incomplete due to a lack in the number of tracked specimens and the number of checks of the same specimens. Tracking of individuals was difficult due to several factors; first, it was unknown that larvae would burrow into the woody material and therefore initial efforts to locate them were unsuccessful. Also, the fact that larvae burrow into wood makes it difficult to find them without destroying their habitat. Because it was more of an interest to produce F1 adults, laboratory habitats were not disturbed as often as would be the case if more extensive measures of larvae were to be taken. Second, photographs of unrestrained living larvae do not provide the most precise way to measure subjects (see comments above). Third, mixing separate egg clutches within the same container cast uncertainty on the age of the individual being measured.

Because this is a rare to find and difficult species to rear in captivity, efforts to minimize handling are still recommended. Ideally, larvae should be tracked separately; however, it may not be feasible to maintain hundreds of small flow-through containers. Improvements of future investigations to better understand life-histories aspects of this species are recommended to consider the following:

1. *Keep all eggs from the same "clutch" separate so that all larvae from that clutch will be roughly the same age*. Tracking hundreds of larvae independently is possible with enough space and planning. However, it is likely that eggs will be recovered in groups or "clutches" representing a period of time that they were oviposited by a female. Transferring and tracking individual eggs does not seem like an efficient use of time and space since eggs are subject to > 50% mortality. Therefore, eggs from the same "clutch" should be kept together with larvae either residing in the same container or being transferred soon after hatching.
2. *Develop a more repeatable photographing technique that can be used as a standard for all photos*. It is evident that there was an inconsistency in larval measurements due to the fact that larvae were photographed in different positions. The use of a cover slip to secure the larvae on a flat surface was used a few times, but this tended to stretch the animal disproportionately. However, consistency among measures may be better than accuracy. The use of a specific stationary object designed for holding larvae during photographs may also be considered.
3. *Consider tracking larvae within leaf packs alone, without providing woody resources for them to burrow*. It was nearly impossible to track and photograph larvae without destroying their burrow habitats. It is possible that the larvae may survive well enough sandwiched between layers of conditioned leaves and may even be able to form pupal chambers within such a habitat. Retrieving larvae to monitor growth would require separating the leaves by peeling off layers; however, these could be reestablished in a similar manner after the larvae is photographed.

New aquaria

The idea behind the BlackBox design was that females oviposit wherever they may be; however, when they are within interstitial spaces within woody material, these are good habitats for larvae to hatch from eggs and reside successfully to pupation and adulthood and it is noted that other dryopid species may perform a similar production habit (Novakovic et al., 2014).

Care of the *Platanus* saplings indicated that fertilizer, even added in small quantities to standing water, caused them to wilt and in most cases die. After the first BlackBox was launched at the SMARC, it was apparent that the well water and conditioned woody material provided for beetles in the tanks was sufficient to promote sapling growth, even in continuous artificial light (**Fig. 8B**). New root growth was evidentially promoted by shielding the roots in darkness (**Fig. 8A**).

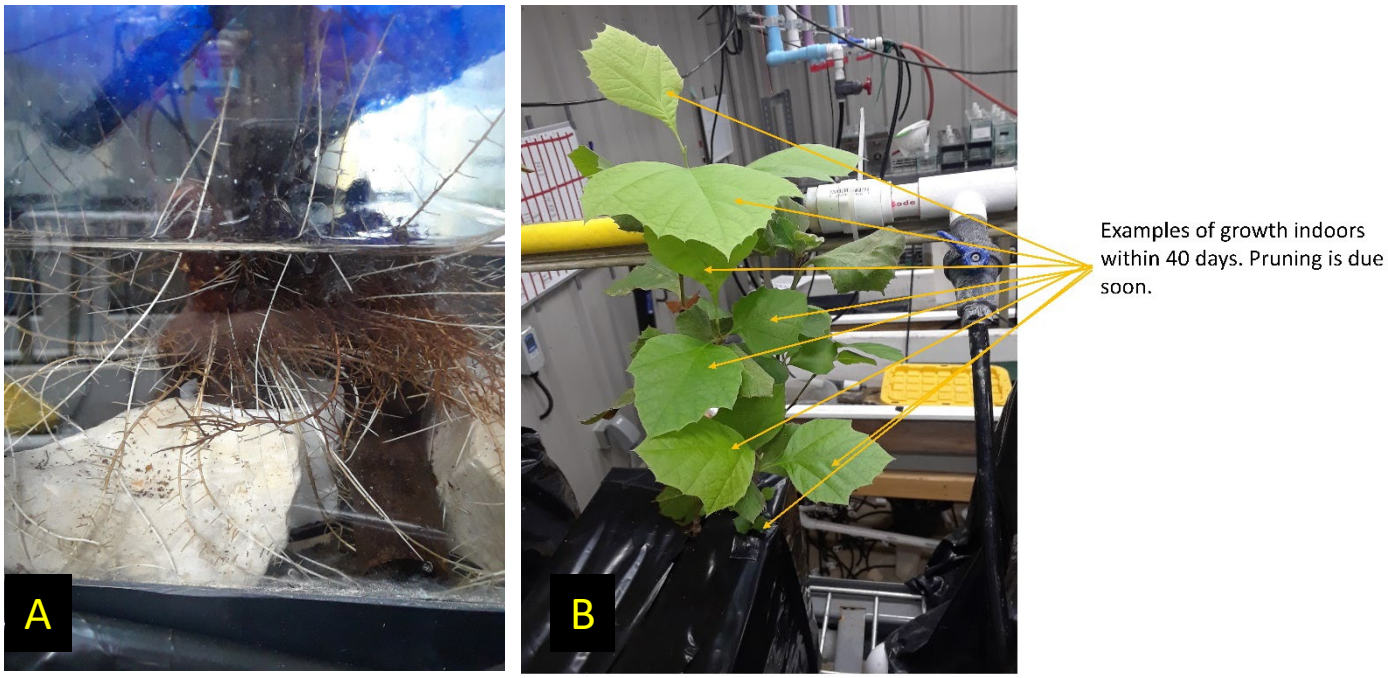

Fig. 8. *Platanus* growth after ca. 40 days in laboratory conditions. Panel A shows new root growth. Panel B shows new leaf growth.

On 27 October 2021, the contents of BlackBox 1 were thoroughly inspected for eggs and larvae. Only two eggs were found in BlackBox 1 along with four living adults and one dead adult. A less thorough, non-destructive search was performed in BlackBox 2; no eggs or larvae were found. The conditioned material, eggs, and adults from BlackBox 1 were transferred to BlackBox 2 since there was a shortage of male subjects. The hope is that additional funding will allow the continued study of these test subjects.

Fig. 9. *Stygoparnus comalensis* producing in aquaria meant to rear all life stages. Panel A shows an adult male within new root growth. Panel B shows two adults clinging to a piece of bark. Panel C shows an egg embedded within a spongy portion of wood.

There was no evidence that adults were feeding on new *Platanus* roots or the exterior of older woody roots. However, at least one male was found in the new root growth when the occupants of BlackBox 1 were transferred to BlackBox 2 (**Fig. 9A**). It seems unlikely that newly hatched larvae could burrow into the root system as well, but more extensive observation is required to make any determination with regard to the usefulness of providing living roots to *S. comalensis*.

Investigation of natural habitat and collecting locations

Surveys were generally unsuccessful. Survey 1 and 2 did not result in any dryopids recovered from lures nor were any observed on naturally occurring woody materials. Survey 3 recovered 1 larva on a lure from Spring Run 3. The larva was returned to the stream margin in plant roots associated with the spring. Survey 4 did not recover any dryopids from locations outside of the known reliable locations at the Spring Island area. Dissecting woody material from the known reliable location revealed two late-stage larvae burrowed in a small scrap of degraded wood ca. 20 x 20 cm area. The wood was found within a spring upwelling that was ca. 1 m below the water surface. These larvae were allowed to burrow back into the woody material and were placed back in the spring.

Concluding remarks

Although there was a great amount of difficulty in studying this species, due to their scarcity in the field and obscurity with regard to rearing conditions, this research represents the most comprehensive study of *S. comalensis* life history to date. It is hoped that future applied research efforts will be able to utilize this information for making improvements to studying this important species.

There is a considerable amount of evidence to indicate that females of *S. comalensis* do not make any special migration to oviposit, contrary to what is believed for other dryopid genera (Brown 1987). Eggs clearly developed even though completely submerged. The experiment involving 12 submerged eggs resulted in 3 viable larvae found in emergent leaf material. Furthermore, well-developed larvae were found in dissected coarse woody material in the field that were found buried within springs. Lastly, eggs produced during 2021 in experimental tanks were found after dissecting submerged wood. Since eggs do not appear to be fixed to substrates, it is evident that females oviposit eggs wherever they may be at the time and that hatchlings will have to make do with that habitat. Larvae hatched within woody material will likely have no problem residing within the wood as their integument is naturally hydrophobic and therefore, they should be able to maintain air pockets while they burrow. Larvae hatching outside of secured woody material will naturally float due to their hydrophobic morphology, and in areas that maintain air pockets or are close to the water's surface, larvae will have a chance to cling onto available organic material and make their way from there. This is not the first species of dryopid to have a submerged larval habitat (Novakovic et al., 2014), and it is likely that other species reside in such habitats but are difficult to study and therefore have gone unnoticed.

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Appendix A. Larval measurements used for estimating instars and tracking growth. DateCheck = date of photo; EggInit = date egg was originally recoded; Days = difference of DateCheck and EggInit; LarvaeCode = the code of potential larvae within the same rearing container; BodyLength = length from the anterior of the head to the posterior of the 9th abdominal segment; PTBL = length from the anterior of the pronotum to the posterior of the 9th abdominal segment; HCW = head capsule width; PrNW = pronotal width; PrNL = pronotal length; MsNW = mesonotal width; MsNL = mesonotal length; MtNW = metanotal width; MtNL = metanotal length; Ab1W = abdominal segment 1 width; Ab1L = abdominal segment 1 length; Ab9W = abdominal segment 9 width; Ab9L = abdominal segment 9 length. Records recorded in red were suspected to represent a separate photo of an individual on a different day, representing the same instar. Na = not available.

DateCheck	EggInit	Days	LarvaeCode	BodyLength	PTBL	HCW	PrNW	PrNL	MsNW	MsNL	MtNW	MtNL	Ab1W	Ab1L	Ab9W	Ab9L
25-Jul-18	20-May-18	66	L-1	1.669	1.66	0.2667	0.227	0.23	0.2478	0.1375	0.2371	0.1272	0.205	0.1019	0.15	0.17
07-Aug-18	20-May-18	79	L-1	1.925	1.78	0.171	0.22	0.25	0.228	0.14	0.239	0.126	0.245	0.13	0.194	0.194
30-Jul-18	20-May-18	71	L-1	1.93	1.79	0.219	0.259	0.285	0.261	0.121	0.247	0.102	0.229	0.107	0.176	0.174
20-Aug-18	20-May-18	92	L-1	2.368	2.2	0.258	0.285	0.299	0.284	0.147	0.289	0.148	0.265	0.152	0.22	0.236
01-Nov-18	17-Aug-18	76	L-10	2.052	1.94	0.209	0.252	0.219	0.246	0.123	0.246	0.143	0.244	0.149	0.178	0.155
01-Nov-18	17-Aug-18	76	L-11	na	na	na	na	na	na	na	na	na	na	na	0.153	0.181
01-Nov-18	17-Aug-18	76	L-12	2.179	2.06	0.188	0.24	0.258	0.241	0.117	0.242	0.156	0.215	0.133	0.165	0.184
13-Nov-18	21-Aug-18	84	L-13	1.909	1.82	0.205	0.224	0.225	0.234	0.121	0.23	0.112	0.216	0.124	0.159	0.217
03-Dec-18	24-Aug-18	101	L-13, L-14, L-15, L-16, L-17, L-18	1.81	1.68	0.181	0.224	0.259	0.222	0.106	0.219	0.13	0.216	0.121	0.159	0.175
03-Dec-18	24-Aug-18	101	L-13, L-14, L-15, L-16, L-17, L-18	2.038	1.95	0.201	0.23	0.23	0.226	0.143	0.226	0.132	0.23	0.152	0.169	0.191
03-Dec-18	24-Aug-18	101	L-13, L-14, L-15, L-16, L-17, L-18	2.183	1.89	0.217	0.239	0.284	0.254	0.139	0.25	0.143	0.229	0.143	0.168	0.199
03-Dec-18	24-Aug-18	101	L-13, L-14, L-15, L-16, L-17, L-18	2.35	2.15	0.227	0.265	0.271	0.259	0.16	0.258	0.14	0.245	0.122	0.175	0.237
03-Dec-18	24-Aug-18	101	L-13, L-14, L-15, L-16, L-17, L-18	2.417	2.32	0.211	0.289	0.34	0.284	0.179	0.268	0.191	0.262	0.187	0.225	0.247
03-Dec-18	24-Aug-18	101	L-13, L-14, L-15, L-16, L-17, L-18	2.439	2.27	0.238	0.265	0.361	0.229	0.176	0.264	0.173	0.26	0.2	0.221	0.284
30-Apr-19	24-Aug-18	249	L-13, L-14, L-15, L-16, L-17, L-18	7.1	6.8	0.506	0.651	0.761	0.709	0.45	0.658	0.473	0.687	0.486	0.502	0.654
13-Nov-18	21-Aug-18	84	L-14	na	na	na	na	na	na	na	na	na	0.263	0.109	0.123	0.191
20-Aug-18	19-Jun-18	62	L-2	1.848	1.78	0.171	0.21	0.234	0.21	0.164	0.227	0.124	0.222	0.135	0.18	0.19
13-Nov-18	11-Jun-18	155	L-2, L-3, L-1, L-5	3.32	3.06	na	na	0.378	na	0.2	na	0.21	na	0.255	na	0.281
03-Dec-18	11-Jun-18	175	L-2, L-3, L-1, L-5	3.96	3.74	0.363	0.403	0.491	0.389	0.313	0.391	0.264	0.334	0.308	na	na
13-Nov-18	11-Jun-18	155	L-2, L-3, L-1, L-5	4.48	4.36	0.38	0.48	0.526	0.49	0.24	0.54	0.27	0.553	0.31	0.381	0.41
13-Nov-18	11-Jun-18	155	L-2, L-3, L-1, L-5	4.53	4.37	0.384	0.45	0.47	0.485	0.25	0.52	0.3	0.522	0.34	0.41	0.43
12-Apr-19	11-Jun-18	305	L-2, L-3, L-1, L-5	5.8	5.64	0.44	0.53	0.636	0.567	0.378	0.579	0.375	0.552	0.407	0.44	0.46
04-Jun-19	11-Jun-18	358	L-2, L-3, L-1, L-5	6.56	6.4	0.53	0.7	0.75	0.7	0.366	0.677	0.358	0.669	0.425	0.554	0.585

04-Jun-19	11-Jun-18	358	L-2, L-3, L-1, L-5	6.57	6.4	0.57	0.71	0.757	0.709	0.369	0.737	0.402	0.712	0.448	0.54	0.54
12-Apr-19	11-Jun-18	305	L-2, L-3, L-1, L-5	6.9	6.66	0.577	0.725	0.761	0.73	0.479	0.72	0.49	0.715	0.526	0.55	0.562
30-Apr-19	03-Nov-18	178	L-21, L-22, L-23	4.989	4.74	0.38	0.489	0.48	0.492	0.283	0.472	0.299	0.448	0.304	0.343	0.479
29-May-19	13-Nov-18	197	L-21, L-23, L-24	5.879	5.65	0.458	0.61	0.647	0.63	0.37	0.625	0.356	0.603	0.369	0.416	0.402
10-Jan-19	15-Oct-18	87	L-22	2.15	2.01	0.21	0.235	0.245	0.226	0.136	0.223	0.148	0.224	0.14	0.148	0.186
29-Jan-19	13-Nov-18	77	L-23	1.965	1.88	0.197	0.244	0.286	0.239	0.159	0.225	0.155	0.216	0.159	0.16	0.19
26-Mar-20	13-Nov-18	499	L-23, L-24	6.876	6.72	0.526	0.699	0.778	0.744	0.427	0.763	0.429	0.733	0.454	0.564	0.733
17-Dec-19	13-Nov-18	399	L-23, L-24	6.972	6.76	0.476	0.718	0.706	0.735	0.412	0.75	0.431	0.737	0.564	0.582	0.7
29-Jan-19	13-Nov-18	77	L-24	2.12	1.86	0.192	0.25	0.257	0.241	0.147	0.233	0.151	0.228	0.177	0.17	0.2
29-Jan-19	13-Nov-18	77	L-25	1.927	1.78	0.211	0.245	0.229	0.247	0.124	0.24	0.129	0.221	0.145	0.182	0.226
08-Feb-19	13-Nov-18	87	L-25, L-26, L-27	1.887	1.79	0.202	0.226	0.217	0.239	0.144	0.215	0.152	0.209	0.121	0.156	0.174
08-Feb-19	13-Nov-18	87	L-25, L-26, L-27	1.9	1.78	0.226	0.255	0.268	0.253	0.153	0.255	0.141	0.245	0.18	0.181	0.211
08-Feb-19	13-Nov-18	87	L-25, L-26, L-27	2.194	1.82	0.207	0.25	0.26	0.2244	0.136	0.246	0.147	0.225	0.157	0.15	0.211
08-Feb-19	04-Dec-18	66	L-29	1.94	1.8	0.204	0.229	0.248	0.192	0.13	0.2	0.131	0.21	0.135	0.143	0.121
20-Aug-18	19-Jun-18	62	L-3	1.751	1.7	0.2	na	na	na	na	na	na	na	na	na	na
14-Feb-19	09-Nov-18	97	L-30	2.034	1.89	0.207	0.236	0.219	0.239	0.145	0.227	0.158	0.217	0.167	0.174	0.177
08-Mar-19	21-Nov-18	107	L-30, L-29	2.887	2.71	0.228	0.258	0.335	0.276	0.203	0.279	0.168	0.284	0.226	0.229	0.252
25-Mar-19	11-Jan-19	73	L-32	2.127	2.03	0.198	0.255	0.242	0.231	0.198	0.229	0.179	0.28	0.158	0.164	0.188
29-Apr-19	11-Jan-19	108	L-32, L-33	2.872	2.75	0.244	0.31	0.33	0.3	0.18	0.29	0.185	0.3	0.248	0.222	0.267
25-Mar-19	11-Jan-19	73	L-33	2.066	1.97	0.202	0.251	0.263	0.248	0.17	0.235	0.153	0.23	0.144	0.197	0.23
11-Apr-19	22-Jan-19	79	L-34	3.229	2.93	0.256	0.289	0.307	0.264	0.184	0.282	0.175	0.313	0.168	0.228	0.313
16-Dec-19	22-Jan-19	328	L-34	7	6.72	0.566	0.76	0.711	0.743	0.414	0.737	0.391	0.777	0.428	0.57	0.655
11-Apr-19	22-Jan-19	79	L-35	2.842	2.68	0.258	0.3	0.33	0.284	0.148	0.289	0.185	0.287	0.167	0.217	0.287
11-Apr-19	22-Jan-19	79	L-36	1.869	1.8	na	na	na	na	na	na	na	na	na	na	na
11-Apr-19	22-Jan-19	79	L-37	1.968	1.9	na	na	na	na	na	na	na	na	na	na	na
11-Apr-19	22-Jan-19	79	L-38	2.007	1.89	0.187	0.225	0.239	0.196	0.17	0.19	0.155	0.186	0.136	0.151	0.15
29-Apr-19	09-Nov-18	171	L-39	2.062	1.93	0.207	0.259	0.275	0.226	0.137	0.239	0.139	0.208	0.142	0.184	0.185
29-Aug-18	19-Jun-18	71	L-4	1.74	1.55	0.222	0.253	0.262	0.243	0.133	0.219	0.144	0.226	0.123	0.18	0.22
03-Dec-18	19-Jun-18	167	L-4, L-6	4.176	3.93	0.38	0.453	0.463	0.45	0.24	0.448	0.227	0.45	0.312	0.38	0.4
03-Dec-18	19-Jun-18	167	L-4, L-6	4.25	4.08	0.37	0.485	0.528	0.487	0.285	0.5	0.334	0.497	0.355	0.399	0.414
30-Apr-19	19-Jun-18	315	L-4, L-6	5.73	5.25	0.53	0.546	0.612	0.548	0.296	0.537	0.281	0.535	0.382	0.398	0.411

13-Dec-19	29-Jan-19	318	L-40, L-41	7	6.9	0.56	0.759	0.674	0.772	0.353	0.756	0.316	0.777	0.368	0.59	0.56
01-Apr-20	29-Jan-19	428	L-40, L-41	7.25	7.12	na	0.738	0.76	0.739	0.42	0.738	0.485	0.771	0.557	0.556	0.74
13-Dec-19	29-Jan-19	318	L-40, L-41	na	7.2	0.647	0.763	0.763	0.783	0.48	0.735	0.394	0.759	0.476	0.58	0.59
29-Apr-19	29-Jan-19	90	L-41	2.988	2.89	0.213	0.294	0.266	0.306	0.172	0.329	0.223	0.32	0.237	0.213	0.288
29-Apr-19	11-Jan-19	108	L-42	1.84	1.73	0.211	0.231	0.239	0.217	0.115	0.213	0.118	0.209	0.11	0.144	0.149
29-Apr-19	11-Jan-19	108	L-43	2.001	1.88	0.206	0.231	0.238	0.216	0.154	0.217	0.14	0.209	0.136	0.174	0.227
24-May-19	08-Feb-19	105	L-44, L-46	3.532	3.46	0.349	0.366	0.489	0.346	0.223	0.346	0.204	0.38	0.203	0.33	0.4
13-Dec-19	20-Mar-19	268	L-44, L-46	6.94	6.98	0.549	0.725	0.696	0.719	0.337	0.693	0.352	0.719	0.496	0.581	0.782
24-May-19	08-Mar-19	77	L-45	2.282	2.18	0.205	0.246	0.29	0.249	0.17	0.25	0.17	0.24	0.17	0.17	0.22
26-Mar-20	28-May-19	303	L-49	7.68	7.29	na	na	0.819	na	0.461	na	0.523	na	0.586	na	0.724
17-Dec-19	12-Aug-19	127	L-50, L-51, L-52	na	4.87	na	0.508	0.521	0.504	0.25	0.477	0.298	0.467	0.361	0.374	0.484
01-Apr-20	12-Aug-19	233	L-50, L-51, L-52	na	6.75	0.563	0.73	0.74	0.722	0.433	0.684	0.407	0.716	0.482	0.541	0.604
01-Apr-20	12-Aug-19	233	L-50, L-51, L-52	na	6.9	0.586	0.695	0.726	0.7	0.44	0.7	0.445	0.647	0.527	0.56	0.631
11-Sep-18	19-Jun-18	84	L-6	2.03	1.87	0.197	0.23	0.241	0.233	0.147	0.229	0.137	0.203	0.13	0.17	0.172
04-Oct-18	20-Jul-18	76	L-7	2.78	2.68	0.27	0.35	0.38	0.35	0.165	0.33	0.18	0.33	0.175	0.246	0.306
29-Apr-19	20-Jul-18	283	L-7	3.264	3.13	0.324	0.412	0.3369	0.403	0.208	0.385	0.204	0.379	0.22	0.33	0.43
12-Oct-18	20-Jul-18	84	L-8	1.991	1.8	0.204	0.21	0.22	0.214	0.12	0.224	0.138	0.234	0.161	0.171	0.178
01-Nov-18	17-Aug-18	76	L-9	2.176	1.99	0.209	0.268	0.248	0.275	0.147	0.25	0.15	0.225	0.166	na	na

Appendix B. Second derivative of PTBL. f(x)'' = second derivative; PTBL.mm = length from the anterior of the pronotum to the posterior of the 9th abdominal segment; Days = days; ln(PTBL) = natural log of PTBL; rank = rank assigned by PTBL.

f(x)''	PTBL.mm	Days	ln(PTBL)	rank		f(x)''	PTBL.mm	Days	ln(PTBL)	rank
-1.84E-05	1.552	71	0.439544	1		-0.01477	2.679	79	0.985444	35
-0.01003	1.659	66	0.506215	2		-0.01735	2.68	76	0.985817	36
-0.0075	1.68	101	0.518794	3		-0.00319	2.714	107	0.998424	37
-0.00157	1.7	62	0.530628	4		0.002623	2.751	108	1.011964	38
-0.00333	1.73	108	0.548121	5		0.005273	2.888	90	1.060564	39
-0.00508	1.779	87	0.576051	6		0.007923	2.931	79	1.075344	40
-0.00462	1.784	62	0.578858	7		0.013504	3.06	155	1.118415	41
-0.00225	1.79	87	0.582216	8		0.018669	3.125	283	1.139434	42
-0.00102	1.8	66	0.587787	9		-0.00094	3.456	105	1.240112	43
0.001142	1.8	79	0.587787	10		-0.00685	3.74	175	1.319086	44
0.003306	1.8	84	0.587787	11		-0.01276	3.931	167	1.368894	45
0.002459	1.819	84	0.598287	12		-0.00541	4.08	167	1.406097	46
0.002663	1.82	87	0.598837	13		-0.0009	4.36	155	1.472472	47
-0.0033	1.862	77	0.621651	14		0.003141	4.37	155	1.474763	48
-0.00305	1.874	84	0.628075	15		0.007185	4.735	178	1.554982	49
-0.00279	1.876	77	0.629142	16		0.00518	4.868	127	1.582683	50
-0.00024	1.877	108	0.629675	17		-0.00366	5.248	315	1.657847	51
-7.08E-05	1.886	101	0.634458	18		-0.00547	5.64	305	1.729884	52
0.001676	1.888	79	0.635518	19		-0.0045	5.651	197	1.731833	53
0.002164	1.89	97	0.636577	20		-0.00353	6.08	499	1.805005	54
0.002653	1.9	79	0.641854	21		-0.01359	6.4	358	1.856298	55
-0.0002	1.926	171	0.655445	22		-0.00588	6.4	358	1.856298	56
-0.00014	1.938	76	0.661657	23		-0.0096	6.658	305	1.895819	57
0.000329	1.95	101	0.667829	24		-0.00627	6.719	499	1.904939	58
0.000801	1.969	73	0.677526	25		-0.00294	6.72	328	1.905088	59
0.00163	1.986	76	0.686123	26		0.000409	6.75	233	1.909543	60
0.002212	2.012	87	0.699129	27		0.002691	6.756	399	1.910431	61
0.006496	2.025	73	0.70557	28		0.001577	6.8	249	1.916923	62
0.002673	2.056	76	0.720762	29		0.001843	6.9	318	1.931521	63
-0.00115	2.15	101	0.765468	30		0.002109	6.9	233	1.931521	64
0.002064	2.179	77	0.778866	31		0.002664	6.983	268	1.943479	65
0.009878	2.2	92	0.788457	32		-0.00011	7.12	428	1.962908	66
0.02071	2.274	101	0.82154	33		-6.31E-05	7.195	318	1.973386	67
0.002972	2.32	101	0.841567	34		-1.97E-05	7.291	303	1.986641	68

Appendix C. Second derivative of principal component 1. f(x)'' = second derivative; Days = days; PC1 = principal component 1; PTBL = length from the anterior of the pronotum to the posterior of the 9th abdominal segment; pca.rank = rank assigned by PC1.

f(x)''	Days	PC1	PTBL	pca.rank		f(x)''	Days	PC1	PTBL	pca.rank
9.04E-05	108	-2.251312537	1.73	1		-0.018211358	76	-0.945699905	2.68	32
-0.043039409	66	-2.125012777	1.659	2		-0.002794576	101	-0.945698014	2.274	33
-0.020678533	66	-2.112618381	1.8	3		0.019206158	107	-0.911908466	2.714	34
0.005998318	101	-2.101943201	1.68	4		0.060211914	90	-0.856118871	2.888	35
0.018839453	84	-2.079252754	1.819	5		0.090084751	108	-0.844276722	2.751	36
-0.002838	76	-2.03494915	1.938	6		0.0651286	155	-0.512513905	3.06	37
-0.005795805	84	-1.972472869	1.8	7		0.040172448	283	-0.353840954	3.125	38
-0.00875361	87	-1.965964666	1.79	8		0.071860465	105	-0.085108221	3.456	39
0.001797954	84	-1.935576363	1.874	9		-0.054122509	167	0.347325968	3.931	40
-0.006403449	76	-1.904855785	2.056	10		-0.104511326	155	0.665814185	4.36	41
-0.0080578	101	-1.881764458	1.95	11		-0.064666079	155	0.834947126	4.37	42
0.006345819	62	-1.876979124	1.784	12		-0.027480724	178	0.984184591	4.735	43
0.000534582	87	-1.863970598	2.012	13		0.00970463	127	1.130320543	4.868	44
-0.005276655	71	-1.826398621	1.552	14		0.089094097	167	1.235267238	4.08	45
0.000243482	171	-1.824660721	1.926	15		0.151185018	315	1.337131889	5.248	46
0.008847414	79	-1.81570006	1.888	16		-0.08715196	197	1.923336616	5.651	47
0.000495372	97	-1.785947225	1.89	17		0.011705411	318	2.131627167	6.9	48
-0.003234573	108	-1.756497637	1.877	18		-0.007655392	305	2.255245926	5.64	49
0.005379622	101	-1.746178259	1.886	19		-0.027016195	358	2.742277715	6.4	50
0.013993816	87	-1.72391751	1.82	20		-0.075504142	358	2.901876551	6.4	51
0.003432084	101	-1.680777908	2.15	21		-0.058339872	328	3.140618782	6.72	52
-0.009032562	77	-1.636797801	1.862	22		0.03047544	268	3.169119392	6.983	53
-0.016683957	87	-1.592437202	1.779	23		0.013753532	233	3.385694637	6.75	54
-0.002261374	77	-1.590615435	1.876	24		-0.027706239	318	3.517204285	7.195	55
0.019369059	73	-1.558801303	1.969	25		-0.069166011	233	3.728328745	6.9	56
0.040999493	92	-1.549045092	2.2	26		-0.028990356	499	3.744691099	6.719	57
0.039785696	73	-1.45299241	2.025	27		0.020746814	399	3.798769154	6.756	58
0.039884164	77	-1.378257579	2.179	28		0.062981309	249	3.877223877	6.8	59
-0.039515704	79	-1.171199279	2.679	29		0.159351994	305	3.962039544	6.658	60
-0.043189326	79	-1.05646628	2.931	30		0.095859659	428	4.224958182	7.12	61
-0.030700342	101	-1.038530649	2.32	31		0.032367324	303	4.709055476	7.291	62

Assessing the effect of *Staphylococcus* exposure on Comal Springs riffle beetle captive survival and propagation

2021 Interim Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

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U.S. Fish and Wildlife Service

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Background

Performed in conjunction with the U.S. Fish and Wildlife's Refugia Program, Dr. Camila Carlos-Shanley's (Texas State University) work revealed variances in wild versus captive microbiomes in Comal Springs riffle beetle (CSRB) (Mays 2021). The research team cultured and identified bacteria to the genus level from CSRB and water samples collected from Comal Springs and the San Marcos Aquatic Resources Center (SMARC). Dr. Carlos-Shanley found that *Staphylococcus* bacteria were more prevalent in the captive population that the wild population (Mays 2021). Although this is a harmless form of *Staphylococcus* for humans, the effects of this bacterium on CSRB are unknown. If the exposure to *Staphylococcus* spp. causes reduced survival or pupation in CSRB larvae, we can use this information to modify standard operating procedures to eliminate CSRB exposure in the refugia.

Objective

The objective for this project was to determine if *Staphylococcus* sp. exposure has an effect on CSRB larvae survival and pupation in captivity.

Methods

Staphylococcus Exposure

The survival of late-instar CSRB larvae exposed to high bacterial loads was examined using two bacterial strains; *Staphylococcus aureus* (Simpson's Index of Diversity; SID-278) and *Bacillus subtilis* (SID-166). The *B. subtilis* group represented the effects of increased bacterial exposure, that might occur in a captive setting, has on survival and pupation, while the *S. aureus* exposure group represented increased pathogenic bacterial loads on survival and pupation.

Because there was a risk that captive-reared CSRB larvae at the SMARC were already exposed to *Staphylococcus* sp., wild CSRB larvae were collected and immediately transferred to Texas State University (TSU). Larvae were collected from cloth lures and *in situ* woody debris by SMARC staff using refugia CSRB lure collection standard operating procedures. All equipment was disinfected with 70% ethanol and staff wore gloves disinfected with 70% ethanol during collection to prevent contamination of wild larvae. Larvae were acclimated at TSU for at least two weeks prior to the start of the experiment. Ten larvae were sacrificed before each experimental trial to confirm the absence of *S. aureus 278* in their gut before exposure. Each treatment received 30 individual larval replicates between two trials. Each trial consisted of 15 replicated larvae per treatment (i.e., 1 larva per replicate, 30 larvae per treatment, 15 larvae per treatment per trial; n = 90).

Food items were conditioned at the Freeman Aquatic Biology building (FAB) on TSU campus to prevent contamination from SMARC water or staff. The FAB is supplied with Edwards Aquifer water from an artesian well on campus. All trials used water from the artesian well. For the first trial, Sycamore leaves were collected from the areas surrounding Spring Runs 1-3 using the same precautions as larvae collections (i.e., disinfection, gloves). The Sycamore leaves were immediately transferred to the FAB and placed in a conditioning container to build biofilm. After having trouble finding recovering larvae from the conditioned leaves in the first trial, cloth was used for the second trial. Two-hundred thread count 60% cotton, 40% polyester blend cloth was cut into approximately 9.5 cm x 24.5 cm pieces. The cloth pieces were washed to remove any contaminants from the manufacturing process and were soaked in 70% ethanol for disinfection. After all cloth pieces dried, they were put in a container at the FAB to build biofilm. All leaves and cloth conditioned for 30-45 days before being used in a trial.

Forty-five cylindrical containers were prepared to hold larvae for the duration of the bacteria exposure. Containers were 16 mL and 4.5 cm height x 4.3 cm inner diameter. The container lids were outfitted with inflow hoses and outflow barbs (Figure 1) to prevent cross contamination among containers and treatments. A 250 µm nylon screen was placed between the lid and jar to prevent escape. Containers were distributed across four flow-through flowbars.

Staphylococcus aureus (SID-278) and *Bacillus subtilis* (SID-166) were isolated from strains found in SMARC CSRB and were used to inoculate agarose placed into containers for each bacterial exposure treatment. Jars and lids were sterilized by autoclave and were aseptically moved to a level-2 biosafety enclosure. Jars were separated into three groups of 15 according to their treatment designation. Three hundred mL of 0.8% agarose (Sigma-Aldrich CAS: 9012-36-6) was prepared using the aquifer water that is pumped into the FAB and sterilized by autoclaving. Agarose was evenly divided between three sterile beakers. One beaker was inoculated with *S. aureus*, another was inoculated with *B. subtilis*, and the last was not inoculated. All cultures grew in brain heart infusion (BHI) broth then washed in sterile spring water prior to use. Enough washed bacteria were added to each respective treatment to create a final absorbance at 600 nm of 0.05. An overnight 18-hour culture of *B. subtilis* was added to the second treatment and a 32-hour culture of *S. aureus* to the third treatment as the staph treatment. After each solution was prepared, a serological pipette was used to add 5 mL of solution to each jar in that treatment. All jars with solution were stored in a 4°C refrigerator overnight until use.

Larvae were randomly placed in containers with small amounts of food items to examine individual survival. Each container held one larva and either pieces of conditioned leaves or cloth for the first and second trials, respectively. Conditioned leaves of approximately 5 cm x 5 cm were used in each container for the first trial, and 9.5 cm x 24.5 cm cloth pieces were used for the second trial. The cloth pieces were larger than the leaves because cloth takes much longer to break down and had less potential to negatively affect water quality. The cloth provided larvae with spaces to ‘burrow’ into by being folded several times, similar to the cloth lures used for CSRB collection.

Larvae holding conditions at the FAB were as similar to wild conditions as possible to monitor survival and pupation for 45 days. At the FAB, aquifer water is pumped into a sump held at a constant 23°C, which then flows through each container and back out to a drain. Dividers between treatments prevented any contamination from splashing. Shade cloth placed over all containers provided a dark environment for the larvae. All equipment and supplies were cleaned and disinfected (70% ethanol or autoclaving) after each trial to ensure residual bacteria did not contaminate the next trial.

Mortality was monitored for 45 days for each trial. Larvae were checked for mortality daily for the first trial. Larvae were checked for mortality weekly during the second trial to reduce the potential for larvae escape that occurred in the first trial by opening the containers less frequently. All containers were checked daily for adequate flow during both trials. Gloves were worn and disinfected with 70% ethanol while handling containers and cleaned or changed between containers. All negative control containers were checked first, followed by *Bacillus* containers, and last the *Staphylococcus* containers to prevent contamination.

After a subset of larvae were sacrificed for testing, the remaining larvae were moved to the SMARC for long-term monitoring at the conclusion of the exposure experiment. Ten larvae from each treatment group from each trial were tested to determine if the *Staphylococcus* exposed larvae contain *S. aureus 278*. in their gut and confirm that the *Bacillus* and control larvae did not contain *Staphylococcus*. The number of larvae sacrificed for testing varied based on the number of mortalities collected during the trial (i.e., the number of collected mortalities plus the number of sacrificed larvae = 10 for each treatment). All living larvae that were not sacrificed were brought back to the SMARC, where they were placed in holding tubes by treatment type and trial and grown out in the SMARC refugia. Holding tubes with larvae from the first trial were connected to a partially recirculating system. Due to flow issues from the partially recirculating system, tubes with larvae from the second trial were connected to a flow-through system. We conducted an inventory of all holding containers monthly to assess long term survival, pupation, and eclosion of treatment groups.

Kaplan-Meier survival curves were constructed (Goel et al. 2010) and a survival analysis was conducted, examining treatment and tank effects. The curves displayed survival over time using weeks since initial exposure as the time increment, and CSRB larvae that survived the duration of the exposure period were right censored. Only the survival data collected during the exposure experiment were used to create the survival curves (i.e., we did not use data recorded after larvae were transferred to the SMARC). Once the curves were created, we tested the null hypotheses that survival was not affected by the tank in which larvae were held or by adding *S. aureus* or *B. subtilis* using the log-rank test comparing the survival curves. The analyses were conducted in the

“survival” package (Therneau 2020) in the program R 4.0.3.

Metagenomic comparison of larvae and adults

A total of nine larvae (one uninoculated, four *Bacillus*, four *Staphylococcus*) were sacrificed in 95% ethanol for downstream applications. Photographs of each larva were taken and labeled prior to DNA extraction. DNA was extracted using the QIAmp BIOstic Bacteremia DNA kit with the addition of Zymo Spike-in Control II and quantified with a Qubit-4 fluorometer. All DNA was within an acceptable concentration for sequencing (≥ 2.0 ng/µl) except for 2 samples from the *Staphylococcus* treatment. DNA was sent to the Microbial Genome Sequencing Center in Pittsburg, Pennsylvania for genome sequencing.

Results

Staphylococcus Exposure

The first trial proceeded successfully, but no larvae pupated and some went missing (Table 1). Missing larvae were not assumed to be alive or dead. At least one larva had escaped to the lip of their container and was crushed in the threads when the lid was removed or replaced on the container. These individuals were not included in analyses. Some larvae that survived the first trial were sacrificed for *Staphylococcus* infection testing.

Long-term monitoring for the first trial lasted three months, at which point all larvae were dead (Table 1). Several instances of low or no flow occurred during long-term monitoring at the SMARC. Low- and no-flow events occurred due to calcification and debris buildup associated with the partially recirculating system in which the larvae were held. Additionally, the tube screens had to be cleaned every day to maintain or resume appropriate flow conditions. No larvae from the first trial pupated.

The second trial proceeded successfully with one minor setback associated with calcium debris reducing flow, and no pupation occurred (Table 1). Significant calcium deposits were found on and cleaned from the screens of containers in Tank 2 on September 1, 2021. These deposits may have flowed into the containers from the pipes. The deposits decreased flow to some containers and notes were made to account for those differences. No larvae went missing during this trial. Some larvae that survived the second trial were sacrificed for *Staphylococcus* infection testing by Dr. Carlos-Shanley.

Long-term monitoring for the second trial resulted in four pupation events, and two of the subsequent adult beetles are being held at the SMARC at the time of this report. No low- or no-flow events occurred, but a high-flow event occurred within the first month the larvae were at the SMARC. Several mortalities occurred during the first month (Table 1), but the state of several dead larvae (crushed against the outflow screen) indicated the high-flow event might have contributed to some of those mortalities. One adult Comal Springs riffle beetle was found in each of the negative control and staph tubes after their first month at the SMARC. Two additional pupations were recorded in the *Bacillus* treatment after their second month housed at the SMARC. No living larvae remained at 2-months post transfer, but all living adults were returned to their tubes for continued monitoring. Two adult beetles remained at 3-months post transfer.

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

Metagenomic comparison of larvae and adults

The samples sent for metagenomic sequences were lost in transit. Thus, genetic confirmation of Staphylococcus presence in the guts of CSRB larvae will not be discussed further. The remaining samples from Trial 1 will be re-submitted for metagenomic sequencing in 2022. Previous effort investigating the metagenomic composition of larvae and adults across Comal Springs, the Uvalde National Fish Hatchery (UNFH) and the SMARC (Table 2) show that adults and larvae harbor a distinct microbiome (PERMANOVA, Bray-Curtis dissimilarity, $F=12.69$, $p < 0.0001$). It is important to highlight that *Staphylococcus* sp. for experimental manipulation was chosen based on the work of Mays et al. (2021) with adult CSRB. New analyses show that the relative abundance of *Staphylococcus* sp. in the larvae does not differ across locations. Many microbial genera were found to be more abundant in larvae from the SMARC facility when compared to wild and UNFH larvae (Table 3), however most of them are hard to be cultivated in laboratory conditions. Except for *Tsukamurella* spp. (Figure 3), for which there are currently three strains isolated from CSRB in the culture collection.

Discussion

Understanding the effects of exposure to human-introduced bacteria on CSRB is important for managing the Refugia population. This study found no statistically significant difference between the staph-exposed larvae survival and that of the two non-staph groups. Although survival was not statistically different for staph-exposed larvae, there are potential biological implications. It might be more appropriate to use a value of 0.1 for α here, because type II error (accepting a false null hypothesis) is more dangerous for CSRB than type I error (rejecting a true null hypothesis) in this case. If an α value is set to a higher level (0.1), the staph treatment larvae would have statistically significantly lower survival than the *Bacillus* group and higher survival than the negative control group. Because CSRB is endangered, it might be advantageous to interpret the results of this study more cautiously (Martínez-Abraín 2008) and consider the biological relevance to the organism. Any decrease in survival of an endangered and sensitive species could result in harm to the Refugia population.

Decreasing the frequency of inventories and more thoroughly training observers could result in fewer escaped or missing larvae. Several larvae were missing by the end of Trial 1, but no larvae went missing during Trial 2. Larvae were checked daily during Trial 1, providing many opportunities for the larvae to spill from or crawl out of containers or observers to incorrectly replace the mesh that prevented escapes. Inventories were decreased to weekly for the second trial, reducing the number of opportunities seven-fold. It is possible some larvae were mistaken for other items (e.g., leaf ribs, other invertebrates) and passed over during the first trial inventories and these mistakes decreased for Trial 2 due to increased experience. The proposed CSRB propagation handbook would be beneficial in training new employees to reduce errors associated with inexperience.

No pupation occurred in either trial while they were housed at FAB on a sump system or in Trial 1 while it was housed at the SMARC on the partially recirculating system. Conversely, two pupations occurred in Trial 2 within a month of being transferred to a flow-through system at the SMARC. These results support previous research that determined flow-through water is preferred by CSRB under lab conditions (Cooke et al 2015), but that study involved only adult beetles. Additional evidence is provided by pupation rates at the SMARC. Very few pupations (<1%) occurred in the SMARC Refugia population in 2021, but four pupations (22%) occurred in Trial 2 and many pupations occur in BIO-WEST holdings at the SMARC (Kosnicki 2020). The Refugia population was held in partially recirculating systems for all of 2021, but there are other differences that could account for some lack of pupation (e.g., holding boxes are used instead of tubes). However, Trial 1 and Trial 2 were held in the same conditions except Trial 1 was provided partially recirculating water.

Increasing biosecurity measures, performing fewer inventory events, and moving CSRB to flow-through systems could provide higher survival and pupation for CSRB and fewer escaped larvae. Staph bacteria could be harmful for CSRB and introducing measures to decrease CSRB exposure could increase survival in the Refugia population. If the goal is to reduce *S. aureus* exposure for CSRB, our findings suggest increasing biosecurity measures like disinfecting items more regularly and wearing more personal protective equipment (e.g., lab coats, face shields) during inventory events could benefit the program. Performing fewer inventories on the Refugia population could also decrease CSRB exposure to *S. aureus* by reducing human handling of items in the CSRB environment and reduce the opportunities for individuals to escape.

Tables and Figures

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

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

Table 2. The number of adult and larval Comal Springs riffle beetle *Heterelmis comalensis* collected from three different locations, Comal Springs, the Uvalde National Fish Hatchery (UNFH), and the San Marcos Aquatic Resources Center (SMARC).

Location	Larvae	Adults
Comal Springs	6	7
UNFH Facility	4	4
SMARC Facility	6	3

Table 3. The differential abundance of microbial genera in Comal Springs riffle beetle *Heterelmis comalensis* larvae among the three different locations (Comal Springs, the San Marcos Aquatic Resources Center (SMARC) and the Uvalde National Fish Hatchery (UNFH)) ($p < 0.005$).

Genus	P-values	Mann-Whitney statistics	Overrepresented
Methylovulum	0.00165	12.812	Comal Springs, UNFH
Monascus	0.00165	12.812	Comal Springs, UNFH
Eremococcus	0.00200	12.426	SMARC
Idiomarina	0.00263	11.882	Comal Springs
Propionimicrobium	0.00320	11.489	SMARC
Pluralibacter	0.00340	11.368	Comal Springs, UNFH
Microbacterium	0.00343	11.353	SMARC
Tsukamurella	0.00361	11.250	SMARC
Kingella	0.00361	11.250	Comal Springs, UNFH
Albimonas	0.00433	10.882	Comal Springs, UNFH
Intestinimonas	0.00433	10.882	Comal Springs, UNFH
Thiothrix	0.00462	10.754	Comal Springs, UNFH
Candidatus Endolissoclinum	0.00484	10.662	SMARC
Cryobacterium	0.00498	10.607	SMARC

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

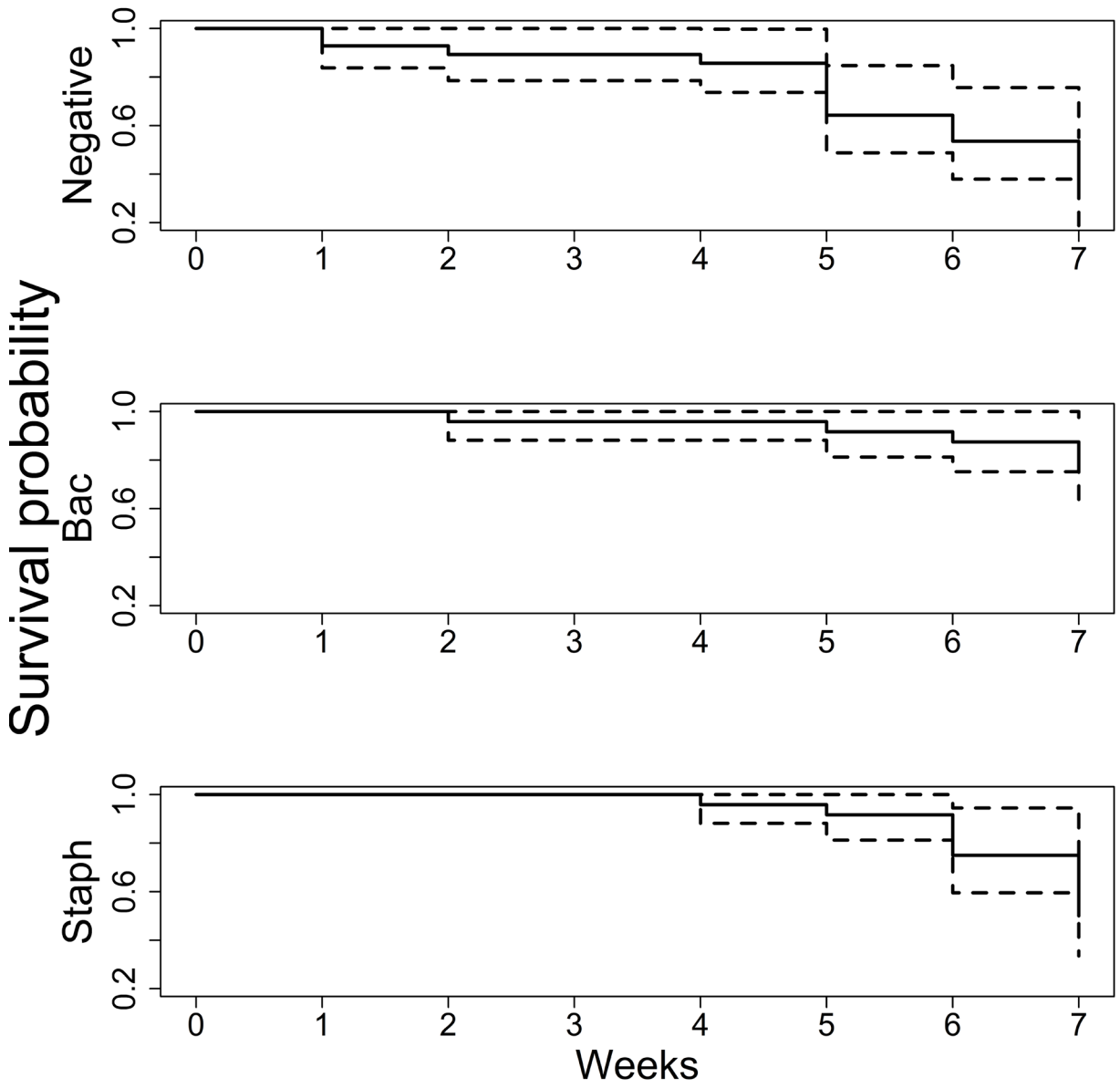

Figure 2. Kaplan–Meier survival curves developed for Comal Springs riffle beetle *Heterelmis comalensis* larvae exposed to *Staphylococcus aureus 278* (staph), *Bacillus subtilis* 166 (Bac), and no bacteria (negative). All groups were held in the same conditions except agarose in their containers contained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

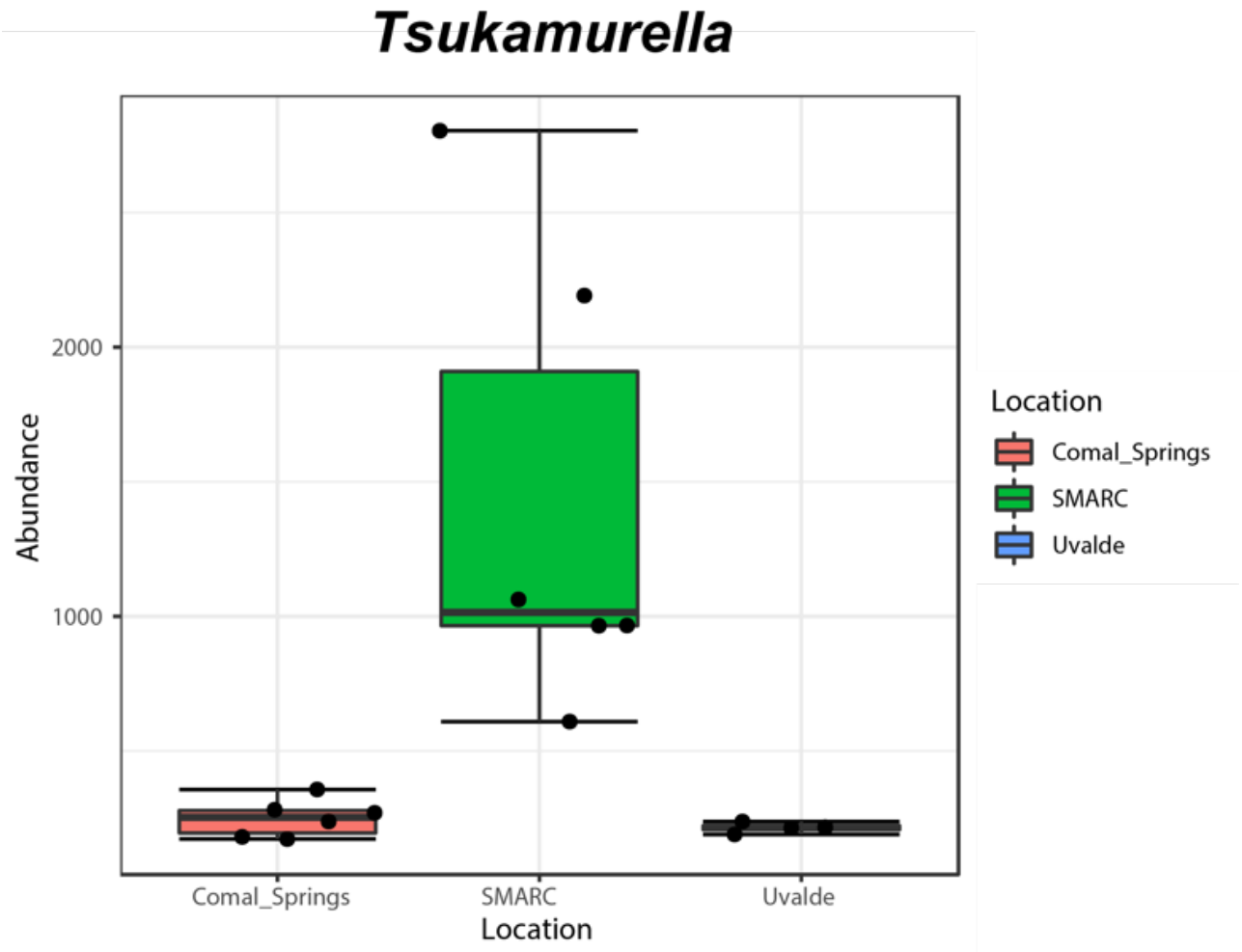

Figure 3. Box-plot of *Tsukamurella* sp. bacteria abundance in larvae from different locations. Locations from where adult and larval Comal Springs riffle beetle *Heterelmis comalensis* were collected are listed on the x-axis, while relative abundance of *Tsukamurella* sp. is on the y-axis. The Comal Springs location is in red, the San Marcos Aquatic Resources Center (SMARC) is in green, and the Uvalde National Fish Hatchery (Uvalde) is in blue.

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Interim Report
Comal Springs Riffle Beetle Research 2021 - 2022: Increasing Comal Springs riffle beetle (*Heterelmis comalensis*) F1 adult production at the Refugia level

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Introduction

The Comal Springs riffle beetle *Heterelmis comalensis* (Bosse et al. 1988) is a beetle in the family Elmidae (Coleoptera), known from Comal Springs, Comal County and San Marcos Springs, in Hays County, Texas (Gibson et al. 2008). It is a federally protected species (USFWS 1997) and has 22 ha of designated critical habitat (USFWS 2013). *Heterelmis comalensis* faces numerous threats to its ecosystem related to pumping of water, pollution, and competition from exotic species (Bowles and Arsuffi 1993). Having self-propagating functional refuges that contain captive populations of *H. comalensis* is a requirement of the Edwards Aquifer Habitat Conservation Plan (EAHCP), and therefore a better understanding of the pupation process leading to healthy adults is essential.

The underlying biology of *H.* comalensis requires air for pupae to respire. Previous work has found that late-instar larvae placed within flow-through tubes had higher pupation rates if given more air resources (Kosnicki submitted). However, it is of interest to further investigate aspects of this previous work to improve refuge production. The overall goal of this study is to increase production of *H. comalensis* at the San Marcos Aquatic Resources Center (SMARC) and Uvalde National Fish Hatchery (UNFH). We will accomplish this goal with three objectives presented as phases:

> Phase I. Determine if the tube design can be modified as a small rectangular flow-through box that can maintain or improve upon measured pupation/eclosion rates.
>
> Phase II. Determine if higher larval densities in a flow-through system (i.e., selected tube or box from objective 1) can maintain or improve upon measured pupation/eclosion rates.
>
> Phase III. Based on optimization of above factors, determine if adding wild cultivated biofilm (on leaves, wood, and cloth) for larvae will improve pupation/eclosion rates.

Phase I

Two rectangular flow-through boxes were constructed as replicas of a prototype box already constructed and tested in 2020 (**Fig. 1**). An additional mesh screen (250 µm) was placed towards the outflow of each box to capture sediment that may clog the outflow screen.

Phase I was initiated in late May 2021. Larval test subjects were supplied from the production of a colony of wild adults that were used as part of a luring study during the end of 2020 and early 2021 (EAA contract 20-014L-TES). Larvae were in later stages of maturation; however, some may have been close to the end of their life expectancy for that life stage.

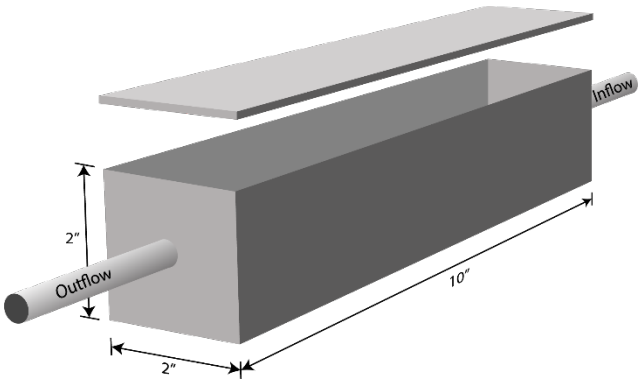

Fig. 1. Schematic of flow-through box for *Heterelmis comalensis* pupation enhancement.

The first of three replicates was retrieved on 9 August 2021 because the larvae were estimated to be the oldest test subjects. Eleven larval carcasses were recovered along with one living but sluggish larva. No pupae or adults were recovered; thus, eight test subjects were unaccounted. The remaining individual was placed into a small flow-through tube and was inspected until it was determined to be dead on 24 September.

The second replicate was retrieved on 26 August 2021. Three living larvae were recovered along with two larval carcasses, leaving 15 test subjects unaccounted. No signs of pupation events were discovered. The remaining individuals were placed into a small flow-through tube and were inspected until all individuals were determined to be dead on 24 September.

The third replicate was retrieved on 27 August 2021. Seventeen living larvae were recovered along with one adult male. Larvae appeared to be viable and still capable of completing their life cycle and therefore were placed back into a cleaned flow-through box with replenished leaf material and the original wood and cloth resources. On 24 September, a teneral female (recently eclosed) was recovered from the box along with nine living larvae. These larvae were re-launched in the flow-through box and were not inspected thereafter up to the time of this report.

Discussion points from Phase I

The first retrieved replicate clearly had flow issues, ranging from 4.9 - 23.1 mL/sec; target flow based on results of Kosnicki (submitted) should range from ca. 9 – 16 mL/sec for 2” flow-through tubes. The flow had to be reset six times during the test run due to clogging of the outflow screen. Even more, one of the clogging events resulted in overflow. Furthermore, test subjects used for this replicate may have already been past their prime as their estimated age was ca. 260 days. Therefore, it is likely that this replicate was unsuccessful due to a number of issues.

The second retrieved replicate also had flow issues but tended to maintain a more consistent discharge, ranging from 7.1 – 19.6 mL/sec; however, flow was reset six times during the trial. The outflow screen was clogged three times and there were three overflowing events.

The third retrieved replicate appeared to have the least amount of flow issues; the outflow screen was clogged one time, and this was also responsible for the only overflow event. Discharge ranged from 9.9 -

20 mL/sec apart from one occasion, where it was noted to show almost no flow. This was the only time the flow was reset. This replicate resulted in a 10% pupation rate. If better control measures could be implemented, it is possible that the flow-through box design might work as well as flow-through tubes.

All trials revealed notable signs of feeding on the leaf and wood resources, and therefore it is likely that the larvae were healthy for at least a period of time during the trial. It was also noted that there were a number of active physid snails occupying the boxes that were likely feeding on similar resources, though they probably did not contribute to gallery formation that is a notable *H. comalensis* larvae feeding behavior. The snails were likely invading from the well water as the inflow connection was not screened off. It is also possible, though not probable, that larval or adult beetles crawled out of the boxes through the flow lines.

Issues related to flow included clogging of the outflow screen and sometimes this resulted in overflow events. Other issues are related to the varying conditions of the partial-recirculation system; the flow was probably too variable, and it has been noted that low-flow conditions can be detrimental to larvae. The flow bars feeding the box inflows tended to calcify and clog. Going forward it was decided that a direct flow-through system needed to be used for the remaining phases of this study.

Results of this trial are inconclusive. The flow-through tubes used in cooperative agreement F19AC00072 underwent several test trials before adequate conditions eliciting adequate pupation events (> 20 %) were identified; it is likely that additional trials would find better conditions for larvae with the flow-through style boxes. Modifications to improve flow-through boxes should include larger outflow drains, screens on the inflow bulkheads, more secure lids, and pipe stands as emergency drains to help prevent overflow events. Slightly larger box dimensions could also be considered. Regardless, the research team has decided to utilize flow-through tubes with the same design as those used in cooperative agreement F19AC00072. The rationale is that data already exist for these types of holding containers, and they are already proven to have a level of success.

A discussion among research team members regarding what constitutes a dead larva was initiated after the initial results of Phase I. It has been noted from previous studies and other researchers that mature larvae may remain in a state of life that is, for lack of better term, lifeless. These larvae may show a number of deleterious effects, such as disease and lack of movement, but still physically respond if prodded (**Fig. 2**). Inactive larvae have been observed to endure for ca. a year with no visible signs of feeding (Kosnicki personal observation). A preliminary key for determining whether a larva should be considered “dead” or “alive” has been initiated. Living larvae are active, moving and/or clinging to substrate, and potentially feeding. Dead larvae are defined here as individuals that are 1) not responsive when prodded with forceps, or 2) inactive larvae are responsive when prodded, but display two of the following three characteristics:

1. C-shaped body posture and lethargic; hardly moving.
2. Show visible signs of disease, usually in the form of brown spots or external fungal growth.
3. Not capable of clinging and holding on to a substrate.

More investigation is needed to characterize healthy, unhealthy, and terminally ill larvae and the potential for individuals assigned to these conditions to pupate.

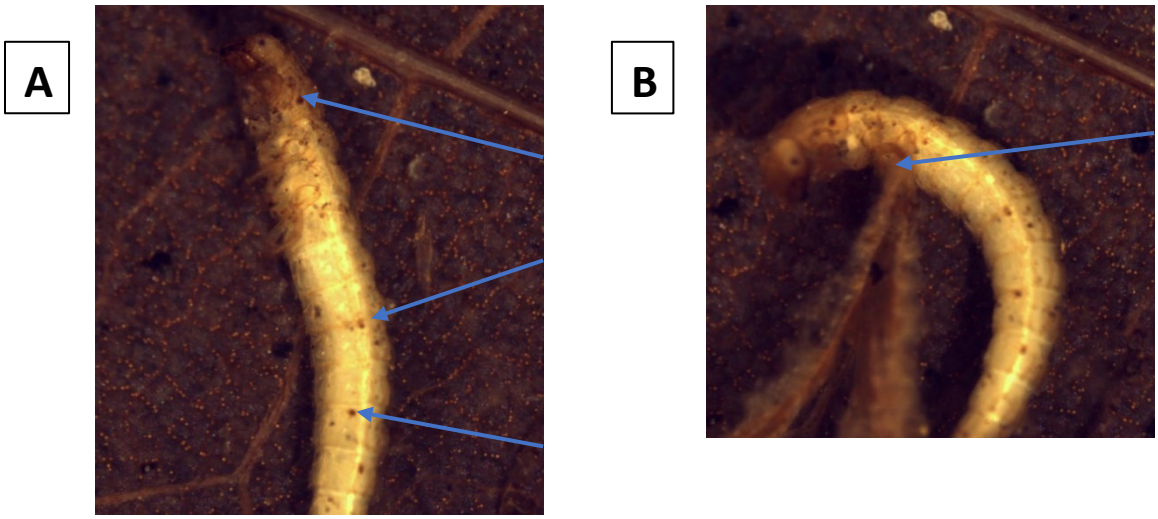

Fig. 2. Panel A, languid larva with brown spots indicating signs of disease; Panel B, same larva given a piece of leaf and responding by weakly clinging. Age of larva estimated to be > 9 months.

Phase II

Phase II of this project has had to undergo adaptive management measures due to a lack of late-instar F1 larvae (test subjects) available from the refuges. Because of the small number of available F1 larvae, it was decided that a new collection of ca. 60 adults was required to produce a sufficient stock of larvae. To stay close to schedule, the research team decided that we would implement Phase II with early-instar larvae. This would also serve as a more holistic test, allowing a broader set of larvae to be exposed to density conditions, rather than utilizing late-stage larvae which are already the fittest survivors of a cohort reared under artificial-habitat conditions.

Adults for the rearing of F1-test subjects were collected on wood from the Comal Springs on 30 September 2021. A total of 71 adults were launched within three separate 2" flow-through "breeding" tubes, packed with conditioned cloth, wood dowels, and *Platanus* sp. leaves with a plastic mesh roll in the center of the tube to help promote flow. On 28 October 2021, we examined one of the three breeding tubes for early-instar F1 subjects to be used for Phase II of the study and a couple of issues were noted. First, it appeared that the cloth was bunched up within the tube, creating an anoxic pocket (**Fig. 3B**); no larvae were found on the cloth and only a couple of adults were found on it, presumably around the perimeter. The cloth was discarded. Second, and more important, eight *Microcylloepus* adults were found within the tube, thereby making it near certain that some of the larvae were not our target species. More than 80 1st – 2nd instar elmid larvae were counted from this tube; however, there was not very strong confidence separating these to genus (**Fig. 3B**). Our decision at the time was to remove the adult *Microcylloepus*, add back the adult *Heterelmis* and wait for the larvae to mature before separating the genera for the experiment. The other tubes were later inspected, resulting in a total of 20 *Microcylloepus* adults among the 71 elmid adults launched for breeding purposes. Although the difference between *Microcylloepus* and *Heterelmis* is distinct, working with live animals that are ca. 2 mm in length can be challenging, especially for novice workers. Future collections will ensure that test subjects and target specimens are double verified by two team members, with the team's most experienced aquatic-insect specialist being one of those doing the verifications. The current standing for Phase II is to wait for F1 larvae to mature so that they can be identified to genus, slated for early 2022.

Launching of various density treatments will likely ensue, depending on the numbers of F1 larvae available per check.

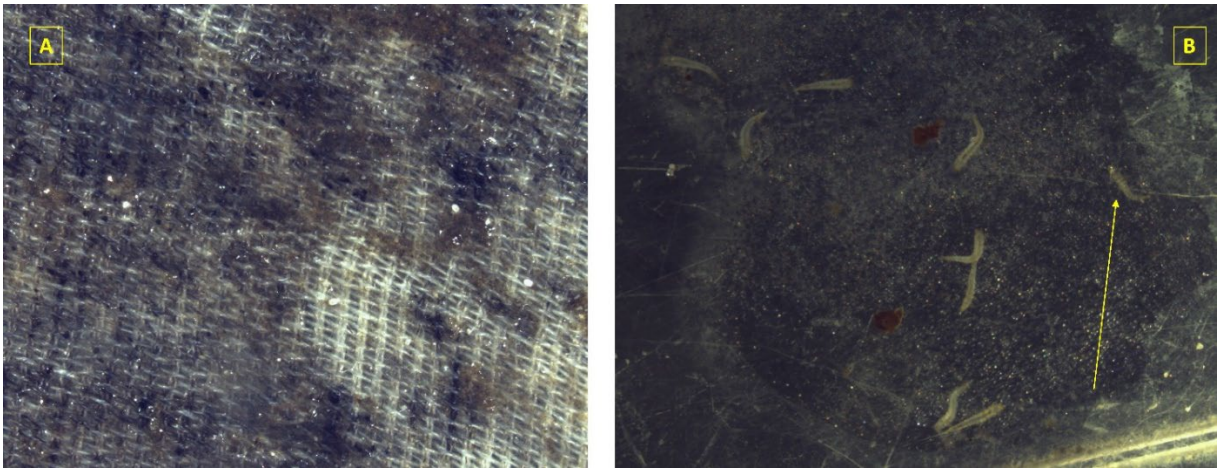

Fig. 3. Panel A, clumps of "slime" on the surface of conditioned cloth; Panel B, illustrating the difficulty in separating early-instar riffle beetle species. The arrow indicates a suspected 1st instar *Microcylloepus* among *Heterelmis*.

Phase III

Wood-dowel resources were placed at the head of Spring Run 3 of the Comal Springs in mid-August and will remain there until Phase III is ready to proceed. Cloth lures and *Platanus* sp. leaves will be placed at the spring head for conditioning ca. one month before the launch of Phase III. Once resource materials are ready for experimentation, they will be inspected roughly for invertebrates, including *Heterelmis*, that will be removed and placed back into the spring before transporting the materials to the SMARC. Conditioned resources will be kept within a recirculation system of Comal Springs water that will be replenished as needed with fresh Comal Springs water collected close to a spring source (*e.g.,* head of Spring Run 1). Comal Springs conditioned resources will be inspected a second time superficially on their surfaces and any invertebrates found will be removed before resources are used for experimentation. Space for setting up a recirculation system for storing conditioned materials has been identified and the system will be set up in 2022. The rate of our production of F1 larvae and their development will determine the basis on which Phase III will be initiated.

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Final Report

For USFWS

and

Edwards Aquifer Authority

Factors Affecting Pupation in the Endangered Comal Springs Riffle Beetle

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Background and Introduction:

In 2013, the United States Fish and Wildlife Service (USFWS) issued an Incidental Take Permit (ITP) to the Edwards Aquifer Authority (EAA), Texas State University (TXSTATE), the City of San Marcos, the City of New Braunfels, and the San Antonio Water System (SAWS) for the use of the Edwards Aquifer and its spring-fed ecosystems. The ITP is maintained through the Edwards Aquifer Habitat Conservation Plan (EAHCP) and the organisms covered by the ITP are the fountain darter (*Etheostoma fonticola*), Texas wild rice (*Zizania texana*), the Comal Springs riffle beetle (*Heterelmis comalensis*), the San Marcos salamander (*Eurycea nana*), the Texas blind salamander (*Eurycea rathbuni*), the Peck's Cave amphipod (*Stygobromus pecki*), the Comal Springs dryopid beetle (*Strygoparnus comalensis*), the Edwards Aquifer diving beetle (*Haideoporus texanus*), the Comal Springs salamander (*Eurycea* sp.), the Texas troglobitic water slater (*Lirceolus smithii*), and the San Marcos gambusia (*Gambusia georgei*; assumed extinct).

The Edwards Aquifer Recovery and Implementation Plan (EARIP) currently sets the long-term mean and minimum daily discharge objective for Comal Springs at 225 cfs (cubic feet/second) and 30 cfs, respectively. However, modeling results from Phase 1 of the EAHCP predict that the mean and minimum daily discharge will be 197 cfs and 27 cfs, respectively (EARIP 2012). Thus, there is currently concern about the impacts of lower spring flows on populations of species covered by the ITP. Historical data and modeling results indicate potential loss of habitat and habitat degradation of these species associated with the reduction in spring flows. It has been observed that Spring Runs 1 and 2 generally cease to flow when total Comal Springs flow is ~130 cfs and Spring Run 3 generally ceases to flow when Comal Springs total flow is about 50 cfs (LBG Guyton 2004). Modeling results suggest that discharge will be less than 120 cfs for a total of 127 months and less than 45 cfs for a total of 7 months during a repeat of the drought of record (in the 1950s) with Phase 1 of the HCP implemented (EARIP 2012). Modeling efforts also indicate that a repeat of the drought of record (with Phase 1 of the HCP fully implemented) will lead to the total flows in the Comal Springs system to be < 30 cfs for a two-month period (EARIP 2012). If flows drop below 30 cfs, it is expected the main spring runs in the system (Spring Runs 1 through 6) will be dry for a considerable time period and the

remaining aquatic habitat within the Comal Springs system will be limited to portions of Landa Lake and the Spring Island area. Cumulatively, this information indicates that it is possible for several if not most of the spring runs in the Comal system to cease flowing for extended periods of time (from months to years) and for a significant reduction of aquatic habitat to occur if there is a recurrence of the drought of record. In order to prevent permanent losses of these protected, sensitive, and geographically limited populations associated with very severe drought periods or environmental incidents (e.g., spill of toxic materials) in the Comal Springs system, the USFWS and the EAA have been tasked with the responsibility of maintaining populations of these organisms off-site; these populations can be used for reintroduction if the Comal Springs system is severely impacted. These "refuge" populations are also used to study aspects of the life history, habitat requirements, and ecology of these spring- and groundwater-dependent species.

The San Marcos Aquatic Resources Center (SMARC) is operated by the USFWS maintains populations of many of the aforementioned species and has performed a variety of research projects on maintaining captive populations and propagating them for long-term refuge purposes. However, there are still several substantial questions and issues associated with many of these taxa which currently impede the ability to maintain captive populations. Specifically, the USFWS can successfully hold Comal Springs riffle beetles (CSRB) in captivity but has experienced difficulties in refugium with low numbers of beetle larvae successfully pupating into adults. The USFWS (and other researchers) can maintain populations of both adults and larvae in captivity, but pupation rates are low and represent a substantial "bottleneck" in the production of F_1 individuals in refuge populations (i.e., captive populations are not self-sustaining). In addition, short-term (~2 month) experiments conducted by my lab in collaboration with BIO-West, Inc. indicated that pupation rates in captivity are typically low (<15% of late instar larvae pupate over this period). Given the need to maintain sustainable captive populations, there is clearly a need to examine factors which may contribute to the successful pupation of CSRBs in captivity.

CSRB Life History and Ecology

Riffle beetles (family Elmidae) are relatively small aquatic beetles that generally occupy well-oxygenated swift water habitats (i.e., riffles) which exhibit low water temperature variation. Larvae respire via gills and adults respire using a plastron (Brown 1987; White and Roughley 2008; Elliott 2008a) and this group is often considered to be sensitive to pollutants or environmental degradation (i.e., used as a water quality indicator group; Elliot 2008a). The CSRB is currently listed as federally endangered due to its limited geographic distribution and potential threats caused by declines in groundwater discharge through drought and/or groundwater extraction (USFWS 1997). CSRB adults are considered flightless due to vestigial/truncated wings and are therefore thought to have limited adult dispersal ability (Bosse et al. 1988). Thus, both larvae and adults largely restricted to crawling or drifting for dispersal, but later larval instars of some elmid species develop tracheal air sacs can control specific gravity and allow drift towards pupation sites (Brown 1987). Drift occurs mostly at night, most likely in response to gaining access to food resources or to escape sub-optimal conditions (Elliott 2008b; Brown 1987; Reisen 1977).

The USFWS has housed the CSRB and worked on establishing a captive breeding program since the mid-1990s; the methodological approach to establishing sustaining captive population has evolved over this time (Fries 2003). In addition, there has been a number of studies conducted in the wild and in the laboratory which have examined the life history (Huston and Gibson 2015; Worsham et a;. 2017), population and conservation genetics (Gonzales 2008), habitat associations (Bowles et al. 2003; Cooke et al. 2015), diet and trophic ecology (Nowlin et al. 2017a; Nair et al. 2021), and environmental tolerances (Nowlin et al. 2016; Nowlin 2017b; Nair et al. in prep). In particular, the study of the life history and trophic ecology of the CSRB has intensified over the last several years and has provided data regarding these aspects of this organism’s life cycle and diet (e.g., Worsham et al. 2017).

The current understanding of the life cycle of the CSRB follows a pattern that is largely consistent with that of other species of elmids (Brown 1987). However, there are several aspects of the CSRB life history which are unique or rare for members of Elmidae. Overall, the CSRB lifespan, from egg to adult is approximately 18 to 24 months (Worsham et al. 2017). In the lab, eggs are preferentially laid by adult females on well-conditioned leaf material, a coarse

particulate organic matter (CPOM) source that is commonly found in their wild habitats (Worsham et al. 2017; Nair et al. 2021). Females lay approximately 1 to 3 clutches of eggs, with each clutch composed of approximately 3 to 18 eggs (Worsham et al. 2017). However, it was found that the presence of poly-cotton cloths (the same cloths used as lures for monitoring populations in their wild habitats) also increased the rate of egg production. Eggs incubate for approximately three weeks and hatched larvae go through a series seven instars before pupation (Worsham et al. 2017). Progression through this series of instars takes approximately 4 months and captive larvae remain in the last instar stage for an extended period of time before pupation (at least 4 months; Worsham et al. 2017). Staying in a late instar stage for an extended period prior to pupation (e.g., several months to a year) is not unusual for elmids (Brown 1987; White and Roughley 2008). Pupation in the CSRB occurs under water (Huston and Gibson 2015; Worsham et al. 2017); unlike the CSRB, many elmid species exhibit pupation on terrestrial surfaces above the water's surface (Brown 1987; Elliott 2008a). After observing this underwater pupation in CSRB in the wild and in the lab, Huston and Gibson (2015) and Worsham et al. (2007) "forced" underwater pupation in the lab and both studies showed increased success with CSRB pupation.

Despite the fact that larval production is relatively successful in captivity and the realization that CSRB pupation occurs underwater, the rate of pupal production and the emergence/eclosion of adults in captivity remains low (Worsham et al. 2017). Pupation in late-stage CSRB larvae can be extremely slow or pupae die before adult emergence. Given these issues, establishment of a self-sustaining captive breeding program for the CSRB remains elusive. Thus, there is clearly a need to examine factors which could affect the rates and success of CSRB pupation in the lab. This series of experiments focused on two larger aspects of the life history and rearing of the CSRB in captivity. The first set of experiments (Year 1) was related to the supply of dietary items to CSRB larvae during their development in captivity. The second set of experiments (Year 2) examined the types of holding systems and whether developing larvae and pupae need access to a defined air space for successful eclosion to the adult stage.

Objective(s):

We proposed to examine several factors which may contribute to successful pupation and emergence of adult CSRB in a captive setting. Specifically, we propose to examine several factors in captivity and has two main research goals in Year 1:

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

2) Does the presence of conspecifics (CSRBs) on conditioning litter affect the quality and ultimately the pupation and adult eclosion rates of CSRBs in captivity?

In Year 2 of the project, we again had two main research questions:

1) Does the configuration and type of incubation chamber affect pupation rates and larval survival of the CSRB in captivity?

2) Does the frequency of handling of larvae and pupae of the CSRB increase larval and pupal mortality?

Significant Deviation(s):

There have been two lengthy and substantial delays during the two years of the work. During the first, a suspension of federal work (furlough) led to substantial delays in the start date of the project due to hiring full-time personnel. Due to university-dictated hiring timelines, I could not hire full-time personnel until the end of May 2020. Work began in earnest on Year 1 in late April 2020. We revised the schedule of deliverables and the project timetable in December 2019, with the intent to get all work finished by September 2020. In addition, we proposed a new set of experiments to be conducted during 2020 (Year 2).

However, the start of the COVID-19 pandemic starting in March 2020 caused Texas State University to cease all research activities from mid-March until mid-July 2020. This caused us to pause all remaining work from Year 1 as well as initiation of some Year 2 activities. As soon as

research was “re-opened” by the university in July 2020 and we had university-approved safety protocols, we were able to again resume work on Year 1 and 2 research. However, even though we were able to resume research activities in July 2020, there were strict rules on the number of people allowed in the lab/field at one time (50% reduction) and how students/technicians travel to and from SMARC and the field; the instituted safety guidelines allowed work to proceed, but at a much slower than anticipated rate. These two delays (furlough with hiring delays, COVID-19 pandemic) have cumulatively led to >8 months of “downtime” with little to no work. In addition, supply chain issues brought about by COVID-19 lead to substantial delays in the ordering and receiving of chemical reagents for biochemical analysis. Finally, the ice and snow storm in February 2021 led to delays in getting larvae for the final set of experiments and observations (i.e., observations of handling on pupae). We were able to obtain enough larvae for experiments by March 2021 to make these observations but had no pupations from March – June 2021. At this point, we needed to end experiments and begin data analysis. Thus, we did not accomplish the last objective due to the cumulative effect of delays and the need for USFWS to use the space at SMARC for other projects.

Methods

The overall goal of this proposed research is to examine how several factors affect pupation rates and the successful eclosion of adult CSRBs in a captive setting. The above questions were addressed in a series of laboratory and field-based experiments to address the four main research goals and questions.

Year 1 Experiments -

The experiments in Year 1 were conducted to specifically examine if the type and how CPOM is conditioned affects larval survival, pupation rates, and adult eclosion rates of CSRBs in captivity. In Year 1, we conducted two experiments. The first experiment examined how specific food types (wood and leaves versus wood, leaves and poly-cotton cloths) affect larval survival, pupation rates, and adult eclosion rates and the second experiment examined whether prior conditioning by conspecifics affected larval survival, pupation rates, and adult eclosion rates. There is growing evidence that in insects, prior conditioning by conspecifics may be important for the utilization of

food resources (Vogel et al. 2017) and that adults can expend substantial resources in parental care (Wong et al. 2013), including the act of prior conditioning of food materials (Capodeanu-Nagler et al. 2016).

Effect of food type and conditioning location

In the first experiment, we examined if the type and conditioning location of CPOM affected pupation rates and adult eclosion success in captive CSRBs. We placed terrestrial CPOM (leaves and wood) and poly-cotton cloths in the native habitat of CSRBs (Comal Springs) and in the SMARC Invertebrate Room to pre-condition prior to feeding to larvae. The experimental design consisted of two levels of food type (leaves and wood versus leaves, wood, and poly-cotton cloth) from two different locations (SMARC versus Comal Springs) that were cross-classified (yielding 4 treatment combinations).

Leaves consisted of pre-dried sycamore (*Platanus occidentalis*) leaves and wood consisted of *Poplar* dowels. Cloth material consisted of a 50-50 polyester-cotton cloth, which is the “standard” lure material used in the field to capture CSRBs. To condition materials prior to experiments, leaf packs (~5g of dried leaves in a 1-cm aperture leaf litter bag), wood (three 10 cm dowels plastic zip tied together), and poly-cotton cloths (enclosed in the standard wire cage used by the USFWS for monitoring and collection) were placed in their respective environments for 5-6 weeks to ensure growth of biofilms and conditioning. Materials in SMARC were housed in a plastic tub flow through chamber, while the “wild” conditioning was performed at several spring openings along Spring Runs 1 and 3 in Comal Springs. A full set of materials (wood, leaves, and poly-cotton clth) were simultaneously deployed at a single spring location. After the conditioning period, all materials were collected and immediately used as food sources in experiments. Materials collected at Comal were thoroughly examined to remove any invertebrates or eggs prior to experiments. A subset of materials was stored at -80^{o}C prior to processing and analyses for nutritional composition and microbial diversity.

As we began to remove food materials from Comal Springs, we anecdotally noticed that there were differing numbers of adults and larvae found on the different material types. Although the study was not initially designed to assess the capture differences among lure material types, we

began to count the numbers of larval and adult CSRBs found on each material type at each spring location. Since the three material types were simultaneously deployed at a single opening (9 spring openings in total) repeatedly over the study period, we decided to assess the effect of lure type on the collection numbers of beetles. In total, we collected 73 lures of each type from a single spring opening over the study period and enumerated the number of larval and adult CSRB found on each lure type at each location.

Three late-stage beetle larvae (5^{th} – 7^{th} instar inferred from head capsule width; Worsham et al. 2017) were placed in flow through PVC incubation chambers. Chambers were constructed of ½" PVC and were 20 cm long. Each of the four treatment combinations were replicated five times (one chamber = one replicate). Beetle larvae were gently placed into each chamber with the food materials and the chamber was sealed. Chambers were checked once per week for a 29-week period from July 2019 to February 2020. At each weekly check, the number of larvae surviving (of the 3 original) were enumerated and any pupae and adults were removed and enumerated. If a larva was "missing" or found dead, it was replaced with another late-stage larvae to keep the amount of interspecific competition consistent. Removed pupae and adults were also replaced with larvae. Food materials were replaced in the chambers every 5-6 weeks with the same type and origin materials.

In addition to the experiment, we also conducted nutritional and microbial analyses of the food sources supplied to the larvae during incubation. Leaves, wood and cloth materials from both locations (SMARC and Comal Springs) were taken to the lab and frozen at -80^{o}C until analysis. Material type from each location was processed for microbial composition and functioning by extracting DNA (using DN-Easy kits) and then Illumina metagenomic sequencing at Texas State University by Dr. Camila Carlos-Shanley.

Dietary material from each location was run in triplicate to analyze for bulk carbohydrate, protein, and lipid content. Different materials needed to be processed in a variety of ways to ensure homogenization and extraction of the biochemical components. Poly-cotton cloth was frozen and stored at -80^{o}C, freeze dried for 48 h. It was then shredded in a IKA sample mill and then ground with mortar and pestle under liquid N_2. *Poplar* wood dowels were frozen and stored

at -80^{o}C and surface shavings were taken from the exterior of the dowel using clean scalpel blades to exterior conditioned wood was removed. Shavings were then freeze dried for 48 h and ground with mortar and pestle under liquid N_2. Sycamore leaves were frozen at -80^{o}C, freeze dried for 48 h, then ground with mortar and pestle under liquid N_2. All processed materials were held at -80^{o}C until analysis.

In addition to food materials, we also analyzed adult and larval beetles from SMARC and from Comal Springs for nutritional status. We collected adult beetles using hand picking around spring openings at Spring Run 1 and 3. We did not find adequate numbers of larval beetles via hand picking in the field, so they were not analyzed from Comal. Adult and larval beetles fed SMARC conditioned materials were held in a plastic flow through chamber for a minimum of 8 weeks and then collected for biochemical analysis. A single sample for carbohydrates, protein, or lipids consisted of 7 adult beetles or 6 larvae and we were able to run 3 replicate samples of adults from SMARC and Comal and larvae from SMARC for each nutritional analyte. Beetles were frozen at -80^{o}C until analysis.

For carbohydrate analysis of food materials, ~20 mg of each dried, ground material was added to 1 mL of 1X Assay Buffer and centrifuged at 4C/10000 rpm for 10 minutes. The supernatant was removed and stored at -20C until analysis. Total carbohydrates were quantified using a CellBiolabs Total Carbohydrate Assay Kit. For each adult sample (*n* = 7 individuals) and larval sample (*n* = 6 individuals), tissue was homogenized in phosphate buffer solution (pH = 7.4) in triplicate and total carbohydrates were quantified using a CellBiolabs Total Carbohydrate Assay Kit.

Protein was extracted from food materials (~25 mg material per replicate) according to Rinalducci (2011). The resulting protein pellet was stored at -80^{o}C until analysis. Prior to analysis, the protein pellet was resuspended in 1,200µL of 1xPBS (7.4 pH). A Bradford protein assay kit (Thermo Fisher Scientific) was used to analyze the resulting pellets under the “low” working range (1-25 µg/mL). Beetle tissue was homogenized in phosphate buffer solution (pH = 7.4) in triplicate and run using a Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific). Analysis performed using the (Working Range = 125–1500µg/mL).

Lipids from food materials (~25 mg material per replicate) were extracted using a modified Folch method. The extracts were suspended in 100µL of DMSO and quantified using the Cell Biolabs lipid quantification kit (STA-613). Lipid content of adult and larval samples were determined by tissue homogenization in 2:1 chloroform-methanol (v/v) in triplicate and lipids were extracted using a modified procedure by the Folch method and quantified using a lipid quantification kit (Cell Biolabs, STA-613).

Effect conditioning by conspecifics

The second goal will assess if the presence of CSRBs themselves affects effects of food source pre-conditioning in the presence of conspecifics on larval mortality, pupation rates, and adult eclosion rates. Traditionally, CPOM sources and cloths used to feed captive CSRBs are prepared in the lab by conditioning these materials in the lab with no beetles present. Once adequately conditioned (>5 weeks), these materials are provided to larval and adult CSRBs. However, aquatic organisms contain diverse but specific gut bacterial assemblages which are adapted to deal with the food sources of the organism (Ayayee et al. 2018). In addition, the feeding activities of biofilm grazers in streams can alter the structure, composition, and biogeochemistry of the biofilms that they are grazing (e.g., Cooney and Simon 2009; Veach et al. 2018). At the present time, we do not know if the grazing activities of CSRB adults and larvae facilitate their own use of these OM sources. This experiment pre-conditioned food materials in the lab in the presence versus absence of adult CSRB and then fed these materials to late-stage CSRB instars to determine if pupation and adult eclosion rates.

The experimental design consisted of two levels of food type (wood and leaves vs wood, leaves, and poly-cotton cloth) cross-classified with two levels of conditioning (absence of conspecifics versus presence of conspecifics during pre-conditioning). Materials of each type were held in SMARC in the Invertebrate Room and conditioned for a 5–6-week period prior to use in experiments. Food materials of each type were held in plastic flow-through tubs for conditioning. The treatment that had conditioning in the presence of conspecifics was held in a flow-through tub with at least 40 adult CSRBs that were allowed to utilize the material. When material was removed for experiments, it was carefully examined to ensure no CSRBs were attached to

material prior to use in experiments.

Like the previous experiment, three late-stage beetle larvae (5^{th} – 7^{th} instar inferred from head capsule width) were placed in flow through PVC incubation chambers. Chambers were constructed of ½" PVC and were 20 cm long. Each of the four treatment combinations were replicated five times (one chamber = one replicate). Beetle larvae were gently placed into each chamber with the food materials of each conditioning type and the chamber was sealed. Chambers were checked once per week for a 19-week period from December 2019 to late April 2020. At each weekly check, the number of larvae surviving (of the 3 original) were enumerated and any pupae and adults were removed and enumerated. If a larva was "missing" or found dead, it was replaced with another late-stage larvae to keep the amount of interspecific competition consistent. Removed pupae and adults were also replaced with larvae. Food materials were replaced in the chambers every 5-6 weeks with material of the same type and conditioning origin.

Data analyses

Year 1 experiments were assessed through a variety of statistical methods to assess for the effects of food type and conditioning origin on beetle survival and development. For the first experiment (*Effect of food type and conditioning location*) we assessed larval survival by calculating the mean weekly larval mortality for each chamber over the 29-week period (each chamber was an independent replicate). Differences among treatment combinations was assessed with a two-way ANOVA, which yields a main effect of food type and food origin as well as an interaction term. All proportional data were arcsine-square root transformed prior to analysis. The number of pupae and adults produced per chamber in each treatment combination was assessed with a Generalized Linear Model (GzLM) approach because count data are often over dispersed and not normally distributed (Bolker et al 2008; Lynch et al. 2014). The GzLM used a log-link function with a negative binomial distribution (Linden and Mantyniemi 2011). The factors in the analysis were food type and conditioning location. Significance was inferred at $\alpha \leq 0.05$ for all analyses.

Biochemical composition (proteins, lipids, and carbohydrates) of leaves, wood, and poly-cotton

cloth were assessed with a two-way ANOVA for cross-classified factors using food type (leaves, wood, and cloth) and conditioning status and location (SMARC, Comal Springs, and Unconditioned) as categorical factors in the analysis. Data were assessed for normality and homoskedasticity prior to analysis. Significance was inferred at $\alpha \leq 0.05$ for all analyses. Beetle biochemical content was assessed with a one-way ANOVA comparing Comal Springs adults, SMARC larvae, and SMARC adults. Data were assessed for normality and homoskedasticity prior to analysis and significance was inferred at $\alpha \leq 0.05$.

The number of CSRB adults and larvae found on different lure types simultaneously deployed at spring locations were analyzed with a GzLM approach due to the large number of zeros in the data and overdispersion. The GzLM used a log-link function with a negative binomial distribution and the factors in the analysis were lure type (wood, leaves, poly-cotton cloth) and spring run location (Spring Run 1 versus Spring Run 3). If a significant effect of a factor was detected, post-hoc pairwise comparisons were conducted to determine where the differences lay. Significance was inferred at $\alpha \leq 0.05$ for all analyses.

For the second experiment (*Effect conditioning by conspecifics*) we assed larval survival by calculating the mean weekly larval mortality for each chamber over the 19-week period (each chamber was an independent replicate). Differences among treatment combinations was assessed with a two-way ANOVA, which yields a main effect of food type and food origin as well as an interaction term. All proportional data were arcsine-square root transformed prior to analysis. The number of pupae and adults produced per chamber in each treatment combination was assessed with a GzLM with a log-link function and a negative binomial distribution. The factors in the analysis were food type and conditioning status (presence versus absence of conspecifics during conditioning). Significance was inferred at $\alpha \leq 0.05$ for all analyses.

Year 2 Experiments -

The experiments in Year 2 were conducted to specifically examine if the type and handling frequency of larvae in captivity affected larval survival, pupation rates, and adult eclosion rates

of CSRBs in captivity. In Year 2, we conducted two sets of longer-term experiments: (1) the effect of a presence of an air space at the top of the larval rearing chamber, and (2) the frequency with which larvae are checked for survival and the presence of pupae and adults. These questions were addressed in laboratory experiments conducted at SMARC.

Effects of air spaces on CSRB development

This experiment examined if CSRB larvae had pupation and pupal survival rates when provided with access to an air-waiter interface within growth chambers. Late-instar CSRB larvae were placed into two types of chambers: chambers with a defined air-water interface versus more standard growth chambers that do not have defined air-water interface areas. The experimental design consisted of a comparison of two treatments: larvae reared in a more “traditional” flow through chamber versus larvae reared in a growth chamber that was specifically designed to have a top area that facilitated the presence of air-filled gaps and spaces.

Three late-stage beetle larvae (5^{th} – 7^{th} instar inferred from head capsule width) were placed in flow through PVC incubation chambers. All chambers were constructed of ½” PVC and were 20 cm long (Fig. 1). The more traditional chamber had flow coming up from the bottom of the 20 cm long chamber (simulating an upwelling) and flowing up and out of the top of the chamber. The ends of the chamber were covered with 100 μm mesh to prevent the escape or loss of CSRBs. The air interface chamber also simulated an upwelling, but the outflow of the chamber was at a 90^{o} angle (perpendicular) to the flow and there was a space created by rolling and wadding up 1 cm mesh plastic netting and 100 μm Nitex netting to create air voids at the top of the chamber (Fig 2). The two treatments were replicated five times (one chamber = one replicate). Beetle larvae were gently placed into each chamber with food materials (a mix of leaves, wood, and poly-cotton cloth pre-conditioned at SMARC) and the chamber was sealed. Chambers were checked once every two weeks for a 20-week period from July 2020 to November 2020. At each bi-weekly check, the number of larvae surviving (of the 3 original) were enumerated and any pupae and adults were removed and enumerated. If a larva was “missing” or found dead, it was replaced with another late-stage larvae to keep the amount of interspecific competition consistent. Removed pupae and adults were also replaced with larvae. Food materials were

replaced in the chambers every 6 weeks with the same type and origin materials.

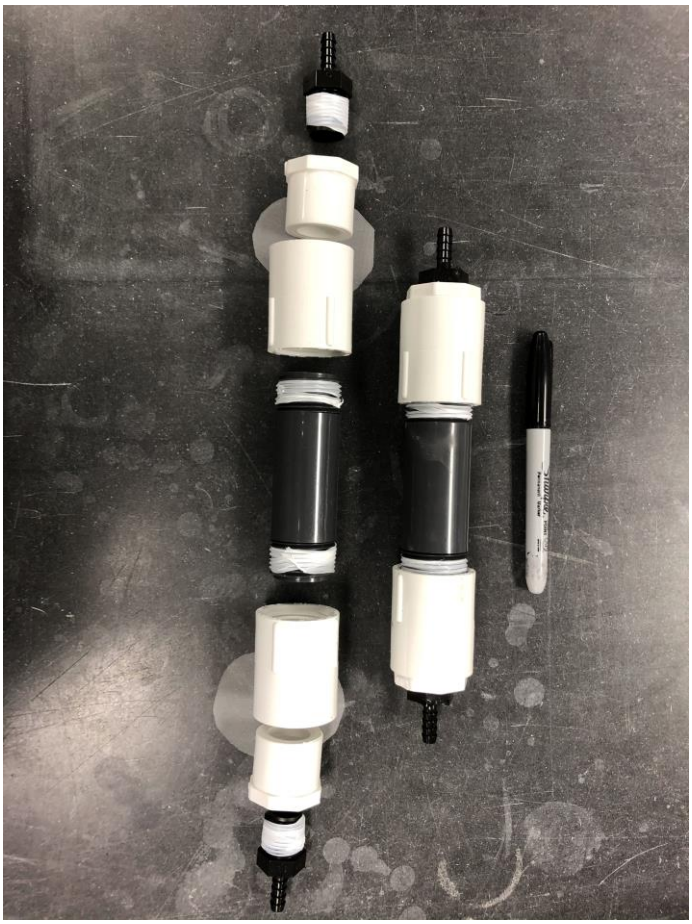

Figure 1. Structure and size of an upwelling flow-through chamber used in the air space access experiments. Note the pieces of 100 mm Nitex mesh used at the ends to prevent escape or loss of CSRBs.

Figure 2. Structure and size of an upwelling flow-through chamber that maintained an air-water interface used in the air space access experiments. Note the pieces of 100 mm Nitex mesh used at the ends to prevent escape or loss of CSRBs and to create an air-water interface.

Effects of frequency of handling on CSRB development

The second experiment examined if the frequency of handing and assessing beetle larvae and adults affected their survival and development, particularly whether this affected the number of pupae and adults produced in captivity. The experimental design consisted of two treatments: (1) larvae that were housed in a flow through chamber that were checked once every two weeks (bi-weekly), and (2) larvae that were housed in a flow through chamber that were checked once every month.

Three late-stage beetle larvae (5^{th} – 7^{th} instar inferred from head capsule width) were placed in

flow through PVC incubation chambers. All chambers were constructed of ½" PVC and were 20 cm long. The 20 cm long chamber had flow coming up from the bottom (simulating an upwelling) and flowing up and out of the top of the chamber. The ends of the chamber were covered with 100 μm mesh to prevent the escape or loss of CSRBs. The two treatments were replicated five times (one chamber = one replicate). Beetle larvae were gently placed into each chamber with food materials (a mix of leaves, wood, and poly-cotton cloth pre-conditioned at SMARC) and the chamber was sealed. Chambers were checked once every two weeks or once a month for an 18-week period from June 2020 to October 2020. At each bi-weekly or monthly check, the number of larvae surviving (of the 3 original) were enumerated and any pupae and adults were removed and enumerated. If a larva was "missing" or found dead, it was replaced with another late-stage larvae to keep the amount of interspecific competition consistent. Removed pupae and adults were also replaced with larvae. Food materials were replaced in the chambers every 6 weeks with pre-conditioned materials of the same type.

Data analyses

Year 2 experiments were designed to assess larval survival and development. For the first experiment (*Effects of air spaces on CSRB development*) we assessed larval survival by calculating the mean weekly larval mortality for each chamber over the 20-week period (each chamber was an independent replicate). Differences among treatment combinations was assessed with a one-way ANOVA. All proportional data were arcsine-square root transformed prior to analysis. The number of pupae and adults produced per chamber in each treatment combination was assessed with ANOVA, with the independent variable in the analysis being larval rearing chamber type. Pupal count data were normally distributed in this experiment and we did not have many zeros in the data, thus we elected to use ANOVA rather than a GzLM approach. Significance was inferred at $\alpha \leq 0.05$.

For the second experiment (*Effects of frequency of handling on CSRB development*) we assed larval survival by calculating the mean larval mortality for each chamber during each inter-check time interval over the 19-week period (each chamber was an independent replicate). Differences among treatment combinations was assessed with a one-way ANOVA. All proportional data

were arcsine-square root transformed prior to analysis. The number of pupae and adults produced per chamber in each treatment combination was assessed with a GzLM with a log-link function and a negative binomial distribution. The factor in the analysis was handling frequency (bi-weekly versus monthly checking). Significance was inferred at $\alpha \leq 0.05$.

Results and Discussion

Year 1 Experiments

Effects of food type and conditioning location

Larval survival, pupal production, and adult eclosion - Mean weekly larval mortality varied from ~10-20% across all treatment types [SMARC vs Comal for wood and cloth (WL) and wood, leaves and cloth (WCL)] (Fig. 3). Weekly average larval proportional mortality did not differ between sites and food types (Table 1). Over the course of the experiment, we generated many total pupae (53 pupation events out of ~200 larvae used in experiment); however, there was substantial mortality associated with pupae in captivity and only three viable adult were produced.

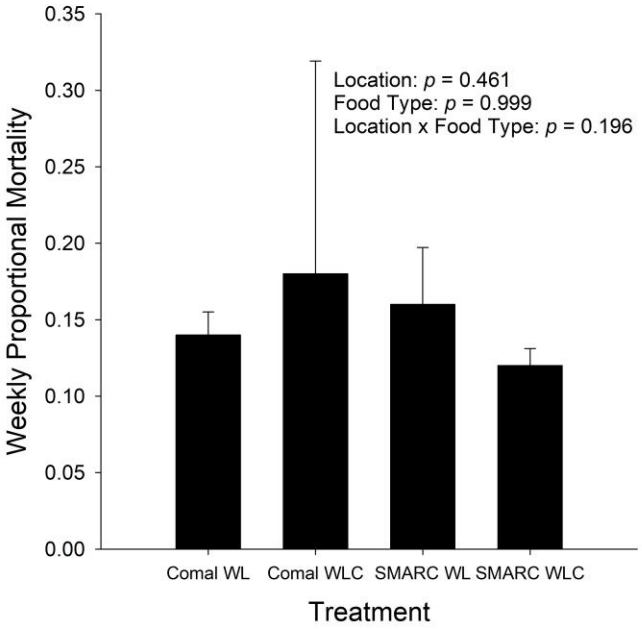

Figure 3. Mean weekly larval mortality (proportion of larvae in a chamber dying) for the four treatment combinations during the *Effects of food type and conditioning location* experiment. WL = Wood and Leaves, WLC = Wood, Leaves, and Cloth.

Table 1. Results of two-way ANOVA on the mean weekly larval mortality (proportion of larvae in a chamber dying) in the Biofilm Origin and Type Experiment.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Site	0.004	1	0.004	0.570	0.461
Food Type	<0.001	1	<0.001	<0.001	0.999
Interaction	0.0115	1	0.012	1.817	0.196
Within	0.102	16	0.006		
Total	0.117	19			

The omnibus test of the GzLM for pupal counts among treatment combinations (comparing the fitted model against the intercept-only model) indicated that there was not an effect of either food type or site of conditioning in predicting the number of pupae produced (Likelihood ratio X^2

= 1.200; *df* = 1; *p* = 0.273). Similarly, there was no effect of food type or site conditioning on the number of adults produced during the experiment (Likelihood ratio X^2 = 0.296; *df* = 1; *p* = 0.586).

Microbial community composition and diversity - Food item microbial metagenomes from the three different substrate types (cloth, leaves, and wood) from three locations (SMARC, Spring Run 1, and Spring Run 3) are presented in Table 2. More than 5,200 microbial genera were detected across all the samples, belonging to 204 different phyla, including Proteobacteria (26% of all sequences), Bacteroidetes (8.2%), Actinobacteria (5.7%), Ascomycota (5.7%), and Planctomycetes (5.4%).

Two-way permutational multivariate analysis of variance (PERMANOVA) of microbial communities on the conditioned food items using Jaccard distance (*J'*) indicated that both substrate type and location contribute to the distribution of microbial genera in the communities (Table 3). Because of the somewhat limited sample size, the interaction between substrate type and location was not significant.

Table 2. Metagenome data set general information for the conditioned food material types from the study locations. Cloth = poly-cotton cloth, wood = Poplar wood dowels, leaf = sycamore leaves. SMARC = conditioned at SMARC, SR1 = conditioned at Spring Run 1 in Comal, and SR3 = conditioned at SR3 in Comal. The total number of reads for each sample are presented.

Sample name	Substrate	Location	Total number of reads
SMARCC1	Cloth	SMARC	8867760
SMARCC2	Cloth	SMARC	5515358
SMARCL1	Leaf	SMARC	6522590
SMARCL2	Leaf	SMARC	3880166
SMARCW1	Wood	SMARC	7933463
SMARCW2	Wood	SMARC	5051864
SMARCW3	Wood	SMARC	9912417
SR1C1	Cloth	SR1	7972914
SR1C3	Cloth	SR1	15243934
SR1L1	Leaf	SR1	10892836
SR1L2	Leaf	SR1	8775399
SR1L3	Leaf	SR1	4876957
SR1W1	Wood	SR1	4270233
SR1W2	Wood	SR1	7719740
SR1W3	Wood	SR1	9119414
SR3C1	Cloth	SR3	6227791
SR3C2	Cloth	SR3	9057926
SR3L1	Leaf	SR3	7807592
SR3L3	Leaf	SR3	12504091
SR3W1	Wood	SR3	6094080
SR3W2	Wood	SR3	11913797
SR3W3	Wood	SR3	12732015

Table 3. Two-way PERMANOVA using Jaccard distances based on shared microbial genera (Permutations = 9,999).

Source	Sum of sqrs	df	Mean square	F	p
Substrate	0.012435	2	0.0062174	1.149	0.0013
Location	0.013812	2	0.0069062	1.2763	0.0001
Interaction	0.002153	4	0.00053825	0.099469	0.1133
Residual	0.070346	13	0.0054112		
Total	0.098746	21			

Comparison of the microbial diversity indicated that materials conditioned on SMARC had a significantly higher Dominance index and lower Shannon index than the biofilms conditioned at Spring Runs 1 and 3 (Fig. 4). This result indicates that materials conditioned in the field had substantially higher microbial diversity that was not dominated by a few taxa.

Using differential gene expression analysis (based on the negative binomial distribution), we found 130 microbial genera that were differentially distributed between the wood biofilms from the three sampling locations. The most differentially abundant genus, *Chrysochromulina*, a eukaryotic phytoplankton belonging to the Haptophyta clade (Fig. 5).

These taxonomic diversity differences translated to differences in the microbial functional diversity at each location. Comparative functional analysis of the genes present in conditioned wood from SMARC versus Comal Springs revealed differences in the nitrogen (N) pathway (Fig. 6). Wood biofilms from SMARC lack genes involved in converting nitrite to nitrogen, but contain the genes involved in the N_2 fixation to ammonia (biological reduction of N_2). If these pathways are active in microbial communities, wood biofilms conditioned at SMARC may have higher nitrate + nitrite concentrations than wood biofilms conditioned at the springs, leading to differences in nitrogen metabolism found at each location.

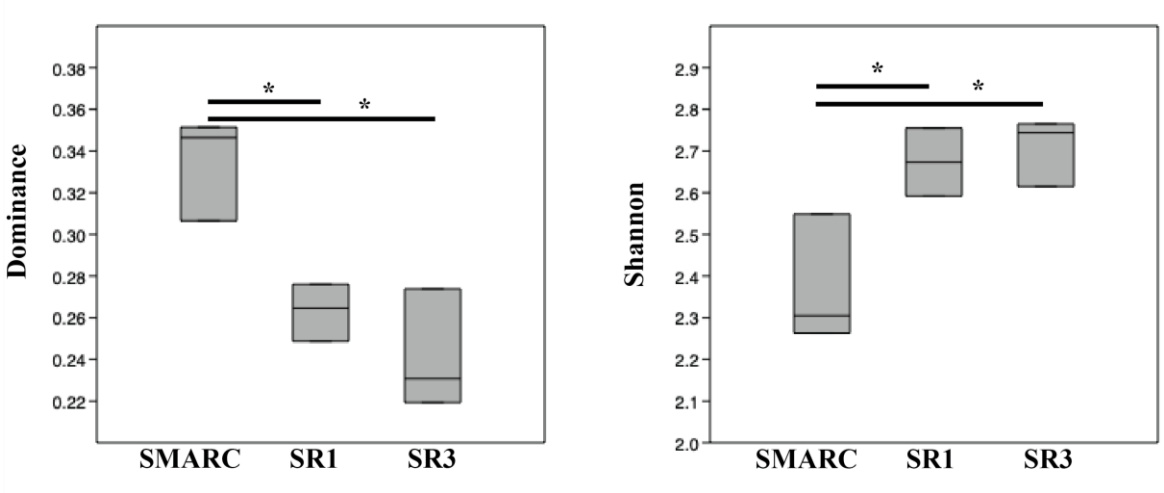

Figure 4. Diversity of microbial genera in wood biofilms conditioned at SMARC, Spring Run 1(SR1) and Spring Run 3 (SR3). Significant comparisons are marked with an asterisks (Tukey's range test, $p<0.05$).

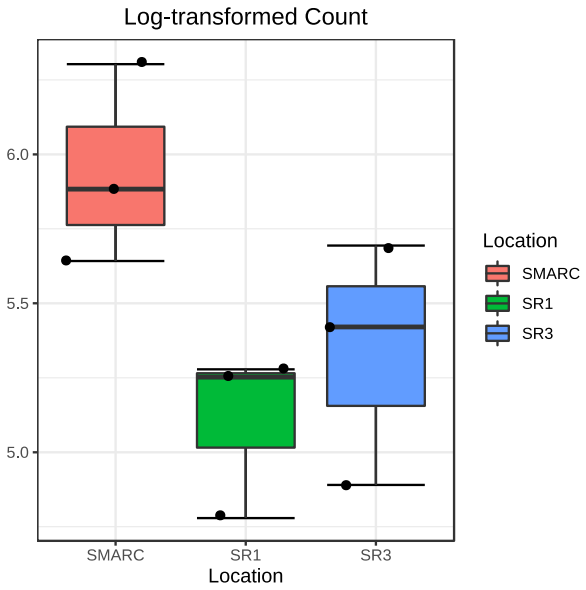

Figure 5. Boxplot of the abundance of *Chrysochromulina* spp. in wood biofilms conditioned at SMARC, Spring Run 1(SR1) and Spring Run 3 (SR3). FDR calculated using DESeq2 is 5.5246E-20.

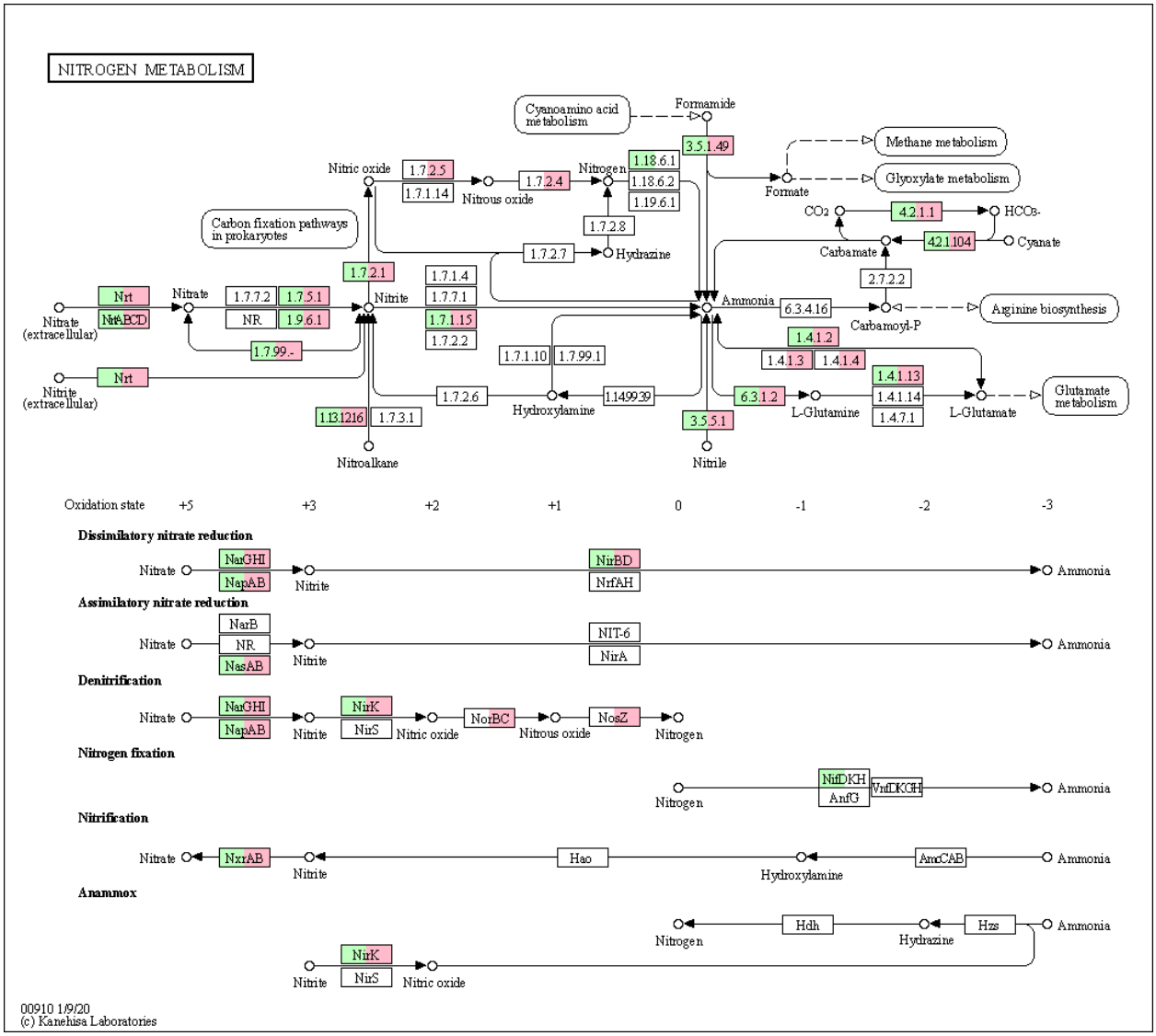

Figure 6. Map of nitrogen metabolism in wood microbial communities. Enzymes encoded by genes present in the wood conditioned at SMARC are shown in green, and enzymes encoded by genes present in the wood conditioned at Comal Springs are shown in red.

The biochemical content of the various potential food items from each location differed substantially (Figs. 7, 8, and 9). Overall, there was a difference among the various food substrate types in their carbohydrate content (Fig. 7), with both leaves and wood having higher carbohydrate content than poly-cotton cloth. However, the carbohydrate content of materials was not affected by the location of the conditioning. There was a significant substrate type x site

interaction, indicating that the carbohydrate content of leaf material greatly declined when it was conditioned, likely through leaching losses or microbial use.

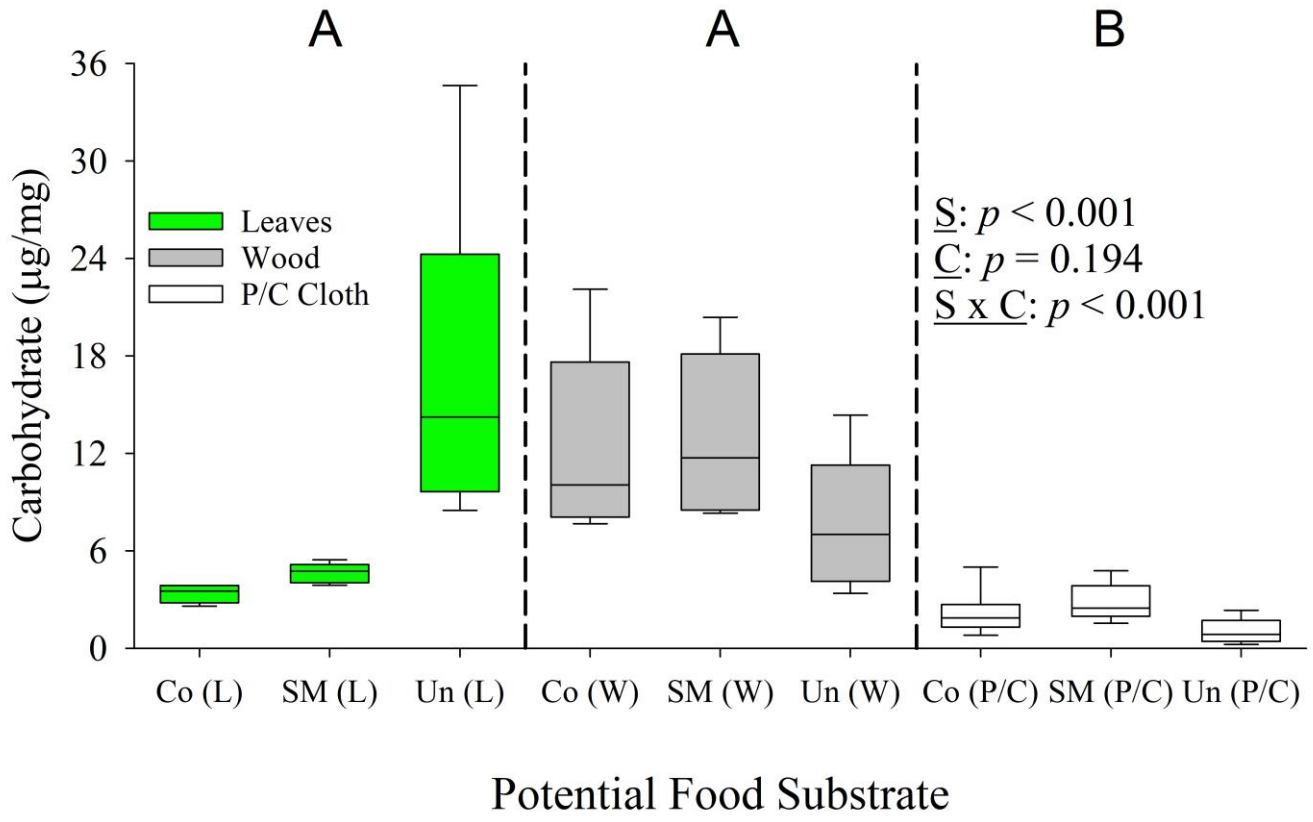

Figure 7. Box plots of carbohydrate content of food substrate materials (L = Leaves, W = Wood, and P/C = Poly-Cotton cloth) from Comal (Co), SMARC (SM), and unconditioned material (Un). Letters above the groupings indicate significant differences and homogenous subsets as determined by post-hoc Tukey's HSD tests. Overall effects of the two-way ANOVA are presented as S = substrate effect, C = Condition effect, and the Substrate x Conditioning interaction (S x C).

Lipid content also differed among the substrate types, with poly-cotton cloth material having little lipid content (Fig. 8). There was not a significant effect of conditioning location/status on lipid content, nor was there an interaction between food substrate type and conditioning location/status.

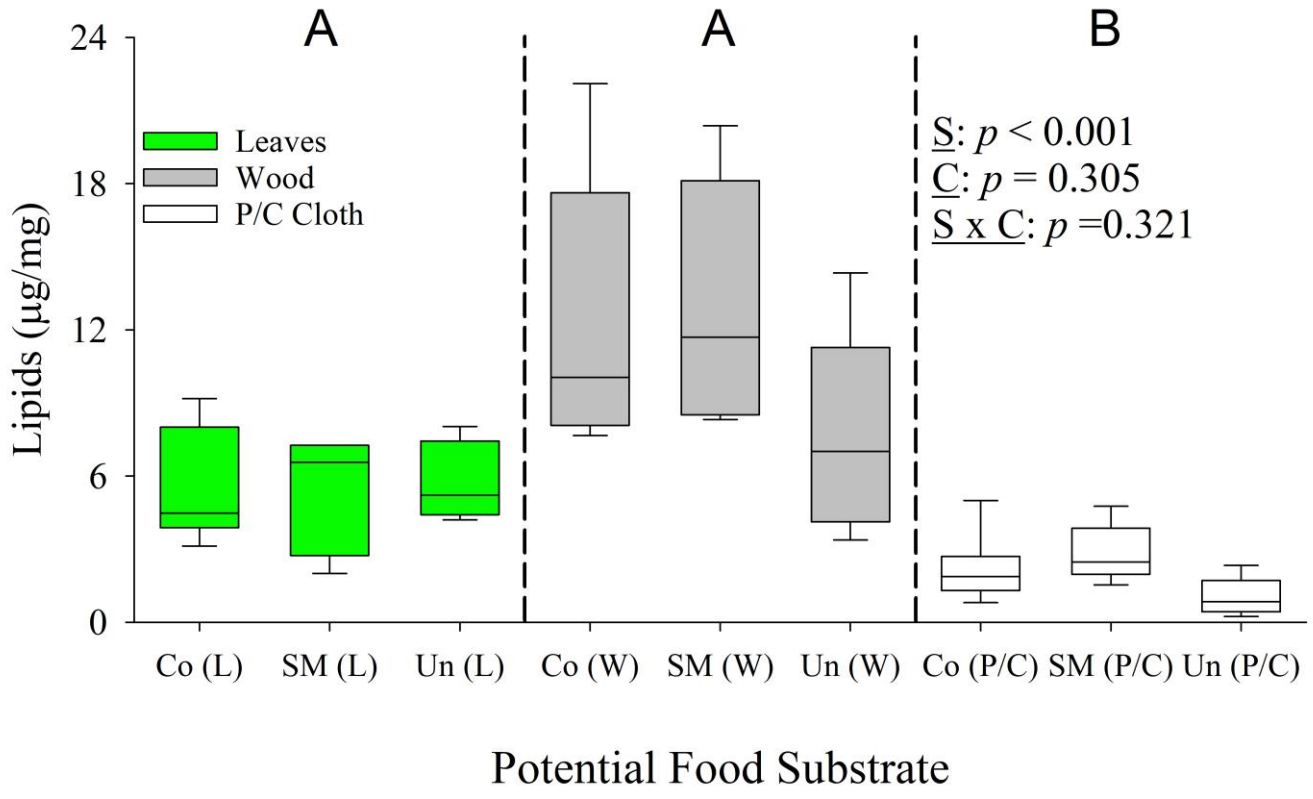

Figure 8. Box plots of lipid content of food substrate materials (L = Leaves, W = Wood, and P/C = Poly-Cotton cloth) from Comal (Co), SMARC (SM), and unconditioned material (Un). Letters above the groupings indicate significant differences and homogenous subsets as determined by post-hoc Tukey's HSD tests. Overall effects of the two-way ANOVA are presented as S = substrate effect, C = Condition effect, and the Substrate x Conditioning interaction (S x C).

Protein content of all the materials was low and was < 0.35 µg/mg for all potential food items (Fig. 9). Protein content differed among the food item types and there was a significant effect of conditioning location/status and interaction effect. Leaves had the lowest protein content of all food items, and it did not vary with conditioning status and location for this food item. However, both wood and poly-cotton cloth demonstrated different response patterns than leaves to conditioning location and status. Conditioning wood material appears to cause a steep decline in

protein content (via leaching and/or microbial use), whereas poly-cotton cloth material appears to have an increase in protein content when it is conditioned (perhaps via microbial colonization of the cloth).

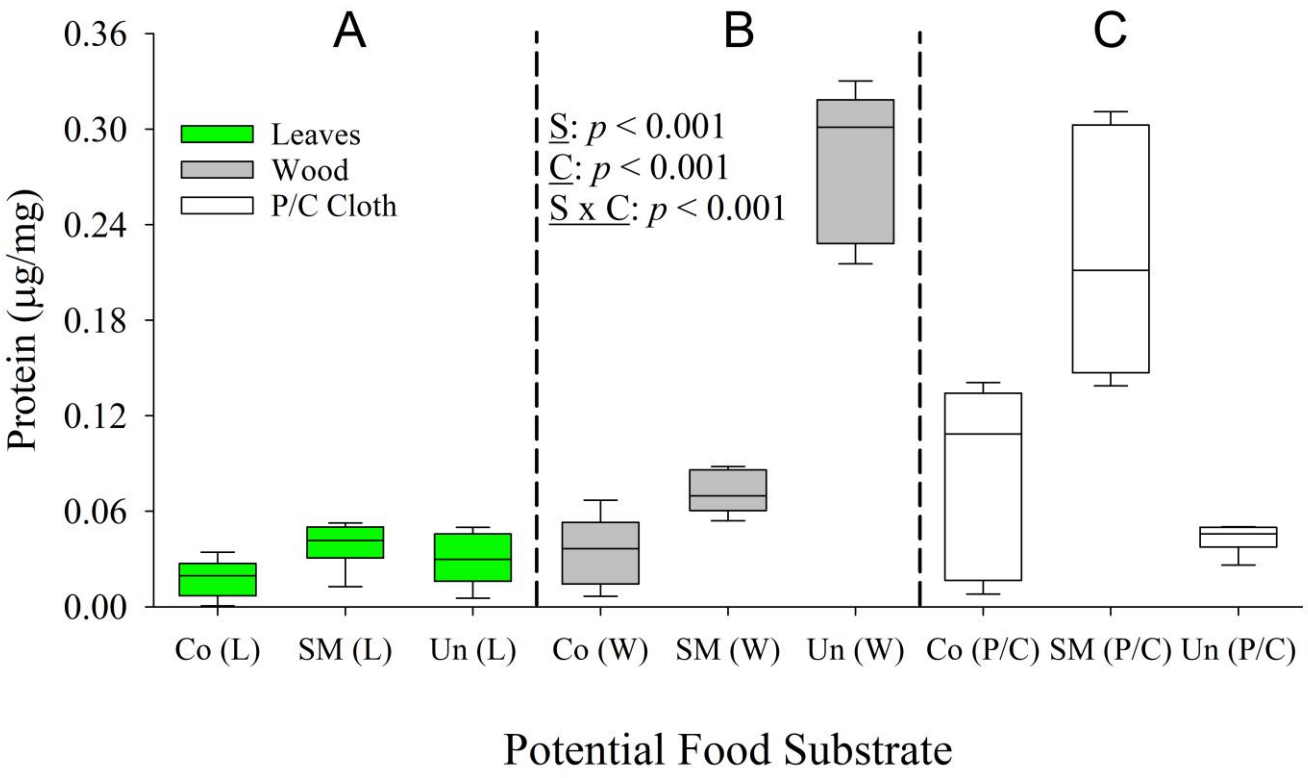

Figure 9. Box plots of protein content of food substrate materials (L = Leaves, W = Wood, and P/C = Poly-Cotton cloth) from Comal (Co), SMARC (SM), and unconditioned material (Un). Letters above the groupings indicate significant differences and homogenous subsets as determined by post-hoc Tukey's HSD tests. Overall effects of the two-way ANOVA are presented as S = substrate effect, C = Condition effect, and the Substrate x Conditioning interaction (S x C).

The biochemical composition of adult CSRBs from Comal and those that had been at SMARC for >8 weeks feeding on SMARC-conditioned materials did not differ in their carbohydrate content (Fig. 10A). In addition, SMARC late-stage larvae did not differ from both adult groups in carbohydrate content.

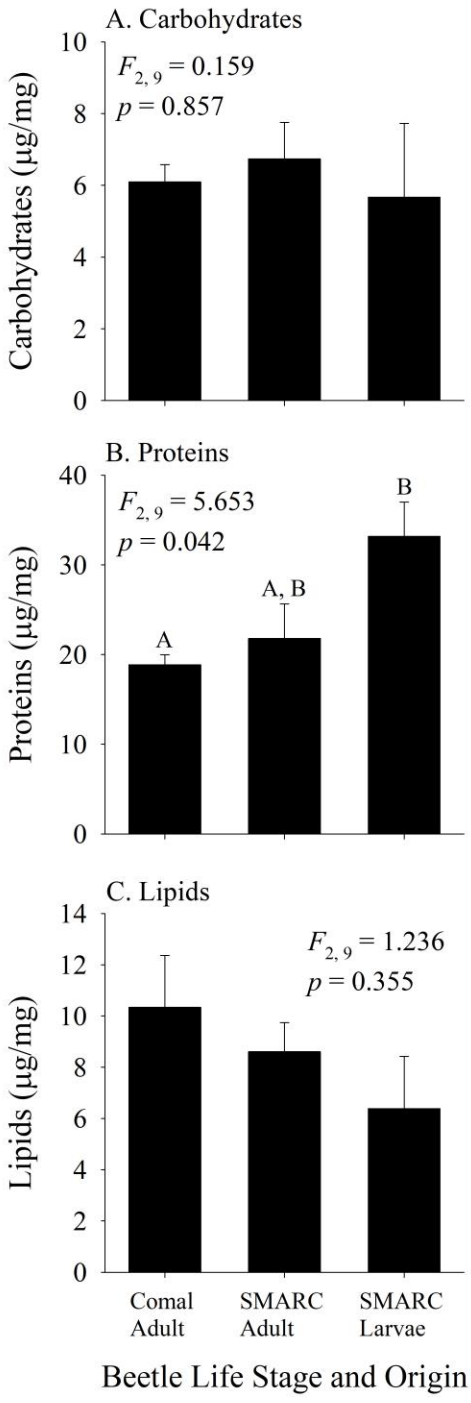

Figure 10. Bar graphs of the carbohydrate content (A), protein content (B), and lipid content (C) of adult beetles from Comal Springs, adults from SMARC, and larvae from SMARC. Overall effects of the ANOVA are presented, including the *F*-ratio, *df*, and *p*-value. Letters above the bars indicate homogeneous subsets determined by post-hoc Tukey's HSD tests.

Protein content significantly differed among the beetle groups, with SMARC larvae having higher protein content than adults found at Comal (Fig. 10B). However, SMARC adults were intermediate in their protein content when compared to Comal adults and SMARC larvae. This result suggests that there might be some site-specific protein differences and future studies should seek out and collect a larger number of late-stage larvae from Comal Springs to determine if larval protein content differs among the larvae held at SMARC. However, hand collection of late-stage larvae is challenging in the field and obtaining adequate numbers without the use of lures presents substantial challenges.

Overall, these results indicate that the inclusion of poly-cotton cloth does not increase or alter the survival of larvae in rearing chambers and that the location of pre-conditioning does not influence those response variables as well. Thus, these results indicate that the use of SMARC-generated organic matter does not have a discernible effect on the ability of larvae and pupae and does not differ greatly in its biochemical and nutritive value when compared to materials conditioned in the wild. However, these results also indicate that the microbial communities found at SMARC are taxonomically and functionally different from those found at Comal and it remains unknown if these compositional and functional differences are influencing the survival and development of CSRB at SMARC.

Use of different lure types to capture CSRB

Although this study did not set out to assess the efficacy of different lure types to capture adult and larval CSRBs in the wild, we opportunistically utilized the design of our field collections to assess if we caught different numbers of beetles on the three food substrate types we used.

When lure type (wood, leaves, poly-cotton cloth) and spring run (SR1 and SR3) are used as factors in a GzLM, the overall omnibus model was significant (Likelihood ratio X^2 = 75.02; df = 3; $p < 0.001$), with both lure type (W_T = 15.73, df = 2, $p < 0.001$) and spring run (W_T = 22.96, df = 1, $p < 0.001$) being significant in the model (Fig. 11). Post hoc comparisons using a Bonferroni correction indicated that leaf lures generated almost 3x as many adult beetles (5.8 adult beetles/lure) as poly-cotton cloth lures (2.22 adult beetles/lure). Poplar dowels were intermediate to leaves and poly-cotton cloth for adult numbers. Interestingly, Spring Run 1

produced almost 3x as many adult beetles (estimated marginal mean = 5.75 adult beetles per lure) across all lure types than Spring Run 3 (estimated marginal mean = 2.18 adult beetles per lure). However, differences among spring runs in Comal were not large focus of this report, so we do not show those results here.

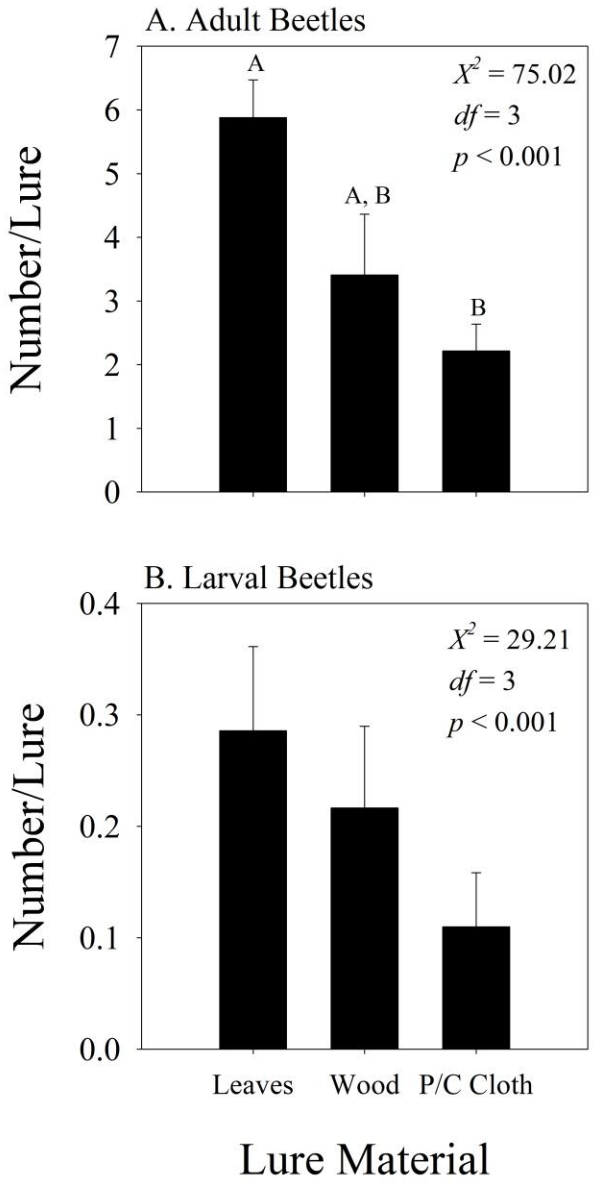

Figure 11. Bar graphs showing the estimated marginal means for adult (A) and larval (B) CSRB found on the three different lure types simultaneously deployed at a spring opening. Omnibus GzLM results are presented, including likelihood X^2 ratio, *df*, and *p*-value. Letters above the bars

indicate homogeneous subsets determined by post-hoc tests.

Effects of grazing by conspecifics on CSRB development

In the second Year 1 experiment, we found that neither the presence of grazing by conspecifics or the food type (presence of poly-cotton cloth) had an effect on larval survival (Fig. 12). In addition, the overall pupation rates and adult eclosion rates were very low; there were only 4 pupae generated across all treatment combinations and only 1 adult beetle emerged during the experiment.

Although pupation rates and adult eclosion were rare in this experiment, we used a GzLM approach (with a binomial distribution with 0 = no pupae or adults produced and 1 = pupae or adults produced) to examine if chamber type affected these responses. Overall, pupation was not dependent upon food type or prior conditioning by conspecifics (Likelihood ratio X^2 = 6.27; *df* = 3; *p* = 0.099) and neither was adult eclosion (Likelihood ratio X^2 = 2.937; *df* = 3; *p* = 0.402).

These results (in combination with the previous experiment) demonstrate three things: (1) the presence of poly-cotton cloth as a food source has no benefit or detriment to the development of beetles, (2) the presence of conspecifics during conditioning of OM does not appear to be necessary for larval development, and (3) beetle pupation and adult eclosion are highly variable among experiments and is hard to predict.

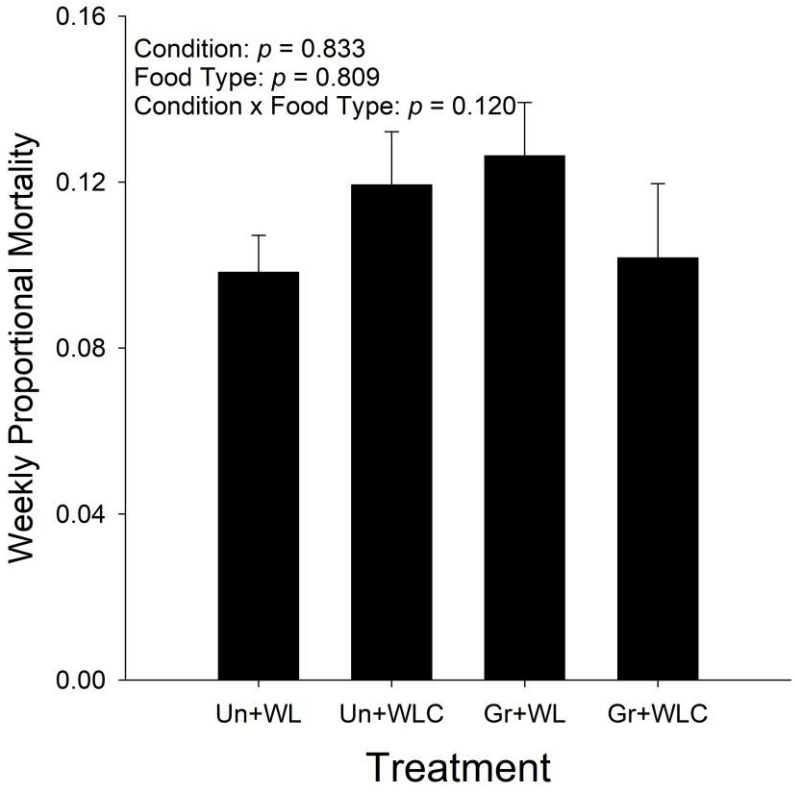

Figure 12. Bar graphs showing mean weekly larval mortality in the experiments examining the effects of food type (WL = wood and leaves, WLC = wood, leaves, and poly-cotton cloth) and prior conditioning (Un = conditioning with no presence of conspecifics, Gr = conditioning in the presence of conspecifics). Two-way ANOVA results are also presented in the figure.

Year 2 Experiments

The experiments in Year 2 examined if the type and handling frequency of larvae in captivity affected larval survival, pupation rates, and adult eclosion rates of CSRBs in captivity.

Effects of air spaces on CSRB development

In experiment examining the effects of the presence of an air-water interface in chambers, pupal mortality rates were considerably higher in both treatments than in the previous experiments in Year 1 (20 – 40% bi-weekly mortality). Although larvae were handled in the same way as they

were in previous experiments, the increased mortality may be due to a variety of reasons, including disease (no obvious physical issues with larvae to myself or students), change in SMARC personnel maintaining larvae, and/or malnutrition. In line with our predictions, the chambers with air-water interfaces had significantly higher larval mortality rates than those held in the “old” chamber types (Fig. 13); the air-water interface chambers had >10% higher bi-weekly mortality than the upwelling chambers with no air-water interface.

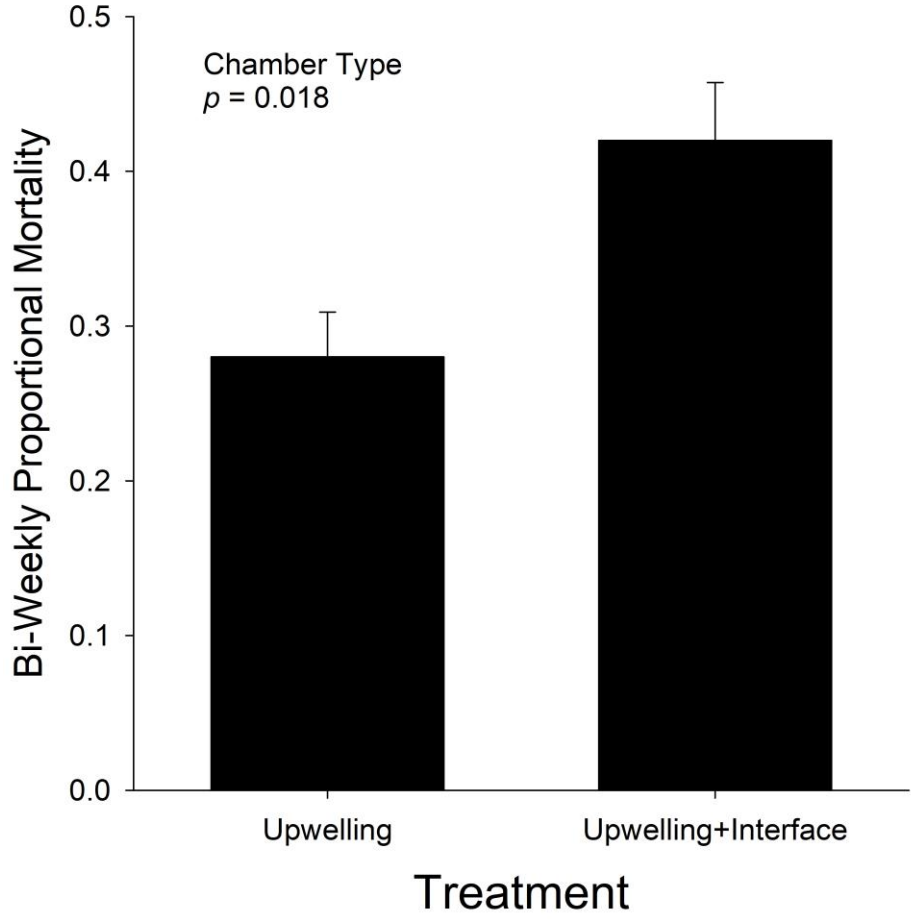

Figure 13. Bar graphs showing mean bi-weekly larval mortality in the experiments examining the effects of chamber type (Upwelling only chambers versus Upwelling Chambers with an Air-Water Interface). One-way ANOVA results are also presented in the figure.

In this experiment, we produced a total of 15 pupae in the chambers with the air-water interface and 6 pupae in the “old” chambers design; Data were more normally distributed, thus we elected to utilize ANOVA to compare pupal and adult counts and found that the air-water interface chambers had significantly greater number of pupae produced across the experiment (F = 8.10; df = 1, 9; p = 0.027); however, the number of adults produced across the experiment did not vary with chamber type, likely because the number of adults produced were low in both treatments (F = 0.40; df = 1, 9; p = 0.141).

The results from this experiment indicate that the use of air-water interface chambers has the potential to increase larval mortality (perhaps through larvae getting trapped in air pockets), but the presence of an air-water interface also increases the number of pupae that are produced. Although these data are limited, it appears that the presence of air “voids” likely allows/facilitates pupation in CSRB or the presence of these spaces may serve as a cue for pupation to begin. Regardless, the low number of adults produced still indicates that adult eclosion is still a substantial challenge in captivity and needs more research.

Effects of frequency of handling on CSRB development

In the experiment in which larvae were checked on a bi-weekly versus a monthly (4-week) basis, we found no effect of handling frequency on larval mortality rates (Fig. 14). In this second Year 2 experiment, we also found slightly increased larval mortality rates when compared to the experiments in Year 1, but the causes remain unknown.

The experiment produced 8 pupae and 4 adults over the time period. The number of pupae produced did not significantly differ between treatments (GzLM omnibus model: Likelihood ratio X^2 = 0.306; df = 1; p = 0.580), indicating that pupation rates were not dependent upon the frequency of handling. However, the number of adults produced was significantly affected by the frequency of handling (GzLM omnibus model: Likelihood ratio X^2 = 4.39; df = 1; p = 0.036), with all adults produced in the monthly handling treatment. This result indicates that handling on a less-frequent basis has a measurable effect on the number of adults produced in captivity.

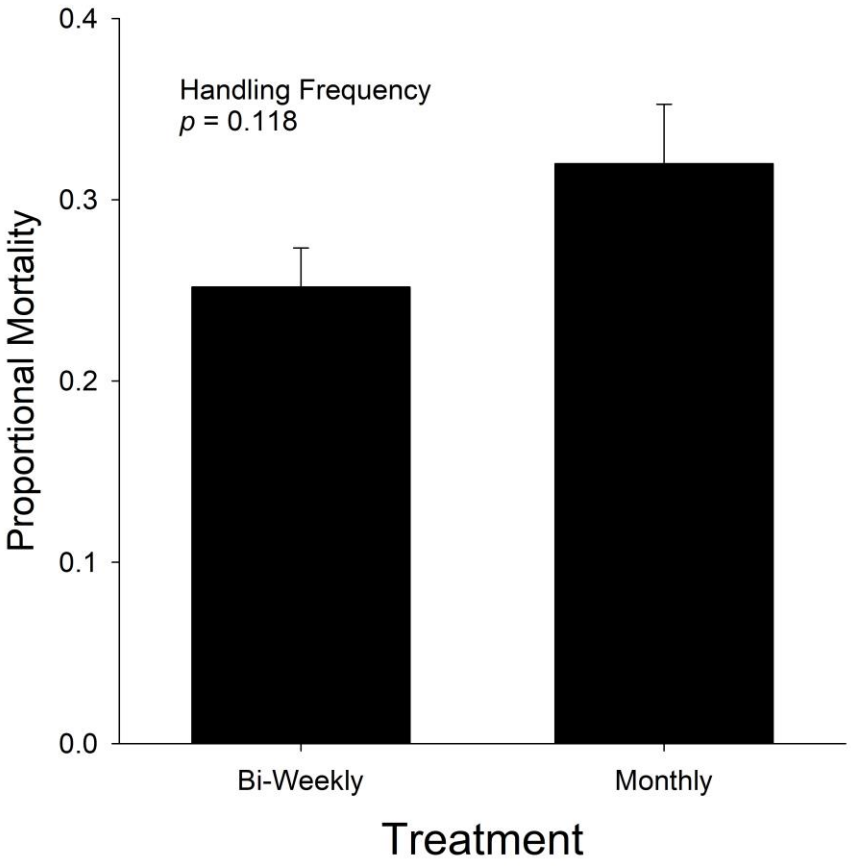

Figure 14. Bar graphs showing mean proportional larval mortality in the experiments examining the effects of handling frequency (bi-weekly checks and counts versus monthly checks and counts). One-way ANOVA results are also presented in the figure.

Summary and Recommendations for Captive CSRB Breeding

Overall, the results of these experiments and observations lead to several specific recommendations about how beetles should be handled in the lab as a part of a captive breeding and refuge program and what areas should be explored in the future.

1) Food sources produced at SMARC versus those in the wild appear to have no effect on

rates of larval survival and the production of pupae and adults. The biochemical composition does not differ between OM sources of the same type that is conditioned in the wild versus at SMARC. The adult beetles at SMARC do not differ in lipid, protein, and carbohydrate content when compared to those found in the wild, suggesting that there is not a nutritional deficiency difference among these two populations. This also suggests that there are no bulk nutritional issues faced by CSRBs developing at SMARC; however, a more detailed effort to examine other nutritional constituents (e.g., essential fatty acids) may be warranted.

2) The inclusion of poly-cotton material (not found in their natural environment) does not confer a benefit or cause an issue in terms of CSRB development in the lab.
3) Microbial communities on OM sources are different between the wild and at SMARC, but it remains unknown if the specific composition of these communities have a substantial effect on the survival and development of beetles in captivity and the water quality/nutrient cycling of SMARC rearing chambers. In our view, this area of research needs further efforts.
4) The presence of conspecifics does not confer any advantage or benefit to CSRB development.
5) The use of poly-cotton lures appears to provide smaller count numbers than using leaf packs. There are many advantages to using poly-cotton cloths (e.g., easy to see beetles on them, can standardize sampling area, consistent with many years of monitoring), but use of cloth lure data to produce a population census will likely underestimate population sizes in the wild.
6) The use of an air-water interface has the potential to increase pupation rates in CSRB in captivity, which is consistent with previous observations of their hydrophibicity and buoyancy. However, larval mortality rates were also higher in the presence of the air-water interface. This represents a trade-off for refuge managers and personnel when designing chambers for various purposes and CSRB rearing.
7) Frequency of handling had an effect on the number of adults produced in captivity. Given that it is often time consuming and tedious to process many hundreds of larvae, this result indicates that infrequent checking (monthly or bi-monthly) is more appropriate.

8) For production of pupae and adults, we would recommend that chambers be built in an upwelling flow-through fashion with clear air voids and spaces (to enhance pupal production) and only be checked once a month (or more). It is relatively easy to produce larvae in the lab (can produce hundreds with a few dozen adults), so mortality caused by any air-water interfaces can be counter-acted by adding new larvae to the chamber on a regular basis.

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Title: Using Molecular Techniques to Assess Genetic Diversity and Population Structure in Texas Wild Rice (*Zizania texana*).

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Executive Summary

Texas wild rice (*Zizania texana*) is a federally endangered plant endemic to the San Marcos River in San Marcos, Texas. The range of the plant is constrained to the upper two miles of the river. To guard against catastrophic losses, an ex situ population was created at the San Marcos Aquatic Resources Center (SMARC) and later expanded to the Uvalde National Fish Hatchery (UNFH). Given the importance of maintaining as much genetic diversity as possible, the captive populations were sampled in 2021 and compared to the wild population to determine if the ex situ populations faithfully capture the diversity seen in the wild. The contemporary genetic analysis was also compared to previous studies that looked at both the wild and captive populations of Texas wild rice. These comparisons revealed that the wild population has not lost genetic diversity since 2012, but previously distinct and spatially separated subpopulations have now been mixed. The comparison also showed that the ex situ populations have become much more representative of the in situ population since the first assessment, but additional work must be done to have adequate representation from all river segments in the ex situ populations. To determine the minimum number of plants to keep in the ex situ populations, genotypes from the in situ population were randomly subsampled four times (representing 25%, 40%, 50%, and 75% of genotypes) and those subsamples were used in one-sided t-tests versus all in situ genotypes to identify significant reductions in allelic richness. Those calculations determined that at least 200 unique individual plants must be kept in the ex situ population to preserve genetic integrity. Additionally, the ex situ populations at the SMARC and UNFH are meant to be redundant failsafe populations, but several plants are not represented in both populations. The plants that are not redundant should be propagated and used to create redundancy between the two refugia populations to protect against catastrophic loss.

Introduction

Evaluating genetic variation and population structure is an essential aspect of conservation biology to determine appropriate management strategies and preserve the biodiversity of native plants (Edwards et al. 2020). Global plant species diversity is dwindling, with recent assessments indicating that approximately 20% of the world's estimated 391,000 plant species are at risk of extinction in the wild (Pimm and Raven 2017; Monks et al. 2018).

One management tool that is used to preserve and safeguard genetic diversity is the formation of ex situ populations, which are utilized to preserve genetic diversity, enhance current population size, keep adaptive traits accessible, and re-establish populations in restoration projects (Richards et al. 2007). These populations must reflect the genetic diversity seen in the wild to be useful management tools (Brown and Hardner 2000). Genetic evaluation of ex situ populations ensures that important genetic variation is preserved and optimizes conservation resources by allowing concise populations to be kept as opposed to maintaining large redundant populations (Richards et al. 2007).

Texas wild rice (*Zizania texana*) has a historically small range, which is confined to the spring-fed upper reaches of the San Marcos River (Emery 1967; Terrell et al 1978). The historically small range, in conjunction with other factors such as habitat degradation and competition with non-native species, led Texas wild rice to be listed as endangered in 1978 (USFWS 1978) under the Endangered Species Act of 1973, as amended (16 U.S.C. 1531-1544, 87 Stat. 884). Texas wild rice is an aquatic perennial grass (lives for more than two years) that exists in the spring-fed headwaters of the San Marcos River in San Marcos, Texas. It is only found in the uppermost two miles of the river, which is almost entirely within the city limits of San Marcos in Hays County, Texas (Poole and Janssen 1997; Pool and Bowles 1998; Poole 2002). Formerly, the species' range extended to the headwaters of the river, which was impounded to create Spring Lake in 1849. The portion of the river occupied by Texas wild rice is divided into segments for monitoring purposes by the Texas Parks and Wildlife Department (TPWD), which began monitoring the area in 1989. These segments are based on physical features such as dams and bridges and do not reflect any sort of plant characteristics or population structure (Figure 1).

Texas wild rice is a habitat specialist. The primary proportion of the plant is completely submerged in water, except for the flowering head which may be emergent. The springs that feed the San Marcos River maintain a constant water temperature, which is instrumental for the healthy growth of Texas wild rice. A few plants do grow in Spring Lake, but plant growth has been severely restricted because of the lake's silty substrate. In some places the lake's depth has also restricted the plant's native range. There are many anthropogenic threats to Texas wild rice including recreational disturbances that disrupt the river bottom, water diversions, urban runoff, and increased pumping of groundwater from the Edwards Aquifer. Other important disturbances that kill plants are flood/scour events such as the major flood event in 2015 that resulted in loss of Texas wild rice coverage among restoration reaches (BIO-WEST and Watershed Systems Group 2016). Sediment deposition is also problematic, because manual sediment removal is an ineffective long term solution since erosion will continually add more sediment to the system (Blanton and Associates, Inc. 2018).

The leaves of *Z. texana* can grow up to 110 centimeters long and the stems can reach lengths of 3.7 meters. The plant has two forms of reproduction. Asexual reproduction is achieved via stolons (runners). Sexual reproduction is achieved via the flowering heads known as culms (above water stems) and panicles (multi-branched inflorescences). Sexual reproduction in the river was once thought to be rare, and flowering in the wild uncommon, but recent mapping

events for the species have found multiple wild *Z. texana* stands actively blooming (Bio-West, Inc. 2016). Flowering has also been observed in refugia, including the San Marcos Aquatic Resources Center (SMARC; San Marcos, TX) (Emery 1977; Power 2001; Richards et al. 2007).

An ex situ population is maintained at the SMARC to mitigate the potential for catastrophic loss of the wild population (USFWS 1995). A second ex situ population is housed at the Uvalde National Fish Hatchery (UNFH; Uvalde, TX) to serve as a failsafe redundancy for the SMARC. The current ex situ populations consist of specimens collected from 9 of the 14 San Marcos River monitoring segments. The ex situ populations were founded via stratified sampling of the wild (in situ) population to minimize collection of clonal plants, thereby maximizing usage of the limited resources available at the SMARC and UNFH. Genetic assessment of these populations was most recently conducted in 2012 (Wilson et al. 2017). Since that time, the ex situ populations have grown in size and many demographic changes have occurred in the wild population. Therefore, this study was commissioned to investigate the current genetic diversity and population structure of Texas wild rice in the wild and refugia populations and compare contemporary diversity to past studies.

The specific objectives of this study were to:

1. Profile the genetic diversity of *Z. texana* plants in the ex situ populations at the SMARC and UNFH.
2. Profile the genetic diversity of in situ *Z. texana* plant stands along the San Marcos River.
3. Compare current in situ *Z. texana* genetic diversity to that of previous studies (Richards et al. 2007; Wilson et al. 2017).
4. Compare the genetic profiles of the ex situ and in situ *Z. texana* to see if the ex situ accurately reflects that of the in situ.
5. Re-evaluate if 430 ex situ plants are sufficient to conserve biodiversity, or if this number should be altered.
6. Determine if any ex situ plants with unique or rare alleles should be considered for propagation and replanting efforts.

Methods

Sample Collection

Captive population- We collected tissue samples from all *Z. texana* plants in the captive populations at the SMARC (N = 212) and UNFH (N = 180). Samples consisted of a ≥ 12 cm segment of a leaf blade with little to no browning or damage when possible. Each sample was labelled with the identification number of the plant from which it was collected. Plants lacking identification numbers were labelled as unknown (UNK) and provided a unique single-digit number. Samples were stored at -20°C at the SMARC until sent to the Southwestern Native Aquatic Resources and Recovery Center (SNARRC; Dexter, NM). Samples were shipped frozen overnight in a cooler with ice packs to preserve sample integrity.

Wild population- Aerial imagery and shapefiles of *Z. texana* survey data were obtained to visualize the spatial distribution of *Z. texana* stands using ArcGIS. The Meadows Center for Water and the Environment provided the most current (spring 2021) aerial imagery of *Z. texana* available. BIO-WEST conducted surveys of *Z. texana* stands in autumn 2020 and the shapefiles generated from this survey effort were combined with the aerial imagery in ArcMap 10.8.1 to build a *Z. texana* stand map. These data were collected using funding by the Edwards Aquifer Authority. The stand map was used to determine the density of *Z. texana* within each river segment (Figure 1).

Targets were developed for the number of samples collected in each river segment based on the proportion of *Z. texana* and our perceived sampling needs in that segment. The separate shapefiles for each river segment were created using the select tool in ArcMap to determine sampling effort by segment. Then, the spatial coverage of *Z. texana* in each river segment was quantified by summing the area values given to each polygon within that river segment. The proportion of *Z. texana* coverage in each segment was calculated by dividing the area of *Z. texana* for that segment by the total area of *Z. texana* across all segments. A minimum target of 5-10 samples per segment was implemented regardless of proportion of *Z. texana* to increase sampling diversity in segments with low *Z. texana* densities. A target of total samples (i.e., captive and wild) was set at 760, where the number of wild samples collected depended on the number of captive samples. Many stands in segment A (Figure 1) were planted there for conservation purposes and were assumed to be genetically similar. Segment B (Figure 1) contained a high density of *Z. texana* that were assumed to be genetically similar due to clonal reproduction based on previous studies. Under the assumption of high clonal propagation and low genetic diversity, we reduced the total number of samples for Stand A and B relative to *Z. texana* density in the river segments.

Stands of *Z. texana* were randomly selected to collect tissue samples from within each river segment. Using a random number generator, we randomly selected numbers corresponding to unique identification numbers for the *Z. texana* stands. Once a polygon was selected, we estimated the number of samples that would be collected based on the length of the stand. This process continued until the target sample number was reached for each river segment. GPS coordinates were recorded for the upstream end of each stand to facilitate easy location in the field.

The selected *Z. texana* stands were located and tissue was collected in the field (N = 380). Each stand was located using the recorded coordinate data and a handheld GPS unit (Bad Elf GNSS Surveyor, Tariffville, Connecticut, USA). Following methods of Wilson et al. (2017), we collected samples from the middle of stands that were ≤ 2 m in length at the longest measurement. In stands > 2 m in length, samples were taken 2 m apart in upstream to downstream order. We created sample identification codes using the river segment letter (Figure 1), an arbitrary two-digit stand identification number, and a two-digit sample number corresponding to the order of collection (i.e., the furthest upstream sample is 01, next is 02, etc.). Non-emergent plants were sampled using

snorkelers or divers when needed. Samples were collected from the wild population using the same methods (i.e., length and quality of tissue) as described for captive plants. Sample storage and shipment methods also matched those previously outlined for captive plants.

Genetic Sampling

Genomic DNA was extracted using the Nucleospin Plant II Maxi prep kit following the manufacturer's protocol (MACHEREY-NAGEL, Duren, Germany). A subsample of remaining plant tissue was archived at -80°C. All DNA extracts were also archived at -80°C after laboratory procedures were completed.

Ten microsatellite markers were amplified for this study (Richards et al. 2004, 2007; Quan et al. 2009). To facilitate comparisons to the 2012 analysis, we amplified these markers in samples originally collected for the Wilson et al. (2017) study (Appendix 1). This included re-amplification of all markers previously utilized by both Wilson et al. (2017) and Richards et al. (2007). Three of the ten markers were ultimately discarded from analysis, including Zt-18 which had been utilized in all previous studies (see results section).

Microsatellite amplification occurred in 10 µl multiplexed polymerase chain reactions (PCR) containing the following: 1 µl DNA, 3 µl Qiagen Multiplex Master Mix® (Qiagen, Valencia, CA, USA), 0.2 µl of both forward and reverse primers (final concentration of each individual primer ranged from 0.1 to 0.5 µM), and 5.6 µl of nuclease-free water. Forward primers were labeled with one of four fluorescent dyes (6-FAM, PET, NED, VIC; Applied Biosystems, Inc., Foster City, CA, USA). Amplification for all samples consisted of an initial denaturing step of 95°C for 15 min, followed by 35 cycles of 94°C for 60 s, 56°C for 45 s, and 72°C for 60 s, with a final extension of 10 min at 70°C. Amplified products were processed on an ABI 3500xl Genetic Analyzer using LIZ-500 as an internal size standard. Composite genotypes for individual plants were compiled using GeneMapper^{TM} 4.0 software (Applied Biosystems, Inc., Foster City, CA, USA). A second researcher rescored all the original data and re-extracted and re-amplified 10% of samples for quality assurance/quality control assessment.

Data Analysis

We utilized the same sample naming scheme as Wilson et al. (2017), in which samples were identified by the river segments from which they were originally collected (river segments A – K and S; Figure 1). For ex situ samples, this designation was used in conjunction with the letter R to denote that it is a refugia sample (e.g., AR = refugia samples from river segment A). Samples for which collection site data have been lost are referred to as UnR for unknown refugia sample.

For consistency and ease of comparison to Wilson et al. (2017), the identical genotypes that occurred within segments or refugia populations were identified and removed. Identical genotypes were identified using the multi-locus matcher function in GenAlEx version 6.5 (Peakall and Smouse 2006; 2012). Departures from Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium (LD) were tested using Genepop version 4.2 (Raymond and Rousset 1995), and alpha (0.05) was adjusted for multiple comparisons using the Benjamini and

Yekutieli (2001) false discovery rate method (Narum 2006). Deviations from HWE due to stuttering, null alleles, and large allele dropout were tested using MICROCHECKER 2.2.3 (van Oosterhout et al. 2004).

Several approaches were used to examine how genetic diversity was partitioned among population segments. The average number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), probability of identity (PI), and the number of private alleles (PAL) were calculated in GenAlEx version 6.5. Allelic richness (A_R) was estimated for all river segments with at least 10 sampled individuals using FSTAT v2.9.4 (Goudet 1995, 2003). An Analysis of Molecular Variance (AMOVA) was also conducted in GenAlEx to examine the amount of molecular variance among populations (Excoffier et al. 1992; Michalakis and Excoffier 1996).

Allelic richness was also utilized to determine the minimum necessary ex situ population size to capture extant diversity from the in situ population. This was accomplished by first using FSTAT to compare A_R values from subsamples of the in situ population. Four subsamples were taken from the in situ plants representing different proportions of the wild population (25%, 40%, 50%, and 75%). The subsampled data were used in a one-tailed t-test against the entire wild population to generate P-values to detect statistically significant differences for A_R. Additionally, HP-Rare (Kalinowski 2005) was used to generate A_R estimates for subsampled data using the statistical technique of rarefaction which compensates for sampling disparity. Those estimates were used to create a visual representation of the changes in A_R for the subsampled wild population.

To calculate the optimal number of plants needed per segment for the ex situ population, the percentage of area that each segment covers in the wild was weighted by the N_A/N_{Atotal} calculated from the genetic assessment of the wild population. Those weighted percentages were used to calculate the recommended ex situ population numbers for various potential population sizes that could be kept in captivity. River segments that were not sampled during the wild collections could not be evaluated.

The genetic structure of a population can broadly be defined as the amount and distribution of genetic variation within and among populations. F-statistics (F_{ST} and F_{IS}) are among the most common measurements of genetic differentiation. The fixation index (F_{ST}) examines how much variation is contained in a population or subpopulation, while the inbreeding coefficient (F_{IS}) estimates the proportion of the variance in the subpopulation contained in an individual. Both F_{ST} and F_{IS} were calculated using FSTAT.

Population structure was assessed through two additional analyses. The first was the program STRUCTURE v2.3.2 (Pritchard et al. 2000; Falush et al. 2003), which uses Markov chain Monte Carlo in a Bayesian framework to assign individuals to genetic clusters without a priori population definitions being specified. The admixture model was used with twenty iterations performed for each K (the number of genetic clusters), with K assumed to be between 1 and 10 genetic clusters. For each run, a burn-in of 100,000

iterations was performed followed by 1,000,000 iterations of data collection. The STRUCTURE results were evaluated in Clumpak (Kopelman et al. 2015) and the delta K method (Evanno et al. 2005) was used to determine the optimal number of genetically differentiated clusters.

Population structure was also assessed through a Discriminant Analysis of Principal Components (DAPC). This commonly used multivariate method identifies clusters of genetically similar individuals and assigns them to groups, while creating a visual representation of between-population differentiation. DAPC was conducted in R (R Core Team, 2021) using the adegenet package (Jombart 2008). The *find.clusters* function was used to detect the most likely number of clusters. The optimal number of subpopulations was determined by the lowest associated Bayesian Information Criterion (BIC) value.

Results

Three microsatellite markers were discarded from analysis. Our study found that Zt-18 was not suitable for analysis, despite being used in previous studies (Richards et al. 2007; Wilson et al. 2017). This marker appears to amplify two loci resulting in unrealistic observed heterozygosity (H_O), which is exhibited in Wilson et al. (2017) with almost every population segment having $H_O = 1$. Several approaches were taken to address the double amplification, including non-multiplexed amplification, testing alternative annealing temperatures, and adding bovine serum albumin to the PCR reagents, but all conditions resulted in the same amplification pattern. Two additional markers (Zm-4 and Zm-26) were discarded because they were monomorphic in all samples.

None of the remaining seven loci regularly deviated from HWE in the refugia or wild populations, so these loci were retained (Table 1). One locus (Zt-23) deviated from HWE in two refugia segment populations. MICROCHECKER 2.2.3 was used to investigate all the loci for each segment. MICROCHECKER indicated there were potential null alleles present due to excess homozygotes at some loci (Zt-1 in F; Zt-13 in AR & C; Zt-16 in B; Zt-21 in B & C; Zt-22 in CR, FR, A & B; Zt-23 in AR, FR & UnR), but these deviations were not consistent across all of the river segments.

The probability of identity (PI) ranged from 2.0×10^{-5} to 7.3×10^{-3} which was comparable to Wilson et al. (2017) and Richards et al. (2007) despite using different markers. The GenAlEx multilocus match function identified 39 genetic clusters of identical individuals with individuals duplicated multiple times in some cases (Appendix 2). The initial dataset consisted of 771 individual genotypes (Appendix 3). The final dataset after removal of duplicates within river segments included 652 individual genotypes. Removal of all duplicate genotypes from the refugia and wild populations both within and across segments would reduce the dataset to 600 unique genotypes across the 771 samples analyzed.

The AMOVA indicated that there was some significant variance contained among individuals within each river segment ($F_{ST} = 0.097$, $P = 0.001$) and among river segments ($F_{ST} = 0.026$, $P = 0.001$); however, 88% of the variation was within individuals not among river segments (Figure 2).

The genetic summary statistics for *Z. texana* were evaluated in two ways. First, the two refugia (SMARC and UNFH) were compared to one another. Secondly, the refugia were combined and compared to the wild population. The first evaluation (i.e., head-to-head comparison of genetic profiles for the SMARC and UNFH ex situ populations) is presented in Table 2. Comparisons of population genetic summary statistics between the two ex situ refugia populations were similar, including observed heterozygosity. For river segments with ≥ 10 individuals, the SMARC samples ranged from H_O = 0.47 (SM_AR) to H_O = 0.56 (SM_ER). The observed heterozygosity for the UNFH ranged from H_O = 0.4 (U_DR) to H_O = 0.51 (U_BR & U_CR). Comparisons of sample groups that originated from the same river segment yielded similar results. For example, the mean H_O for river segment A was similar when comparing the UNFH and SMARC ex situ populations (U_AR = 0.41; SM_AR = 0.47).

Private alleles (PALs) are alleles that are found only in a single population among a broader collection of populations. A comparison of the two ex situ populations identified 15 different private alleles (not listed), the majority of which (13 of 15 PALs) were present at the SMARC. The number of microsatellite alleles per locus (N_A) in the SMARC samples varied from 2 to 13 alleles with an average of 4.2 alleles per locus across the 7 loci. The number of microsatellite alleles (N_A) in the UNFH samples varied from 2 to 9 alleles with an average of 3.6 alleles. While N_A is commonly used to evaluate genetic diversity, it is sensitive to differences in sample size. That is why A_R is preferable for population allelic diversity comparisons because it corrects for sample size differences using rarefaction techniques. The A_R values for the ex situ river segments from the SMARC tended to be similar to corresponding segments at the UNFH (Table 2). However, river segment B was a notable exception (U_BR A_R = 3.73 and SM_BR A_R = 4.23), which indicates a real difference between the two refugia at that segment. The number of UNFH samples (N = 157) is only slightly lower than the number from the SMARC (N = 186), so the lower numbers of both PALs and overall allele counts suggest the SMARC refugium captures more diversity from the wild population than the UNFH refugium.

Across the two refugia populations the inbreeding coefficient (F_{IS}) was generally low and occasionally negative (Table 2). The mean F_{IS} for the SMARC samples varied from -0.04 (SM_CR) to 0.1 (SM_AR). The mean F_{IS} of UNFH samples varied from 0.03 (U_BR) to 0.21 (U_DR).

Following comparison of the two refugia to one another, samples from the ex situ populations were combined and compared to the wild in situ samples (Table 3). The wild population assessment suffered from the same number of sample constraints that several of the ex situ population segments had, where there were not enough samples to calculate reliable population genetic parameters (i.e., N < 10). Fewer than 10 samples were collected from 4 of 10 river segments. Two more segments (D and K) were limited to 11 and 10 samples respectively, meaning values calculated for these segments could also suffer from sampling bias. The observed heterozygosity of the *Z. texana* samples was consistent across the refugia and wild populations (Table 3). The refugia population ranged from H_O = 0.38 (GR) to H_O = 0.54 (UnR) and the observed heterozygosity for the wild population ranged from H_O = 0.4 (A) to H_O = 0.54 (K).

The number of microsatellite alleles in the combined ex situ samples varied from 2 to 15 alleles with an average of 4.6 alleles per locus across the 7 loci. The wild in situ samples were similar to the ex situ with N_A varying from 2 to 15 alleles with an average of 4.3 alleles. The comparison between the mean genetic indices showed several interesting trends. For example, the ex situ river segment ER (N_A = 5.43) is dramatically different from its wild counterpart E (N_A = 3.86), but very few plants were collected from the wild. While the difference is not as dramatic, the wild river segment C (N_A = 5.00) notably differed from its ex situ counterpart CR (N_A = 5.86). The allelic richness was extremely close between the ex situ and in situ river segments with the largest discrepancy between D (A_R = 3.23) and DR (A_R = 3.55). Mean A_R for the combined refugia populations ranged from 3.06 (GR) to 4.04 (UnR). The A_R in the wild population varied by river segment from 3.23 (D) to 4.25 (F).

It is important to understand the minimum number of plants needed in ex situ to conserve the genetic integrity observed in the wild in situ plants. The wild population was subsampled to generate A_R values which were used to determine the minimal ex situ population size needed. Subsamples were randomly drawn from the wild population (25%, 50%, and 75% of all individuals). The subsampled data were used in a one-tailed t-test against the whole wild population to calculate P-values for changes in A_R (Table 4). The subsampling revealed that A_R dropped precipitously between the 25% and 50% subsamples, while A_R estimates for the 50% and 75% subsamples were very similar. Consequently, the wild population was again subsampled to 40% of the original populations and the one-tailed t-test was repeated. After re-analysis, it was concluded that an absolute minimum of 166 individuals (e.g., 50% of total wild samples) is necessary to preserve the majority of observed wild diversity in an ex situ population. However, the visual representation of changes in A_R determined via rarefaction (HP-Rare: Figure 3) also showed that the subsampling of 331 individuals exhibited no obvious plateau in A_R, indicating that maintenance of more than 166 plants in an ex situ population would better capture wild diversity.

The optimal number of *Z. texana* plants from each wild segment that should be kept in an ex situ population depends on the space available for housing and care for the plants. Recommended ex situ population numbers for various potential population sizes that could be kept in captivity were made using the area that each wild segment covers weighted by the N_A/N_{Atotal} calculations (Table 5). That table should serve as a guideline for the number of individuals that should be kept from each segment as the ex situ population grows.

Thirteen samples were detected to have alleles that were private to either the wild or ex situ populations (Table 6). There were 8 PALs in the refugia populations across 5 river segments. There were 3 PALs in the wild populations across 2 river segments. The PAL (175) for Zt-23 found in wild segment B was shared across three individuals but was unique to segment B. Each of the other PALs was detected in a single individual.

Analyses of population structure converged upon three genetic clusters in both the STRUCTURE (Figure 4) and DAPC (Figure 5) analyses. The genetic structure of a population can also be inferred by examining the amount and distribution of genetic variation within and between populations. F_{IS} is the inbreeding coefficient of an individual with respect to the local

subpopulation, and F_{ST} is the fixation index that refers to the average inbreeding coefficient of subpopulations relative to the total population. Across the ex situ and in situ populations the inbreeding coefficient (F_{IS}) was primarily positive, with only a few negative values. The mean F_{IS} of the refugia populations varied from 0.03 (BR) to 0.21 (DR) (Table 3). All but one of the wild mean F_{IS} were positive with a range of -0.06 (K) to 0.18 (A and C). The one negative sample only contained 10 individuals. While a positive F_{IS} means that individuals are more related than one would predict under a model of random mating, a negative F_{IS} specifies that individuals in a population are less related than predicted.

Pairwise F_{ST} values were calculated to compare the combined ex situ refugia populations with their wild counterparts (Table 7). Very little significant genetic differentiation was evident among populations. There were only two significant estimates, and both were from comparisons between the refugia population FR and other populations (B, and BR). These estimates could be the consequence of low number of samples in FR appearing as substructure or since the population FR also had private alleles (PALs), those few individuals with PALs in a small sample group could be driving the structure.

The genetic profile created for the in situ and ex situ plants (Table 3) from this study can be compared to the genetic profile of samples from 2012 (Wilson et al. 2017), which we reanalyzed with our additional microsatellite markers (Appendix 1). Using the GenAlEx multilocus match function there were found to be 28 genetic clusters of identical genotypes in the 2012 samples (Appendix 4). The initial 2012 dataset consisted of 245 individual genotypes (Appendix 5), which was reduced to 176 genotypes after these duplicate genotypes were removed. There are wild population segments with similar genetic profiles across the two temporal samples such as segment F, for which 2012 values (A_R = 4.4, F_{IS} = 0.19) were similar to contemporary values (A_R = 4.25, F_{IS} = 0.15). However, this was not true for all river segments. One example is population segment A having similar A_R, but an F_{IS} almost twice as high in the 2012 analysis compared to our current study. There are several more examples of genetic indices changing across river segments over time, in some cases going up and in others going down.

Not only can the current study and the samples from 2012 be compared, but that information can be used in comparison to Richards et al. (2007). One such comparison that can be made between all three studies is examining the number of alleles detected at each locus (Table 8). There were double the number of samples in this study and Richards et al. (2007) relative to the 2012 analysis, meaning the studies with higher sample sizes had a greater probability of encountering higher numbers of alleles per locus. While the Richards et al. (2007) paper and the current study used different loci, the number of individuals used were similar, and the loci that were shared between the two studies followed similar patterns. Richards et al. (2007) had more alleles at three loci (Zt-

1, Zt-13, and Zt-21), the 2021 analysis had more alleles at one locus (Zt-23), and at a fifth locus the two studies were tied for the highest number of alleles present (Zt-22).

The loci utilized between the three studies are different, but the overall trends in F_{IS} can be compared across the three. While Richards et al. (2007) had many negative F_{IS} scores, both this study and the 2012 samples had few, instead exhibiting positive F_{IS} scores.

Finally, the three studies can also be compared by visually comparing population structure barplots (Figure 6). Each of the three studies conducted a STRUCTURE analysis of the wild in situ *Z. texana* population. The STRUCTURE plot for each study depicts three significantly differentiated genetic clusters (K = 3) with a general trend towards homogenization of clusters across river segments over time. While the 2007 analysis exhibited a trend of spatial structure partially associated with river segments, this structure had largely disappeared by 2021.

Discussion

Genetic diversity is an important aspect of the dynamics of populations, as it is directly related to the evolutionary potential of the population and the deleterious effects of inbreeding. Incorporating population-level genetic analyses in the context of population monitoring for endangered species can provide insight into demographic, ecological, and evolutionary processes that could be hindering recovery (Hartl and Clark 2007). Long-term monitoring is vital to increase the understanding of *Z. texana* and its management. Long-term genetic monitoring must take place for the wild in situ populations as well as the refugia ex situ populations if management of the species is going to be effective (Schwartz et al. 2007).

With that in mind, the first goal of this study was to profile the genetic diversity of *Z. texana* in the ex situ populations at the SMARC and UNFH. Having a redundant ex situ population at the UNFH is an effective way to mitigate the loss of genetic diversity if a stochastic event should affect the population at the SMARC. However, that mitigation only works if the UNFH population is representative of the diversity seen at the SMARC. The river segments with enough individuals to be compared have very similar genetic profiles but are not totally redundant. Overall, the similarity between the two ex situ populations is encouraging, but there needs to be true redundancy between the two populations if they are supposed to be failsafe backups of one another. The two populations can be cross-referenced in Appendix 2, which lists identical genotypes. Individuals that are not listed on the identical genotypes spreadsheet are present in only one ex situ population. Those individuals without redundancy should be cultivated to create a redundant plant to be kept at the other facility.

After profiling the genetic diversity of *Z. texana* in the ex situ populations, the wild plant stands along the San Marcos River needed to be reassessed for genetic diversity. The wild river segments experienced the same sample size constraints as several of the ex situ population segments, where there were not enough samples to reliably calculate some genetic diversity measures such as A_R (i.e., $N < 10$). Despite this issue, the mean genetic diversity indices are similar across all wild river segments with only a few exceptions.

Two previous studies examined the genetic indices of the wild *Z. texana* population (Richards et al. 2007; Wilson et al. 2017). Direct comparison of our contemporary data to the Richards et al. (2007) study is challenging due to different sampling techniques and genetic markers being used. However, direct comparison to the Wilson et al. (2017) study was facilitated by usage of similar methods and access to their genetic samples. This allowed for evaluation of the Wilson et al. (2017) samples with the updated microsatellite panel, which revealed two key points: 1) the ex situ populations need better representation of wild plants from some river segments but are currently more representative of the wild populations than during 2012, and 2) the wild population has experienced shifts in genetic indices like A_R and F_{IS} but has not experienced dramatic losses of genetic diversity.

Comparisons made between the loci common to all three studies revealed that allelic patterns have shifted and changed over time, but there has not been a dramatic loss of alleles. However, the overall trends in F_{IS} across all three studies did change over time. While the Richards et al. 2007 paper had many negative F_{IS} scores, both this study and the 2012 samples used in Wilson et al. 2017 had few, instead exhibiting mostly positive F_{IS} scores. Positive F_{IS} scores indicate that either inbreeding or mixing of previously separated subpopulations has occurred, causing a distortion in allele frequencies (i.e., Wahlund effect). The Wahlund effect is the deviation of genotype frequencies from Hardy-Weinberg expectations due to mixing of genetically differentiated subpopulations within the sample (Hallerman 2003). The mechanism causing positive F_{IS} scores is unclear. It is possible that sexual reproduction between related individuals is partially responsible since flowering and seeding has been repeatedly detected in the wild (Bio-West, Inc. 2016). Another likely cause of the higher F_{IS} is the mixing of previously isolated river segments. Mixing among segments could have occurred through several mechanisms. For example, seeds that originated from crosses between a few plants from segment F were added to segment A in the summer of 1996 (Wilson et al. 2017). Additional possibilities include the planting events that occurred under the Edwards Aquifer Habitat Conservation Plan, the downstream movement of stolons, or a combination of all aforementioned factors. Since so many replanting efforts have taken place, the high F_{IS} appears to be mainly the result of the Wahlund effect rather than inbreeding, especially since no great loss of genetic diversity has occurred.

Another way that the three studies can be compared is by visually analyzing population structure (Figure 6). The STRUCTURE results from this study were consistent with the past studies in finding three significantly differentiated genetic clusters ($K = 3$), indicating that no major changes have occurred. There are most likely not 3 actual different clusters, but rather STRUCTURE is likely detecting relatedness and identifying it as distinct clusters (Anderson and Dunham 2008). Unlike previous studies, the contemporary analysis of the in situ population exhibits little evidence of population structure that is spatially distributed across different river segments. Population assignments of individuals are relatively evenly distributed across all river segments, and the ancestry of many individuals is fractionally assigned to two or more clusters.

Recently, several studies have indicated that STRUCTURE yields poor individual assignments to source populations and gives frequently incorrect estimates of K when sampling is done between unbalanced populations (Neophytou 2014; Puechmaille 2016; Wang 2017). Whether or not it is distinct genetic clusters or relatedness, all three plots compared side by side indicate that the number of wild population clusters (K = 3) has remained stable over the past 15 years.

Since the ex situ refugia populations have expanded so much since the Wilson et al. (2017) study, they had to be reassessed and compared to the wild population. The genetic profile of the combined refugia populations is mostly representative of the wild *Z. texana* population. Comparing the current ex situ to the in situ samples shows that the ex situ populations have improved since their first examination. There are exceptions to these similarities in which wild river segments exhibit lower diversity measures than their ex situ counterparts, but these most likely result from sample size driven biases (example: river segment E). Overall, the F_{IS} scores of the in situ populations tended to be higher than in the ex situ populations. While sexual reproduction is happening in the wild as suggested by the observation of many flowering stands (Bio-West, Inc 2016), the reintroduction of plants or seeds (Power 2001; Wilson et al. 2017; Blanton and Associates, Inc. 2018) into the wild is most likely causing the allelic disturbances.

Space that can be allotted for conservation of any species is finite. One of the best indications on how that space should be used is to examine the genetic diversity of the species being conserved. The space should be partitioned in a way that allows the maximum amount of diversity to be conserved while efficiently utilizing finite resources. Ex situ programs generally try to maximize the genetic diversity of the seed or plant collections while minimizing the cost and effort to collect (Guerrant et al. 2014). In addition, genetically representative collections are essential if they are to be used for recovery and restoration work (Sharrock and Jones 2010; Pritchard et al. 2012). The creation of the *Z. texana* ex situ population at the SMARC occurred prior to genetic evaluation so a clear description of how space should be allocated to plants from each segment was not proposed until Wilson et al. (2017) made recommendations based on the SMARC's ability to accommodate roughly 430 plants. The addition of hatchery space as well as the reassessment of genetic diversity means that a reallocation of resources will be in order to accommodate the diversity of the current wild population. As the hatchery population grows, it should adhere to the listed number of plants put forth (Table 4) while keeping in mind the number of plants should not dip below the 200 plants mark that subsampled A_R values indicated was a critical threshold maintained to avoid a precipitous drop in A_R and create a buffer against loss. This is an absolute minimum, and ideally more individuals should be kept to conserve as much variation as possible.

The wild population of *Z. texana* has been shown to be dynamic, with shifts in allelic frequencies both spatially and temporally. As the wild population shifts and changes, refugia plants can be used to mitigate losses in diversity. In order to do so, individuals in the captive populations with unique genetic profiles were identified. There were 8 PALs detected in the hatchery populations. They originated from 5 different river segments. The individuals exhibiting these PALs are listed in Table 6, along with the locus at which each allele is found,

and the river segment from which each plant originated. Those individuals should be considered for propagation and replanting in the wild.

Limited sampling of some wild segments resulted in difficulties assessing several river segments, which complicates the understanding of how many plants per segment should be kept in ex situ populations. Moving forward, it is important to continue genetic monitoring of the *Z. texana* in situ populations to document spatial and temporal patterns. Whenever possible, future sampling events should collect enough individuals from each segment so that proper recommendations can be made for maintenance of the ex situ population. This may be difficult to accomplish at lower river segments due to reduced plant numbers. If census size allows, then at least 15 plants should be sampled from each river segment. If this minimum threshold is unattainable, then all plants should be sampled. This is so the removal of identical individuals does not hinder the evaluation of the genetic diversity.

Overall, the efforts to create an ex situ population of *Z. texana* have made improvements since the first genetic analysis and are more reflective of the wild population. There is still progress that needs to be made in collecting more samples for some of the population segments that are underrepresented or not represented at all in the ex situ populations. Moving forward, the genotyping of individuals that are being taken from the wild to be placed in the ex situ populations should be done to reduce the occurrence of duplicate plants as there are many duplicate plants in the ex situ populations currently (Appendix 2). Finally, the ex situ populations at the SMARC and UNFH are meant to be redundant failsafe populations, but there are several plants that are not represented in both populations. The plants that are not redundant should be propagated and used to create redundancy between the two refugia populations to ensure that no catastrophic loss takes place.

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Table 1. Test of Hardy-Weinberg equilibrium for *Zizania texana* across each locus by river segment and percent of linked loci (%LD) by river segment. Loci that are significantly out of equilibrium after a Bonferroni correction ($P < 0.0004$) are indicated with an asterisk. River segment names correspond to designations in Figure 1. Refugia sample groups are indicated by an 'R' following the river segment name.

	AR	BR	CR	DR	ER	FR	GR	JR	UnR	A	B	C	D	E	F	G	H	K	S
Locus	n = 38	n = 119	n = 46	n = 16	n = 39	n = 21	n = 11	n = 6	n = 24	n = 32	n = 191	n = 42	n = 11	n = 8	n = 18	n = 6	n = 5	n = 10	n = 8
Zt-1	0.47	0.15	0.74	0.05	0.61	0.96	-	1.00	0.23	0.20	0.05	0.04	1.00	0.18	0.00	-	1.00	0.48	0.44
Zt-13	0.03	0.01	0.76	0.09	0.00	0.98	0.07	0.93	0.22	0.02	0.10	0.00	0.50	1.00	0.21	1.00	0.40	1.00	0.98
Zt-16	0.41	0.82	0.31	0.20	0.26	0.14	1.00	-	0.26	1.00	0.01	0.43	1.00	1.00	0.16	0.27	1.00	1.00	1.00
Zt-21	0.05	0.44	0.12	0.01	0.01	0.04	0.29	1.00	0.36	0.07	0.01	0.01	0.06	0.42	0.05	0.58	0.62	0.67	0.08
Zt-22	1.00	0.21	0.09	1.00	0.41	0.00	-	0.09	1.00	0.03	0.00	0.02	0.04	1.00	0.24	1.00	0.11	1.00	-
Zt-23	0.00*	0.01	0.08	0.03	0.24	0.00*	0.33	1.00	0.03	0.17	0.07	0.07	0.11	0.18	0.05	0.93	0.21	0.50	0.66
Zt-26	0.47	0.79	0.60	0.45	0.44	1.00	0.60	0.27	0.98	0.13	0.07	0.66	0.75	0.22	0.80	0.15	0.24	1.00	0.91
% LD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	0.00	0.00	19.0	0.00	0.00	0.00	0.00	-	0.00	-	0.00

Table 2. Genetic diversity indices for the two ex situ populations of *Zizania texana* at the San Marcos Aquatic Resources Center (SMARC) and Uvalde National Fish Hatchery (UNFH) calculated after removing samples with identical multi-locus genotypes that occurred within segments. N_A = number of alleles, A_R = allelic richness, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} = inbreeding coefficients and the symbol (–) was used in the place of statistics that were not calculated due to small sample size.

Locus	Statistic	Ex situ SMARC Samples										Ex situ UNFH Refugia Samples							
		SM_AR n = 16	SM_BR n = 67	SM_CR n = 27	SM_DR n = 5	SM_ER n = 17	SM_FR n = 15	SM_GR n = 8	SM_HR n = 1	SM_JR n = 6	SM_Un n = 24	U_AR n = 28	U_BR n = 58	U_CR n = 22	U_DR n = 11	U_ER n = 25	U_FR n = 6	U_GR n = 6	U_Un n = 1
Zt-1	N_A	2.00	2.00	2.00	2.00	2.00	2.00	2.00	1.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	1.00	1.00
	A_R	2.00	2.00	2.00	-	2.00	2.00	-	-	-	2.00	2.00	2.00	2.00	2.00	2.00	-	-	-
	H_O	0.38	0.33	0.37	0.20	0.59	0.67	0.13	0.00	0.50	0.38	0.52	0.28	0.36	0.09	0.44	0.33	0.00	0.00
	H_E	0.43	0.35	0.35	0.42	0.46	0.44	0.12	0.00	0.38	0.48	0.50	0.35	0.35	0.24	0.47	0.44	0.00	0.00
	F_{IS}	0.16	0.06	-0.05	0.60	-0.26	-0.47	0.00	-	-0.25	0.24	-0.03	0.22	-0.01	0.64	0.09	0.33	-	-
Zt-13	N_A	7.00	13.00	6.00	5.00	5.00	7.00	4.00	2.00	6.00	8.00	5.00	9.00	7.00	3.00	7.00	4.00	3.00	2.00
	A_R	6.04	5.97	4.84	-	4.63	6.19	-	-	-	6.46	4.82	5.47	5.49	3.00	5.92	-	-	-
	H_O	0.69	0.70	0.81	0.60	0.71	0.93	0.38	1.00	0.83	0.79	0.48	0.64	0.73	0.55	0.64	0.67	0.33	1.00
	H_E	0.76	0.72	0.65	0.72	0.67	0.74	0.66	0.50	0.69	0.76	0.72	0.75	0.74	0.49	0.74	0.60	0.49	0.50
	F_{IS}	0.13	0.03	-0.23	0.27	-0.03	-0.22	0.48	-	-0.11	-0.02	0.35	0.16	0.04	-0.06	0.16	-0.03	0.39	-
Zt-16	N_A	3.00	3.00	5.00	2.00	3.00	3.00	2.00	1.00	2.00	3.00	2.00	4.00	3.00	2.00	3.00	2.00	2.00	1.00
	A_R	2.69	2.77	3.22	-	2.88	2.47	-	-	-	2.71	1.83	2.66	2.88	2.00	2.69	-	-	-
	H_O	0.31	0.43	0.33	0.20	0.59	0.13	0.38	0.00	0.17	0.38	0.12	0.41	0.45	0.27	0.28	0.17	0.33	0.00
	H_E	0.35	0.42	0.37	0.18	0.44	0.13	0.30	0.00	0.15	0.43	0.11	0.38	0.45	0.43	0.40	0.38	0.28	0.00
	F_{IS}	0.15	-0.03	0.11	0.00	-0.30	-0.02	-0.17	-	0.00	0.16	-0.04	-0.08	0.01	0.41	0.32	0.62	-0.11	-
Zt-21	N_A	6.00	12.00	10.00	4.00	9.00	9.00	4.00	2.00	9.00	10.00	9.00	8.00	9.00	6.00	8.00	7.00	5.00	1.00
	A_R	5.66	7.40	7.12	-	7.55	8.56	-	-	-	7.66	7.30	5.67	6.74	6.00	6.43	-	-	-
	H_O	0.88	0.79	0.74	0.60	0.53	0.73	0.63	1.00	1.00	0.83	0.76	0.69	0.77	0.55	0.84	0.67	0.50	0.00
	H_E	0.78	0.82	0.78	0.66	0.79	0.85	0.65	0.50	0.88	0.82	0.83	0.74	0.79	0.75	0.79	0.79	0.67	0.00
	F_{IS}	-0.09	0.04	0.07	0.20	0.35	0.17	0.10	-	-0.05	0.01	0.10	0.07	0.04	0.32	-0.05	0.25	0.33	-
Zt-22	N_A	3.00	6.00	3.00	3.00	4.00	3.00	1.00	2.00	3.00	4.00	2.00	6.00	3.00	4.00	5.00	1.00	2.00	1.00
	A_R	2.38	3.21	2.74	-	3.57	2.87	-	-	-	2.77	1.83	3.08	2.92	4.00	2.76	-	-	-
	H_O	0.13	0.22	0.19	0.40	0.35	0.00	0.00	1.00	0.17	0.21	0.12	0.19	0.18	0.27	0.16	0.00	0.17	0.00
	H_E	0.12	0.25	0.26	0.34	0.35	0.24	0.00	0.50	0.29	0.19	0.11	0.22	0.35	0.25	0.15	0.00	0.15	0.00
	F_{IS}	-0.02	0.10	0.31	-0.07	0.03	1.00	-	-	0.50	-0.06	-0.04	0.16	0.49	-0.05	-0.03	-	0.00	-
Zt-23	N_A	6.00	9.00	8.00	4.00	6.00	7.00	3.00	2.00	5.00	8.00	6.00	7.00	6.00	5.00	5.00	4.00	3.00	2.00
	A_R	5.19	6.09	5.53	-	5.18	6.59	-	-	-	5.54	4.40	5.22	5.20	5.00	4.41	5.69	-	-
	H_O	0.44	0.79	0.81	0.60	0.88	0.53	0.50	1.00	1.00	0.54	0.44	0.69	0.64	0.64	0.60	0.17	0.50	1.00
	H_E	0.63	0.78	0.72	0.58	0.73	0.73	0.55	0.50	0.74	0.73	0.57	0.72	0.69	0.70	0.68	0.68	0.63	0.50
	F_{IS}	0.33	-0.01	-0.12	0.08	-0.18	0.30	0.16	-	-0.28	0.28	0.24	0.06	0.11	0.14	0.14	0.79	0.29	-
Zt-26	N_A	2.00	3.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	1.00
	A_R	2.00	2.16	2.00	-	2.00	2.00	-	-	-	2.00	2.00	2.00	2.00	2.00	2.00	-	-	-
	H_O	0.50	0.43	0.52	0.40	0.29	0.67	0.63	1.00	0.17	0.67	0.44	0.67	0.41	0.45	0.52	0.50	0.17	0.00
	H_E	0.49	0.50	0.44	0.48	0.50	0.44	0.49	0.50	0.38	0.50	0.50	0.50	0.49	0.50	0.49	0.38	0.49	0.00
	F_{IS}	0.02	0.15	-0.15	0.27	0.44	-0.47	-0.21	-	0.62	-0.32	0.13	-0.34	0.19	0.14	-0.05	-0.25	0.71	-
Mean	N_A	4.14	6.86	5.14	3.14	4.43	4.71	2.57	1.71	4.14	5.29	4.00	5.43	4.57	3.43	4.57	3.14	2.57	1.29
	A_R	3.71	4.23	3.92	-	3.97	4.38	-	-	-	4.16	3.46	3.73	3.89	3.43	3.74	-	-	-
	H_O	0.47	0.53	0.54	0.43	0.56	0.52	0.38	0.71	0.55	0.54	0.41	0.51	0.51	0.40	0.50	0.36	0.29	0.29
	H_E	0.51	0.55	0.51	0.48	0.56	0.51	0.40	0.36	0.50	0.56	0.48	0.52	0.55	0.48	0.53	0.47	0.38	0.14
	F_{IS}	0.10	0.04	-0.04	0.22	0.03	0.01	0.12	-	0.00	0.05	0.16	0.03	0.10	0.21	0.09	0.32	0.34	-

Table 3. Genetic diversity indices for the in situ and ex situ populations of *Zizania texana* calculated after removing samples with identical multi-locus genotypes that occurred within segments. N_A = number of alleles, A_R = allelic richness, H_O = observed heterozygosity, H_E = expected heterozygosity, and F_{IS} = inbreeding coefficient. The symbol (–) was used in the place of statistics that were not calculated due to small sample size.

Locus	Statistic	In situ (Wild) Samples										Ex situ (Refugia) Samples									
		A	B	C	D	E	F	G	H	K	S	AR	BR	CR	DR	ER	FR	GR	HR	JR	UnR
		n = 32	n = 191	n = 42	n = 11	n = 8	n = 18	n = 6	n = 5	n = 10	n = 8	n = 38	n = 119	n = 46	n = 16	n = 39	n = 21	n = 11	n = 1	n = 6	n = 24
Zt-1	N_A	2.00	2.00	2.00	2.00	2.00	2.00	1.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	1.00	2.00	2.00
	A_R	2.00	2.00	2.00	2.00	-	2.00	-	-	2.00	-	2.00	2.00	2.00	2.00	2.00	2.00	1.91	-	-	2.00
	H_O	0.28	0.33	0.21	0.45	0.25	0.06	0.00	0.60	0.30	0.38	0.45	0.32	0.37	0.13	0.46	0.57	0.09	0.00	0.50	0.38
	H_E	0.36	0.38	0.32	0.35	0.50	0.31	0.00	0.42	0.38	0.49	0.47	0.36	0.35	0.30	0.46	0.44	0.09	0.00	0.38	0.48
	F_{IS}	0.23	0.13	0.35	-0.25	0.55	0.83	-	-0.33	0.25	0.30	0.07	0.12	-0.04	0.61	0.01	-0.26	0.00	-	-0.25	0.24
Zt-13	N_A	7.00	15.00	9.00	4.00	6.00	5.00	3.00	3.00	4.00	5.00	7.00	15.00	8.00	5.00	8.00	7.00	4.00	2.00	6.00	8.00
	A_R	5.37	5.46	5.26	3.91	-	4.65	-	-	4.00	-	5.13	5.58	5.10	4.45	5.44	5.36	4.00	-	-	6.25
	H_O	0.59	0.71	0.52	0.73	1.00	0.61	0.50	0.60	0.80	0.88	0.58	0.66	0.76	0.56	0.67	0.86	0.45	1.00	0.83	0.79
	H_E	0.77	0.75	0.73	0.69	0.78	0.58	0.40	0.62	0.59	0.69	0.73	0.73	0.70	0.61	0.72	0.71	0.62	0.50	0.69	0.76
	F_{IS}	0.24	0.06	0.29	-0.01	-0.22	-0.02	-0.15	0.14	-0.32	-0.21	0.22	0.10	-0.08	0.11	0.08	-0.18	0.32	-	-0.11	-0.02
Zt-16	N_A	3.00	5.00	3.00	3.00	3.00	3.00	3.00	2.00	2.00	2.00	3.00	4.00	5.00	2.00	3.00	3.00	2.00	1.00	2.00	3.00
	A_R	2.17	3.10	2.24	2.91	-	2.56	-	-	2.00	-	2.21	2.63	3.06	2.00	2.59	2.41	2.00	-	-	2.66
	H_O	0.19	0.40	0.45	0.55	0.63	0.28	0.33	0.40	0.30	0.38	0.21	0.40	0.37	0.25	0.36	0.14	0.36	0.00	0.17	0.38
	H_E	0.17	0.46	0.47	0.42	0.46	0.39	0.49	0.32	0.26	0.30	0.23	0.39	0.40	0.38	0.40	0.21	0.30	0.00	0.15	0.43
	F_{IS}	-0.07	0.13	0.04	-0.26	-0.30	0.31	0.39	-0.14	-0.13	-0.17	0.10	-0.03	0.09	0.36	0.11	0.36	-0.18	-	0.00	0.16
Zt-21	N_A	7.00	12.00	7.00	5.00	6.00	10.00	6.00	4.00	9.00	6.00	8.00	12.00	12.00	6.00	11.00	9.00	7.00	2.00	9.00	10.00
	A_R	5.74	6.53	5.69	4.91	-	8.21	-	-	9.00	-	6.77	6.49	6.89	5.69	6.64	7.94	6.63	-	-	7.41
	H_O	0.66	0.67	0.62	0.55	0.75	0.78	0.83	0.60	0.90	0.63	0.79	0.76	0.76	0.56	0.69	0.71	0.64	1.00	1.00	0.83
	H_E	0.79	0.80	0.79	0.72	0.75	0.85	0.79	0.58	0.86	0.77	0.81	0.79	0.79	0.75	0.80	0.85	0.71	0.50	0.88	0.82
	F_{IS}	0.18	0.17	0.22	0.29	0.07	0.11	0.04	0.08	0.00	0.25	0.05	0.04	0.05	0.28	0.14	0.18	0.16	-	-0.05	0.01
Zt-22	N_A	4.00	7.00	4.00	3.00	3.00	5.00	3.00	3.00	4.00	2.00	3.00	6.00	3.00	4.00	5.00	3.00	2.00	2.00	3.00	4.00
	A_R	2.74	3.32	2.51	2.91	-	4.10	-	-	4.00	-	1.87	3.02	2.70	3.36	2.90	2.46	1.91	-	-	2.64
	H_O	0.09	0.24	0.14	0.18	0.38	0.33	0.33	0.20	0.30	0.13	0.11	0.22	0.17	0.31	0.21	0.00	0.09	1.00	0.17	0.21
	H_E	0.20	0.27	0.18	0.37	0.32	0.38	0.29	0.46	0.27	0.12	0.10	0.25	0.29	0.28	0.21	0.18	0.09	0.50	0.29	0.19
	F_{IS}	0.55	0.14	0.21	0.54	-0.11	0.15	-0.05	0.64	-0.06	0.00	-0.03	0.12	0.40	-0.09	0.05	1.00	0.00	-	0.50	-0.06
Zt-23	N_A	5.00	12.00	8.00	4.00	5.00	7.00	6.00	3.00	5.00	4.00	7.00	10.00	9.00	6.00	7.00	7.00	3.00	2.00	5.00	8.00
	A_R	3.48	5.74	5.51	4.00	-	6.24	-	-	5.00	-	4.53	5.66	5.27	5.35	4.84	6.07	3.00	-	-	5.33
	H_O	0.63	0.68	0.64	0.55	0.63	0.72	0.83	0.60	0.70	0.63	0.45	0.74	0.72	0.63	0.72	0.43	0.55	1.00	1.00	0.54
	H_E	0.59	0.74	0.73	0.66	0.72	0.78	0.69	0.62	0.72	0.58	0.61	0.76	0.71	0.68	0.73	0.73	0.58	0.50	0.74	0.73
	F_{IS}	-0.04	0.09	0.13	0.22	0.20	0.11	-0.11	0.14	0.07	-0.01	0.28	0.03	0.00	0.12	0.03	0.43	0.11	-	-0.28	0.28
Zt-26	N_A	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	3.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
	A_R	2.00	2.00	2.00	2.00	-	2.00	-	-	2.00	-	2.00	2.08	2.00	2.00	2.00	2.00	2.00	-	-	2.00
	H_O	0.34	0.44	0.50	0.45	0.25	0.56	0.17	0.20	0.50	0.63	0.47	0.54	0.48	0.44	0.46	0.62	0.45	1.00	0.17	0.67
	H_E	0.46	0.50	0.49	0.43	0.47	0.50	0.49	0.50	0.38	0.49	0.50	0.50	0.48	0.50	0.49	0.43	0.48	0.50	0.38	0.50
	F_{IS}	0.27	0.12	-0.02	0.00	0.52	-0.08	0.71	0.67	-0.29	-0.21	0.06	-0.07	0.01	0.15	0.07	-0.43	0.11	-	0.62	-0.32
Mean	N_A	4.29	7.86	5.00	3.29	3.86	4.86	3.43	2.71	4.00	3.29	4.57	7.43	5.86	3.86	5.43	4.71	3.14	1.71	4.14	5.29
	A_R	3.36	4.02	3.60	3.23	-	4.25	-	-	4.00	-	3.50	3.92	3.86	3.55	3.77	4.03	3.06	-	-	4.04
	H_O	0.40	0.49	0.44	0.49	0.55	0.48	0.43	0.46	0.54	0.52	0.44	0.52	0.52	0.41	0.51	0.48	0.38	0.71	0.55	0.54
	H_E	0.48	0.56	0.53	0.52	0.57	0.54	0.45	0.50	0.49	0.49	0.49	0.54	0.53	0.50	0.54	0.51	0.41	0.36	0.50	0.56
	F_{IS}	0.18	0.12	0.18	0.10	0.10	0.15	0.14	0.20	-0.06	0.01	0.13	0.04	0.03	0.21	0.08	0.09	0.13	-	0.00	0.05

Table 4. The wild population of *Zizania texana* was subsampled and used in a one-tailed t-test against the original data to examine changes in allelic richness (A_R). The P-values were used to determine the minimal number of individuals needed to conserve genetic integrity in the ex situ population.

% Subsampled	# Subsampled	A_R	P-value
75%	248	8.08	1.00
50%	166	7.65	1.00
40%	132	7.29	0.17
25%	82	6.43	0.08

Table 5. The recommended inventory of *Zizania texana* plants from each wild segment that should be kept in an ex situ population depends on the space available for housing plants. Recommendations for potential population sizes were determined using the percent area covered by each wild segment weighted by the N_A/N_{Atotal} calculations (N_A = number of alleles per river segment; N_{ATotal} = total number of alleles observed across all river segments).

Segment	NA/NAT	NA/NAT %	Wild % cover	Adjusted % wild cover	Inventory of 200	Inventory of 300	Inventory of 400	Inventory of 500	Inventory of 600
A	39/59	0.7	9	10	20	31	41	51	61
B	55/59	0.9	63	51	101	152	203	254	304
C	35/59	0.6	17	18	36	55	73	91	109
D	23/59	0.4	0	2	5	7	10	12	14
E	27/59	0.5	<.1	2	3	5	6	8	10
F	34/59	0.6	8	10	19	29	38	48	57
G	24/59	0.4	0	2	4	6	8	10	11
H	19/59	0.3	1	3	5	8	11	14	16
K	28/59	0.5	1	3	5	8	11	14	16

Table 6. Private alleles (PAL) observed in wild and refugia *Zizania texana*. Eight PALs were detected among the refugia plants and 3 PALs in the wild. The PAL (175) for Zt-23 found in wild river segment B was shared across three individuals but was unique to segment B. Each of the other PALs was detected in a single individual. Single letters (A, B, etc.) represent wild collections from corresponding river segments of the San Marcos River. Letters followed by the letter R (AR, BR, etc.) represent the refugia populations that originated from wild river segments denoted in the first letter.

Collection ID	River Segment	Locus	Private Allele
264B	BR	Zt-13	218
1442B	BR	Zt-26	190
225B	BR	Zt-23	137
9E	ER	Zt-21	204
122011F	FR	Zt-13	238
7175F	FR	Zt-21	198
4J	JR	Zt-13	240
111812	UnR	Zt-23	161
B0705	B	Zt-23	175
B3501	B	Zt-23	175
B1327	B	Zt-22	194
B1348	B	Zt-23	175
F0102	F	Zt-21	174

Table 7. Pairwise estimates of genetic divergence (F_{ST}) between sampled *Zizania texana* groups are reported above the diagonal. Pairwise F_{ST} values that were significant after adjusting for nominal comparisons ($P \leq 0.00024$) are indicated by an (*) while non-significant F_{ST} are denoted by NS. Significance is reported below the diagonal. Single letters (A, B, etc.) represent wild collections from corresponding river segments of the San Marcos River. Letters followed by the letter R (AR, BR, etc.) represent the refugia populations that originated from wild river segments denoted in the first letter.

Population	Ex situ (Refugia) Samples										In situ (Wild) Samples									
	AR	BR	CR	DR	ER	FR	GR	HR	JR	UnR	A	B	C	D	E	F	G	H	K	S
AR		0.001	0.005	0.058	0.066	0.017	0.014	-	0.129	0.024	0.288	0.003	0.001	0.013	0.286	0.004	0.008	0.011	0.011	0.296
BR	NS		0.844	0.343	0.965	0.000	0.009	-	0.392	0.188	0.001	0.441	0.240	0.790	0.446	0.003	0.449	0.141	0.501	0.218
CR	NS	NS		0.284	0.628	0.001	0.003	-	0.773	0.180	0.013	0.046	0.119	0.815	0.557	0.009	0.239	0.242	0.554	0.350
DR	NS	NS	NS		0.804	0.349	0.107	-	0.292	0.204	0.011	0.202	0.631	0.490	0.443	0.250	0.618	0.171	0.293	0.289
ER	NS	NS	NS	NS		0.009	0.045	-	0.379	0.942	0.006	0.664	0.287	0.550	0.835	0.079	0.190	0.114	0.471	0.337
FR	NS	*	NS	NS	NS		0.016	-	0.088	0.074	0.001	0.000	0.001	0.003	0.071	0.041	0.049	0.111	0.018	0.299
GR	NS	NS	NS	NS	NS	NS		-	0.152	0.229	0.002	0.043	0.029	0.001	0.019	0.087	0.141	0.009	0.021	0.002
HR	NS	NS	NS	NS	NS	NS	NS		-	-	-	-	-	-	-	-	-	-	-	-
JR	NS	NS	NS	NS	NS	NS	NS	NS		0.520	0.105	0.168	0.113	0.269	0.720	0.648	0.373	0.572	0.969	0.298
UnR	NS	NS	NS	NS	NS	NS	NS	NS	NS		0.001	0.045	0.073	0.074	0.592	0.030	0.168	0.178	0.540	0.311
A	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		0.001	0.001	0.042	0.348	0.003	0.003	0.033	0.004	0.085
B	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS		0.075	0.543	0.762	0.003	0.368	0.108	0.220	0.204
C	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		0.308	0.605	0.005	0.160	0.171	0.075	0.139
D	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		0.526	0.050	0.329	0.178	0.379	0.148
E	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		0.449	0.085	0.154	0.383	0.693
F	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		0.350	0.159	0.366	0.121
G	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		0.423	0.396	0.153
H	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		0.233	0.162
K	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		0.214
S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	

Table 8. (A) Total number of alleles in wild *Zizania texana* across three different studies. (B) Total number of alleles in ex situ populations as well as the combined ex situ and in situ populations of *Z. texana* across two different temporal samplings. The symbol (–) indicates that the locus was not used in a study.

A.

Locus	Saltzgiver et al 2021 (in situ only) n = 331	Wilson et al 2017 (in situ only) n = 156	Richards et al 2007 (in situ only) n = 298-346
Zt-1	2	3	4
Zt-13	15	9	20
Zt-16	5	4	-
Zt-21	14	13	15
Zt-22	7	5	7
Zt-23	14	9	13
Zt-26	2	2	-

B.

Locus	Saltzgiver et al 2021 (ex situ only) n = 321	Wilson et al 2017 (ex situ only) n = 48	Saltzgiver et al 2021 (ex situ and in situ) n = 652	Wilson et al 2017 (ex situ and in situ) n = 197
Zt-1	2	2	2	3
Zt-13	18	8	18	15
Zt-16	5	3	5	5
Zt-21	18	14	19	15
Zt-22	6	4	7	6
Zt-23	14	7	16	13
Zt-26	3	2	3	2

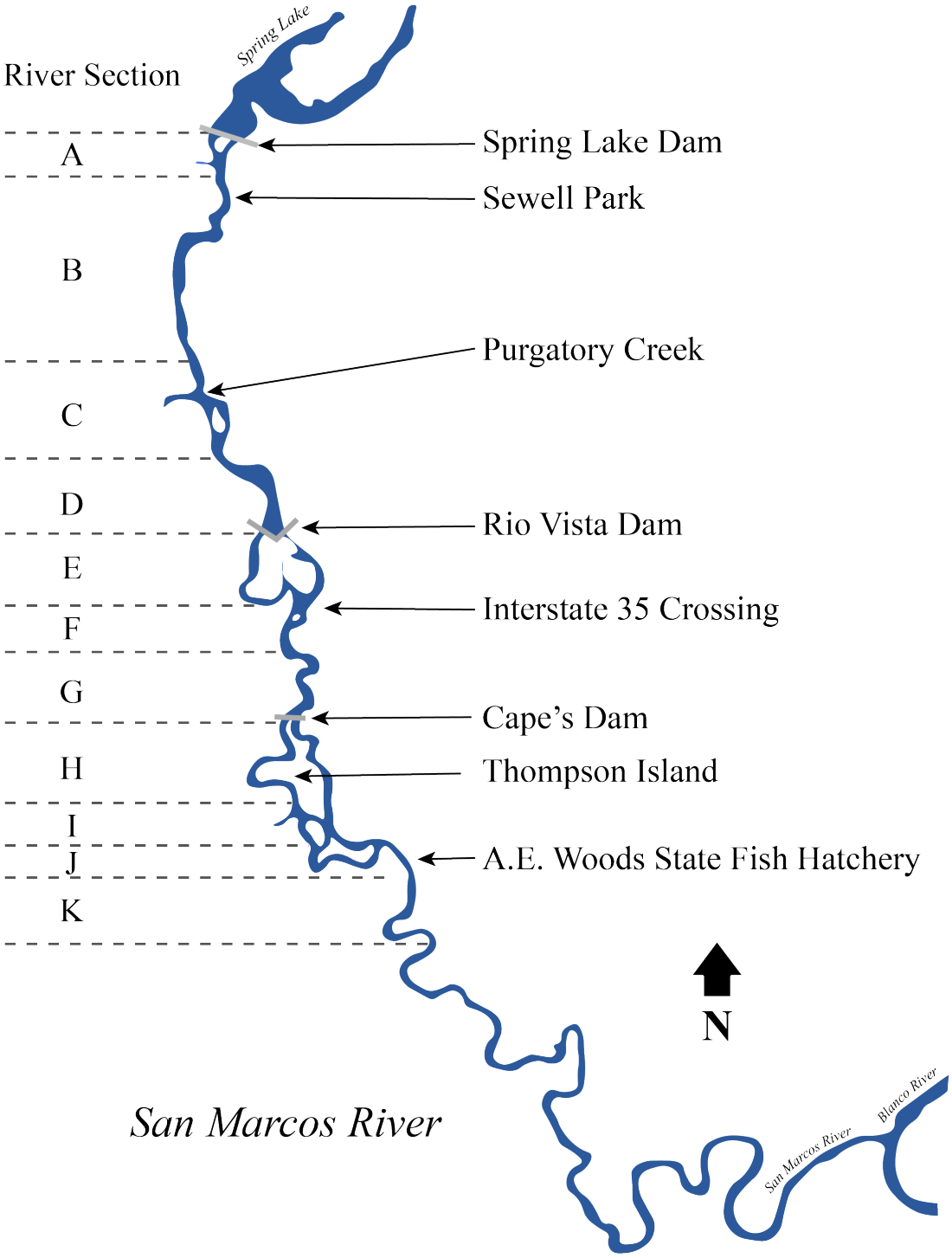

Figure 1. Map of the *Zizania texana* monitoring segments within the San Marcos River in Texas. River segments were designated by Texas Parks and Wildlife personnel. Sampling was conducted randomly within each river segment. Spring Lake was given the segment designation "S" for this project.

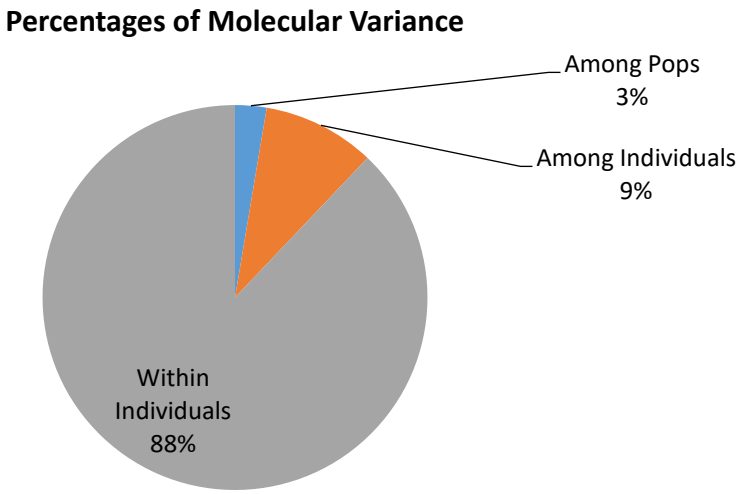

Figure 2. An Analysis of Molecular Variance (AMOVA) conducted on *Zizania texana* showed the amount of molecular variance among populations (i.e., river segments), among individuals and within individuals demonstrated that most variance is observed within individuals and not between populations.

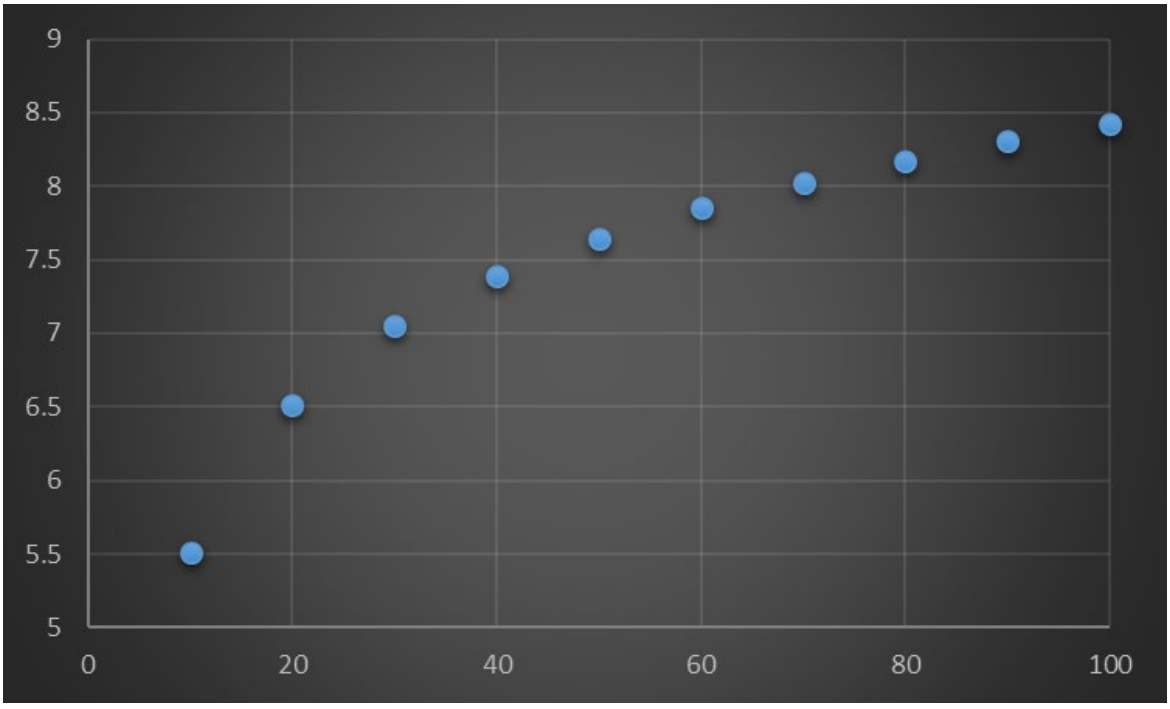

Figure 3. Using the software program HP-Rare v1.1 the data from the wild population of *Zizania texana* was subsampled multiple times to generate allelic richness estimates (y-axis) ranging from 10% to 100% of samples obtained from the in situ population (x-axis).

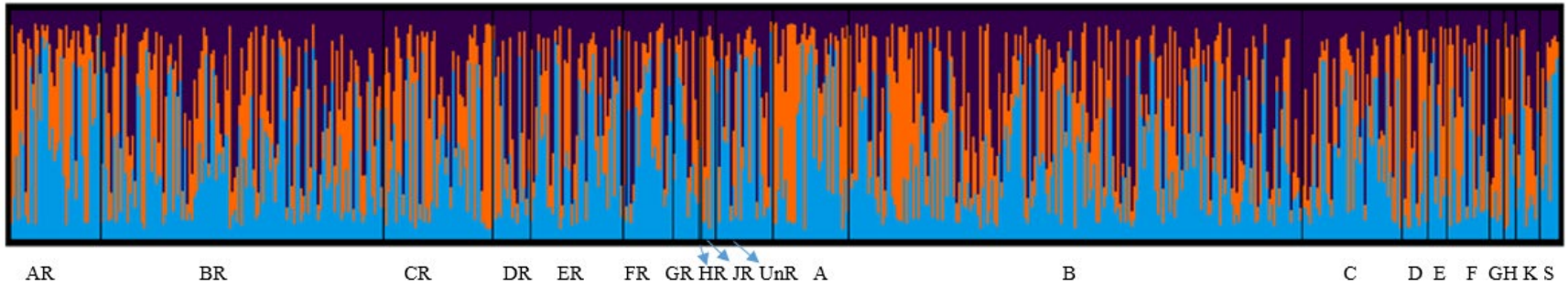

Figure 4. STRUCTURE results for three genetic clusters (K = 3) of *Zizania texana* collected from in situ and ex situ populations. Each individual plant is represented by a single vertical bar, with the proportion of color in each bar representing the estimated proportion of ancestry attributed to each of three genetic clusters. Single letters (A, B, etc) on the X-axis represent in situ river segments where the wild plants were collected. Letters followed by the letter R (AR, BR, etc.) represent the ex situ populations that originated from wild river segments denoted by the first letter.

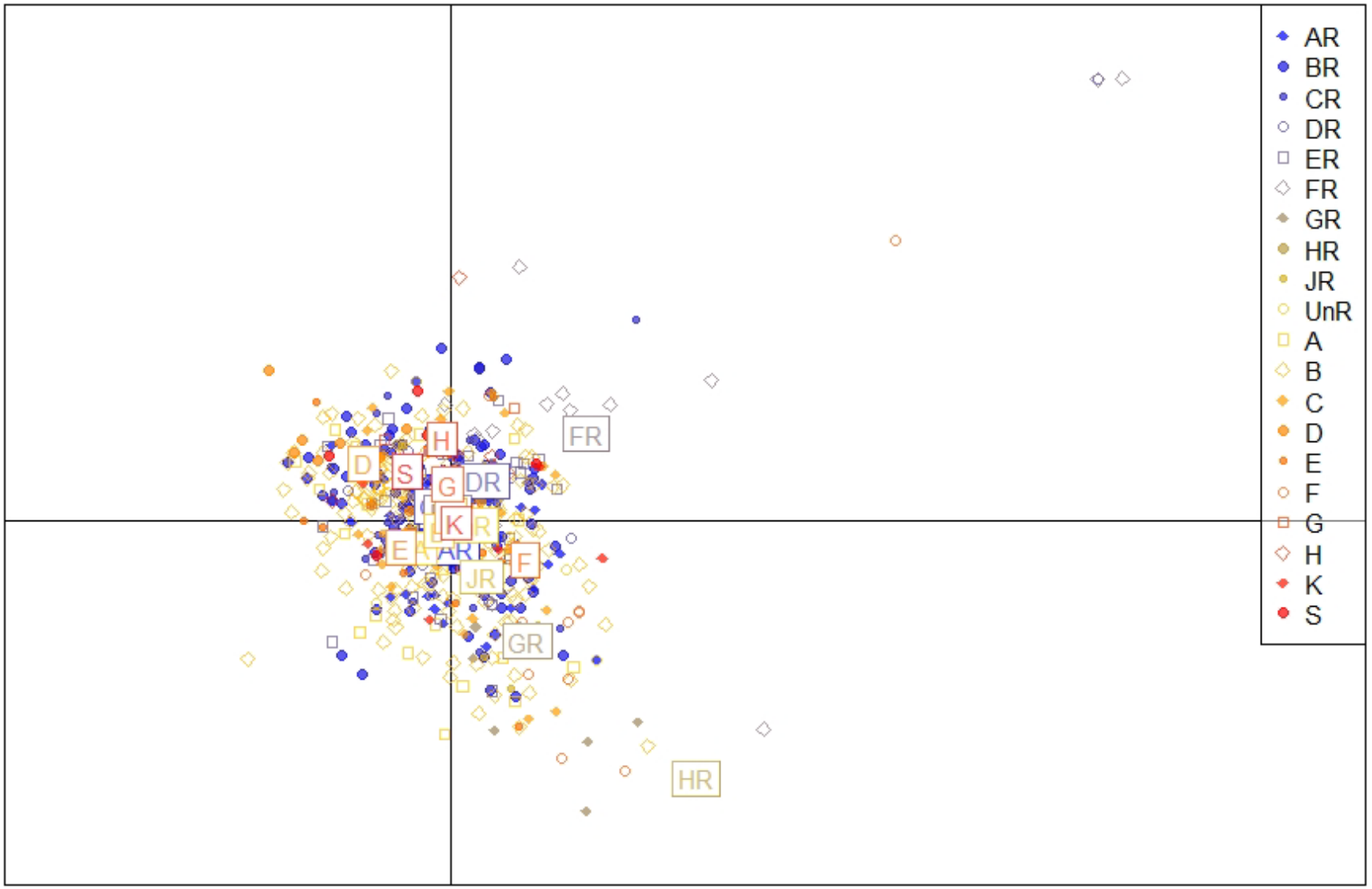

Figure 5. Discriminant Analysis of Principal Components (DAPC) of *Zizania texana* from wild and refugia populations. Single letters (A, B, etc.) represent wild collections from corresponding river segments of the San Marcos River. Letters followed by the letter R (AR, BR, etc.) represent the refugia populations that originated from wild river segments denoted in the first letter.

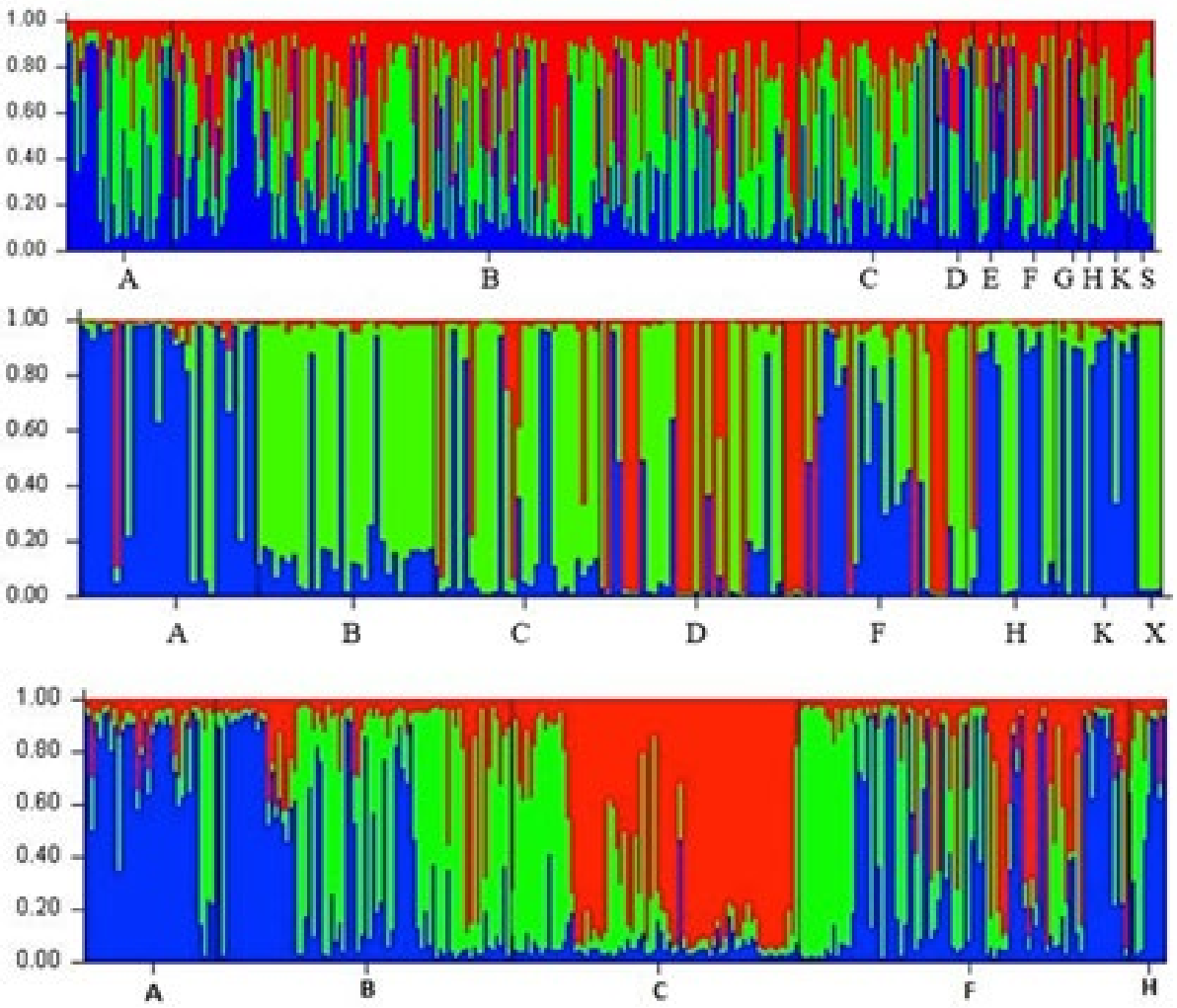

Figure 6. Graphical representations of three different STRUCTURE analyses. The top plot shows the wild population structure of *Zizania texana* analyzed for this study, the middle figure represents the wild *Z. texana* population from Wilson et al. (2017) and the bottom figure represents the Richards et al. (2007) study. Each individual plant is represented by a single vertical bar, with the proportion of color in each bar representing the estimated proportion of ancestry attributed to each of three genetic clusters (K = 3). Single letters (A, B, etc.) represent river segments where the wild plants were obtained. While the three plots use the same color scheme, they were created independently and therefore the colors do not necessarily represent the same three genetic clusters across the three plots.

Appendix 1. Genetic diversity indices for the 2012 in situ and ex situ populations of *Zizania texana*, used in Wilson et al. (2017), that were reamplified using the loci listed below and recalculated after removing samples and with identical multi-locus genotypes that occurred within segments. N_A = number of alleles, A_R = allelic richness, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} = inbreeding coefficients and the symbol (–) was used in the place of statistics that could not be generated due to small sample size.

Locus	Statistic	In situ (Wild) Samples									Ex situ (Refugia) Samples						
		A	B	C	D	E	F	H	K	X	AR	BR	CR	ER	FR	JR	KR
		n = 27	n = 19	n = 25	n = 21	n = 1	n = 27	n =14	n = 12	n = 3	n = 2	n = 20	n = 7	n = 3	n = 11	n = 3	n = 2
Zt-1	N_A	2.00	2.00	2.00	2.00	2.00	3.00	2.00	2.00	1.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
	A_R	2.00	2.00	1.91	1.99	-	2.34	2.00	2.00	-	-	2.00	-	-	2.00	-	-
	H_O	0.33	0.26	0.08	0.29	1.00	0.22	0.14	0.33	0.00	0.50	0.45	0.29	0.67	0.27	0.33	0.50
	H_E	0.49	0.30	0.15	0.24	0.50	0.20	0.24	0.28	0.00	0.38	0.47	0.24	0.44	0.24	0.28	0.38
	F_{IS}	0.34	0.15	0.47	-0.14	-	-0.09	0.45	-0.16	-	-	0.07	-	-	-0.11	-	-
Zt-13	N_A	5.00	7.00	8.00	8.00	1.00	9.00	5.00	5.00	2.00	3.00	5.00	6.00	4.00	3.00	5.00	4.00
	A_R	4.91	5.85	5.54	6.51	-	6.21	4.95	4.83	-	-	4.38	-	-	3.00	-	-
	H_O	0.48	0.89	0.60	0.81	0.00	0.59	0.50	0.75	0.33	1.00	0.45	0.71	1.00	0.73	1.00	1.00
	H_E	0.76	0.71	0.62	0.78	0.00	0.75	0.72	0.60	0.28	0.63	0.54	0.74	0.72	0.60	0.78	0.75
	F_{IS}	0.38	-0.24	0.05	-0.01	-	0.23	0.34	-0.21	-	-	0.18	-	-	-0.17	-	-
Zt-16	N_A	3.00	3.00	4.00	3.00	2.00	2.00	2.00	4.00	2.00	2.00	2.00	3.00	2.00	3.00	1.00	2.00
	A_R	2.39	2.83	3.43	2.95	-	1.94	2.00	3.75	-	-	2.00	-	-	3.00	-	-
	H_O	0.22	0.58	0.48	0.38	1.00	0.19	0.00	0.17	0.67	0.50	0.25	0.29	0.33	0.36	0.00	0.50
	H_E	0.26	0.54	0.58	0.42	0.50	0.17	0.24	0.23	0.44	0.38	0.29	0.62	0.28	0.38	0.00	0.38
	F_{IS}	0.15	-0.04	0.19	0.11	-	-0.08	1.00	0.31	-	-	0.16	-	-	0.08	-	-
Zt-21	N_A	9.00	6.00	8.00	13.00	2.00	12.00	5.00	8.00	3.00	3.00	8.00	8.00	4.00	6.00	6.00	3.00
	A_R	6.48	5.24	6.21	10.26	-	9.18	4.57	7.66	-	-	7.21	-	-	6.00	-	-
	H_O	0.48	0.63	0.64	0.76	1.00	0.67	0.57	0.83	1.00	1.00	0.80	1.00	1.00	0.91	1.00	0.50
	H_E	0.78	0.69	0.75	0.88	0.50	0.88	0.62	0.79	0.61	0.63	0.78	0.81	0.72	0.74	0.83	0.63
	F_{IS}	0.40	0.11	0.17	0.16	-	0.26	0.11	-0.01	-	-	0.00	-	-	-0.18	-	-
Zt-22	N_A	4.00	3.00	4.00	5.00	2.00	4.00	4.00	3.00	2.00	1.00	2.00	3.00	2.00	4.00	1.00	1.00
	A_R	2.62	2.16	3.04	3.81	-	2.62	3.74	2.83	-	-	1.55	-	-	4.00	-	-
	H_O	0.07	0.11	0.20	0.33	1.00	0.07	0.14	0.08	0.33	0.00	0.05	0.29	0.33	0.55	0.00	0.00
	H_E	0.17	0.10	0.25	0.37	0.50	0.17	0.36	0.16	0.28	0.00	0.05	0.36	0.28	0.58	0.00	0.00
	F_{IS}	0.58	-0.01	0.23	0.11	-	0.58	0.63	0.50	-	-	0.00	-	-	0.10	-	-
Zt-23	N_A	6.00	6.00	7.00	7.00	2.00	9.00	4.00	7.00	3.00	2.00	6.00	4.00	4.00	6.00	4.00	4.00
	A_R	4.43	5.14	6.06	6.04	-	6.54	3.96	6.75	-	-	5.09	-	-	6.00	-	-
	H_O	0.44	0.63	0.72	0.57	1.00	0.70	0.36	0.75	1.00	0.50	0.65	0.29	0.67	0.82	0.67	1.00
	H_E	0.57	0.68	0.74	0.77	0.50	0.79	0.69	0.74	0.61	0.38	0.75	0.60	0.67	0.71	0.67	0.75
	F_{IS}	0.24	0.10	0.05	0.28	-	0.13	0.51	0.03	-	-	0.16	-	-	-0.10	-	-
Zt-26	N_A	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	1.00	2.00
	A_R	2.00	2.00	2.00	2.00	-	2.00	2.00	2.00	-	-	2.00	-	-	2.00	-	-
	H_O	0.41	0.26	0.40	0.24	1.00	0.41	0.07	0.25	0.33	0.50	0.25	0.14	0.67	0.36	0.00	0.50
	H_E	0.48	0.49	0.49	0.50	0.50	0.49	0.48	0.41	0.28	0.38	0.44	0.34	0.44	0.40	0.00	0.38
	F_{IS}	0.16	0.49	0.21	0.54	-	0.19	0.86	0.43	-0.29	-	0.45	-	-	0.13	-	-
Mean	N_A	4.43	4.14	5.00	5.71	1.86	5.86	3.43	4.43	2.14	2.14	3.86	4.00	2.86	3.71	2.86	2.57
	A_R	3.55	3.60	4.03	4.79	-	4.40	3.32	4.26	-	-	3.46	-	-	3.71	-	-
	H_O	0.35	0.48	0.45	0.48	0.86	0.41	0.26	0.45	0.52	0.57	0.41	0.43	0.67	0.57	0.43	0.57
	H_E	0.50	0.50	0.51	0.56	0.43	0.49	0.48	0.46	0.36	0.39	0.47	0.53	0.51	0.52	0.37	0.46
	F_{IS}	0.32	0.07	0.15	0.17	-	0.19	0.49	0.05	0.15	-	0.15	-	-	-0.05	-	-

January 2021 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

February 10, 2021

Task 1 Refugia Operations

Staff

We have reorganized our Edwards Aquifer Refugia Program into two teams: a Research Team and a Husbandry/Collections Team.

Within the Research Team, we have two positions, both at the San Marcos Research Center (SMARC). One of those positions (the Lead Researcher) remains unfilled. The U.S. Fish & Wildlife Service (USFWS) received 99 applications for the position. Our Human Resources department estimated that they would have a list of names for us to evaluate after they process the applications and verify basic qualifications. The other research position was filled this month with Desiree Moore, who comes to us from Oklahoma State University, where she recently completed her Master's Degree.

Adam Daw leads our Husbandry/Collections Team. He oversees two biological technicians each at Uvalde National Fish Hatchery (UNFH) and the SMARC. The USFWS conducted interviews for a lead biological technician at UNFH. We approved the promotion of Jennifer Whitt to the lead biological technician position under Mr. Daw next month. We plan to hire a GS-05 biological technician this year to fill the vacancy Ms. Whitt will leave when she accepts her promotion. When this occurs, UNFH will have three biological technicians.

Species Collection

On January 22, 2021, refuge staff from Uvalde and San Marcos collected 16 Comal Springs riffle beetles (CSRB) from downed wood at Spring Island, New Braunfels. Of the individuals collected, 15 were transferred to UNFH and one was released.

Organisms that were transferred out of quarantine and into the refuge population during January include 173 Comal Springs Riffle Beetles (40 - UNFH, 133 - SMARC), 28 San Marcos salamanders at SMARC, and 13 Texas wild rice (TWR) at SMARC.

Husbandry

Uvalde

Staff conducted maintenance on refuge and quarantine systems to remove calcium buildup and add dividers to tanks. Staff began construction on a new hospital system in the refuge room and began cleaning and reconfiguring a previous experimental tank system for experiments being planned for 2021.

We installed the shade cloth canopies over the TWR. We drained and pressure washed one of the four rice tanks.

We constructed a stand for the invertebrate room chillers, placed outside the Invert Room with assistance from facilities personnel. Moving the chillers outside will prevent waste heat in the Invert Room. We disassembled one invertebrate rack system. We began rust removal from this rack in preparation for reinstallation.

During invertebrate inventories, we found six CSRB larvae that had pupated in one of the larval boxes. We did not notice anything unique about the system they were in, compared to our other larvae boxes.

Cultures of *Moina* sp. and rotifers were brought in to evaluate their potential use as live food for refuge species at UNFH.

SMARC

Staff repotted and transferred around 20% of SMARC TWR into a cleaned tank and added TWR that came out of their 30 day quarantine period. We finished one of the racks for the new invert system in the refuge room. We constructed a new rack for F1 Texas blind salamanders. And, staff trained the new biologist (Desiree Moore) on husbandry methods.

We have observed consistently high mortalities in one fountain darter tank in the refugium. We treated these fish with a 1% salt solution at the beginning of the month and treated them with chloramine-T at the end of the month. We are preparing to move these fish to a tank in the quarantine room for further

evaluation/treatment. We plan to sterilize the tank in which they were previously held.

Task 2 Research

Desiree Moore, our newly hired research biologist spent time getting familiar with the facility and organisms, doing new hire training, reading literature, and planning for the proposed projects for 2021. She assisted our Husbandry and Collection Team in the collection of Comal Springs riffle beetles and learned to identify the larvae and adults. She began working with UNFH biotech Jennifer Whitt and Dr. Carlos-Shanley at Texas State University to develop a more detailed proposal for a an extension of our Comal-Springs-riffle-beetle microbiome project. She also began s modifying the proposal for increasing CSRB F1 production. She also began planning for collecting leaf cuttings from each of the Refugia Texas wild rice plants for a genetics assessment.

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 meetings or presentations were conducted this month.

Table 1. New collections and total census in ~~December~~ 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.

January 2021										
Species	Kept		Released	Total Collected	Incorporated		Mortalities		Census	
	SMARC	UNFH			SMARC	UNFH	SMARC	UNFH	SMARC	UNFH
Fountain darters (San Marcos)	NT	NT	--	--	0	0	75	11	526	473
Fountain darters (Comal)	NT	NT	--	--	0	0	1	2	171	25
Comal Springs riffle beetles	0	15	1	16	133	40	0	0	133	55
Comal Springs dryopid beetles	0	0	0	0	0	0	0	0	1	0
Peck's Cave amphipods	NT	NT	--	--	0	0	56	28	209	294
Edwards Aquifer diving beetles	NT	NT	--	--	0	0	--	--	0	0
Texas troglobitic water slaters	NT	NT	--	--	0	0	--	--	0	0
Texas blind salamanders	NT	NT	--	--	0	0	1	0	268	29
San Marcos salamanders	NT	NT	--	--	28	0	27	5	227	192
Comal Springs salamanders	NT	NT	--	--	0	0	4	0	118	49
Texas wild rice plants	NT	NT	--	--	13	0	2	--	217	174

Summary of ~~December~~ January Activities

January 22, 2021 - Collected Comal Springs riffle beetles at Spring Island, New Braunfels

Pictures

Figure 1. Jennifer Whitt, Braden West and Desiree Moore sorting Comal springs riffle beetles during a collection at Spring Island, New Braunfels, Texas.

Figure 2. Jennifer Whitt and Benjamin Thomas assisting with the construction of the new hospital rack in the UNFH refuge room.

Figure 3. The completed canopies over the Texas wild rice tanks at UNFH.

Figure 4. New invertebrate racks at SMARC under construction.

February 2021 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Adam Daw and David Britton

San Marcos Aquatic Resources Center
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Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

Traps were set for Texas blind salamanders at Primer's fissure and Johnsons well on February 1, 2021 and removed on February 22, 2021. The trap removal date was scheduled for the 16th but due to the winter storm we were not able to reach the site unit the 22nd. Eight TBS were captured at Primer's fissure with three retained and three were captured at Johnson well with 1 retained. The TBS are being quarantined at SMARC

On February 26th, 2021 refuge staff from UNFH and SMARC collected Texas wild rice from the San Marcos River (section A). Fifty one tillers where collected from five locations and transported to UNFH for quarantine.

Husbandry

Uvalde

Staff conducted maintenance on refuge and quarantine systems, including cleaning systems and adding dividers to tanks. The new hospital system has being constructed in the refuge room and is undergoing a system test before we start placing animals in the system. The experimental tank system is being constructed for a salamander spawning habitat experiment to be conducted in the summer of 2021.

The invertebrate rack that was previously undergoing rust removal has been placed back in the invert room and being prepped for plumbing with external chiller.

The inventory of the Texas wild rice tanks was completed with 35 mortalities, which is the number of mortalities since the last full inventory in May, 2020.

Aquariums are being cleaned and setup to display refuge animals in the UNFH lobby.

The *Moina* sp. culture was scaled up slightly (5 gallons) to evaluate their potential culture and use as a live food item. When offered to Fountain darters they were actively consumed.

During the winter storm the power to UNFH was out for roughly 4 days. The refuge generators functioned as intended and provided power to all systems within the refuge and quarantine buildings with the exception of two chillers in the refuge system that were connected to the main buildings generator. The UNFH main pump lost power for 1 day due to the generator running empty on fuel during which time the refuge systems relied on recirculated water. Refuge staff were able to access the facility during the entire time. We did not observe any adverse effects on the animals during this period.

SMARC

Staff conducted maintenance on refuge and quarantine systems. Work on the new invertebrate system continued. Staff oversaw the checking of the TBS traps every 2-3 days during deployment.

The fountain darters that were having high mortality last month were moved into the quarantine room and treaded with formalin and 1% salt solution. The mortality rate has decreased after treatment.

During the winter storm power was out at SMARC for roughly 5 days, during this time the generators worked as intended and powered the refuge building. Six refuge chillers were broken as a result of water freezing inside of them. Although the chillers and some pumps froze/broke water flow was maintained through well water inflow for most systems. Refuge staff were unable to access the facility for 3 days during this period due to the roads being impassible. We observed high than normal mortality in the invertebrate system after the storm which is most likely caused by the system loosing water flow. The design of the systems are being evaluated to minimize the potential impact of future extreme events.

Staff

Task 2 Research

Biologist Desiree Moore completed Texas wild rice (TWR) genetic clips from the SMARC population and obtained the aerial photographs for wild TWR. She worked on research proposals and began construction of the rectangular holding boxes for the Comal

Springs riffle beetle project. Rectangular box construction should be completed within the next week. MS. Moore also assisted with collection of Texas Blind Salamanders and TWR in the field.

Unfortunately, the winter weather delayed some February goals. The Uvalde staff have almost completed clipping the Uvalde TWR population for genetic analysis. Selecting TWR stands to sample in the wild was delayed. The Covid-19 pandemic, which has prevented annual SCUBA and snorkel certifications, has also affect our collection schedule. However, these delays should not affect the completion time for the project. As long as all sampling is completed by June, Geneticist Steven Mussmann (USFWS's Southwest Native Aquatic Resources and Recovery Center) assures us he can stay on schedule.

Task 4 Species Reintroduction

No reintroduction activities occurred this month.

Task 5 Reporting

David Britton finalized and submitted an Annual report to the EAA's Chief Science Officer.

Task 6 Meetings and Presentations

Adam Daw gave a presentation to the Des Moines Central Campus high school marine biology and aquarium science classes about the refuge program.

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.

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	48	23	478	450
Fountain darter: Comal	NT	NT	--	--	0	0	0	0	171	25
Comal Springs riffle beetle	NT	NT	--	--	0	14	109	0	24	69
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	0	1	0
Peck's Cave amphipod	NT	NT	--	--	0	0	56	36	209	258
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	--	--	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	--	--	0	0
Texas blind salamander	4	NT	4	11	0	0	5	0	263	29
San Marcos salamander	NT	NT	--	--	0	0	5	0	222	192
Comal Springs salamander	NT	NT	--	--	0	0	1	1	117	48
Texas wild rice plants	NT	5	--	5	0	0	1	35	216	139

Summary of january Activities

Feb 1, 2021 – Feb 22, 2021 – Collected Texas blind salamanders from Johnson well and Primer's fissure

Feb 26, 2021 – Collected Texas wild rice from the San Marcos River (section A)

Pictures

March 2021 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Adam Daw and Desiree Moore

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

Task 1 Refugia Operations

Species Collection

On March 18, 2021, refuge staff from SMARC collected San Marcos salamanders below the dam at Spring Lake, San Marcos River. An estimated 126 were observed with 51 captured and transported to SMARC and are undergoing quarantine.

On March 25, 2021, refuge staff from SMARC and UNFH collected invertebrates and salamanders by hand from the Spring Island area (east of the island) of the Comal River in New Braunfels. Animals collected included 34 Comal springs riffle beetles (SMARC), 13 Peck's Cave amphipods (SMARC), 1 Comal springs dryopid beetle (UNFH), and 15 Comal springs salamanders captured of the estimated 45 observed (UNFH). Organisms are undergoing quarantine at their respective facilities.

Husbandry

Uvalde

Staff conducted maintenance on refuge and quarantine systems. A rack of tanks that will be used for future experiments is under construction. Work continues on the Texas wild rice housed at UNFH, including repotting/reorganizing the plants and modifications to the tank layout to allow easier cleaning of the tanks. Leaf clippings were taken from TWR in the refuge for a genetic research study. Staff moved 44 Texas blind salamanders from SMARC refuge stock to UNFH. Two aquariums setup to display refuge animals in the UNFH lobby are currently being nitrogen cycled before animals are added.

SMARC

Staff conducted maintenance on refuge and quarantine systems. Work on a new invertebrate system was completed. Some of the invertebrates were moved to the new system. Staff removed some of the chillers broken during the heavy freeze last month. Staff repositioned working units to critical systems. Three new chillers have been ordered along with parts to repair the broken ones.

Five San Marcos River Fountain Darters from UNFH, and five San Marcos River and ten Comal River Fountain Darters from SMARC were sent to the FWS Southwestern Fish Health Unit for parasite enumeration. The San Marcos River Fountain Darters from both facilities had minimal parasites. For the Comal River Fountain Darters two of ten had *Centrocestus formosanus* and four of ten had *Ichthyoboda* sp. observed on the gills. We have not had any abnormal mortalities this month with Comal River Fountain Darters. Currently, we have decided not to treat them, although we may in the future as a precaution.

Staff at both SMARC and UNFH (Adam Daw, Benjamin Thomas, Thomas Funk and Braden West) completed their snorkel tests with Jennifer Whitt and Desiree Moore to complete later. Jennifer Whitt and Benjamin Thomas conducted a portion of the heavy equipment training, which will be completed in April.

Task 2 Research

Texas Wild Rice Genetics

This year we are conducting a genetic assessment of the captive and wild Texas wild rice (TWR) populations to determine how well our captive population reflects the genetics in the wild, where we should collect new rice plants from to close any gaps, and the number of plants needed to maintain a fully functional refugia population. Desiree Moore collected 36 tissue samples from TWR across nine stands from the first section of the San Marcos River and sent the tissue samples we had (including all refugia plants and section A of the river) to the Southwestern Native Aquatic Resources and Recovery Center for genetic analysis. In April, Ms. Moore plans to collect more TWR from the wild population, depending on availability of divers and kayak training.

Comal Springs Riffle Beetle Pupation

BIO-WEST and SMARC will assess husbandry changes to holding containers, densities, and wild versus captive biofilm used for food to determine if these changes will increase pupation of CSRB larvae in the refugia population. Dr. Ely Kosnicki (BIO-WEST) and Ms. Moore prepared a system to test pupation of Comal Springs riffle beetles (CSRB) in rectangular boxes. Once they have the F1 larvae needed, they will start that experiment. In April, Ms. Moore will help Dr. Kosnicki begin experiments examining CSRB pupation.

Comal Springs Riffle Beetle Exposure to Staphylococcus

In previous research, Dr. Carlos-Shanley (Texas State University) found SMARC CSRB have *Staphylococcus* spp. (Staph) in their gut but wild CSRB do not. We will expose wild CSRB larvae to Staph to determine if survival and pupation are affected by these bacteria at SMARC. Ms. Moore purchased all of the supplies needed for a Staph exposure experiment and started building the system. She will test whether Staph has an impact on the survival of CSRB in captivity. While waiting on ordered parts, Ms. Moore began conditioning leaves at the Freeman Aquatic Building (FAB, Texas State University) to ensure they will be ready for the experiment and prevent Staph contamination. She plans to help collect at least 50 wild CSRB and finish setting up the system at the FAB for the Staph exposure experiment. Adam Daw and Ms. Moore transferred ten adult CSRB and ten larvae CSRB to Dr. Carlos-Shanley from Uvalde to test for Staph and other potentially harmful bacteria.

Captive Habitat for San Marcos Salamanders

This year we are conducting an experiment to examine the effects of darkened tanks, textured tank bottoms, and a combination of the two on reproduction of San Marcos Salamanders to determine if we can use these conditions to promote reproduction when needed. Ms. Moore purchased and prepared habitat items for the San Marcos Salamander habitat modification project. She also prepared Standard Operating Procedures and data sheets for the habitat modification project to reduce any confusion for Uvalde staff. In April, Ms. Moore plans to prepare the pond liner for the San Marcos Salamander habitat modification project and help the Uvalde staff prepare the system and randomly place salamanders in tanks.

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

Adam Daw, Desiree Moore, and David Britton contributed to the monthly report and the annual work plan.

Task 6 Meetings and Presentations

Only general discussions with EAA staff were conducted this month. We had no formal meetings or presentations.

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 Mar Kept	UNFH mar Kept	Released	Total Collected	SMARC Mar Incorporated	UNFH Mar Incorporated	SMARC Mar Mortalities	UNFH Mar Mortalities	SMARC Mar Census	UNFH Mar Census
Fountain darter: San Marcos	NT	NT	--	--	0	0	24	26	423	424
Fountain darter: Comal	NT	NT	--	--	0	0	11	1	160	24
Comal Springs riffle beetle	34	NT	--	34	0	14	NA	33	24	36
Comal Springs dryopid beetle	NT	1	--	1	0	0	NA	--	1	0
Peck's Cave amphipod	13	NT	--	13	0	0	102	24	107	234
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	218*	73*
San Marcos salamander	51	NT	75	51	0	0	7	1	215	191
Comal Springs salamander	NT	15	30	15	0	0	1	0	116	48
Texas wild rice plants	NT	NT	--	--	0	0	0	3	216	136

*44 Texas blind salamanders transferred from SMARC to UNFH

Summary of March Activities

March 18, 2021 - Collected San Marcos salamanders from the area below Spring dam of the San Marcos River.

March 25, 2021 - Collected event at the Spring Island area of the Comal River for Peck's cave amphipod, Comal springs dryopid beetle, Comal springs riffle beetle and Comal salamander

Pictures

Figure 1. Benjamin Thomas and Jennifer Whitt preparing for snorkel test at UNFH.

Figure 2. Thomas Funk and Braden West collecting San Marcos salamanders in the San Marcos River for the SMARC refuge.

April 2021 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Adam Daw and Desiree Moore

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500 East McCarty Lane
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Task 1 Refugia Operations

Species Collection

On April 1, 2021, refugia staff checked lures placed by Dr. Ely Kosnocki (BIO-WEST) in Spring Run 3 of Landa Park, New Braunfels; 28 Comal Springs riffle beetles and four Peck's Cave amphipods were captured. Only the Comal Spring riffle beetles were retained for incorporation into the refugia at SMARC after quarantine.

On April 13, 2021, Desiree Moore and Dr. Ely Kosnocki checked lures placed by Dr. Ely Kosnocki in Spring Run 3 of Landa Park, New Braunfels; 623 juvenile Comal Springs riffle beetles were captured with 84 retained to be used in a research experiment. Ninety-nine adult Comal Springs riffle beetles were captured and 78 retained for incorporation into the refugia at SMARC after quarantine. Sixteen Peck's Cave amphipods were captured and released.

On April 22, 2021, refugia staff from SMARC and UNFH collected Texas wild-rice tillers from sections D and E of the San Marcos River. A total of 216 tillers from 22 distinct stands (2 – D, 20 - E) were harvested and transported to UNFH and are undergoing quarantine.

During the period of March 26-28, 2021, refugia staff from SMARC and UNFH picked up fountain darters collected during the BIO-WEST biomonitoring survey of the San Marcos River. A total of 148 fountain darters collected from the middle section of the river on March 26 and 27 were transferred to refugia staff and transported to SMARC. On March 28, 147 darters collected from the lower section of the river were transferred to refugia staff and transported to UNFH. All fountain darters underwent a formalin dip except for 52 at SMARC that will be sent to the FHS Southwest Fish Health Unit for parasite and virology analysis.

On April 29, 2021, UNFH refugia staff set eight lures for Comal Springs riffle beetles and Peck's Cave amphipods in Landa Park, New Braunfels (Spring Run 3). Lures will be harvested in May.

Husbandry

Uvalde

Staff conducted maintenance on refugia and quarantine systems. Adam Daw finished construction on the rack of 44 tanks that will be used for the upcoming salamander habitat experiment. Work continues on the Texas wild rice housed at UNFH, with over eighty of the plants repotted in April. Twenty additional Comal springs riffle beetles (10 adults, 10 larvae) were taken to Dr. Camila Carlos-Shanley of Texas State University to test for *Staphylococcus* as part of a research project. The pump room connected to the refugia room was cleaned out and a

Virkon and freshwater wash bath were constructed and placed inside to allow easier disinfection and washing of equipment. A red composting worm farm was setup to use as feed for the Texas blind salamanders. The chiller to Tank 1 in the refugia room, which has been malfunctioning over the last few months, was removed and replaced with a chiller that was not being used in the quarantine building. Racks 6 and 8 in the quarantine building were put into operation to house Comal and San Marcos fountain darters collected in April and May. Benjamin Thomas and Jennifer Whitt continued with heavy equipment training. Jennifer Whitt completed her snorkel training.

SMARC

Staff conducted routine maintenance and husbandry for the refugia systems. In addition to assisting with the field collection of refugia organisms, staff assisted Desiree Moore with collecting Texas wild-rice for a genetics research project. Staff worked on cataloguing the Texas blind salamander inventory at SMARC by tag, sex, collection location and currant tank. Thomas Funk and Braden West completed the online non-motorized boat training course and will complete the field portion of the training in May.

Staff

Task 2 Research

Texas Wild Rice Genetics

Desiree Moore, assisted by Braden West and Tommy Funk, collected 98 tissue samples from TWR across 17 stands from three sections (B, D, and E) of the San Marcos River. She also worked with Kristy Kollaus to collect four tissue samples from TWR plants creating inaccuracies in the velocity data collected by the USGS stream gage in the first section of the river. The genetic data obtained by those four samples will help determine the best locations to potentially relocate those plants and improve data measurements. Desiree also assisted with rice collection for the Uvalde refugia population and clipped tissue samples from those new plants. Ms. Moore, completed the online portion of non-motorized boat training. She will complete the field portion of the training to be able to use kayaks to reach more TWR plants in May.

Comal Springs Riffle Beetle Pupation

Dr. Ely Kosnicki (BIO-WEST) and Ms. Moore collected 50 adult Comal Springs riffle beetles (CSRB) for the SMARC refugia in hopes that an increase in F1 larvae will be produced. Unfortunately delays in larvae availability have delayed in the start date for experiments examining CSRB pupation. In May, Ms. Moore will help Dr. Kosnicki begin those experiments.

Comal Springs Riffle Beetle Exposure to Staphylococcus

Dr. Ely Kosnicki (BIO-WEST) and Ms. Moore also collected 89 Comal Springs riffle beetles (CSRB) larvae held at the Freeman Aquatic Building (FAB, Texas State University) until the Staph exposure experiment begins. Ms. Moore transferred 13 larvae CSRB to Dr. Carlos-Shanley (Texas State University) to test to confirm these newly collected larvae to not contain Staphylococcus. Ms. Moore finished constructing the system and wrote the Standard Operating Procedures for the experiment. Dr. Carlos-Shanley began bacteria cultivation and is on track to begin the experiment on time. Adam Daw and Ms. Moore transferred another ten adult CSRB and ten larvae CSRB to Dr. Carlos-Shanley from Uvalde because the first test for Staph and other potentially harmful bacteria failed, possibly due to contaminated reagents that have since been replaced. Ms. Moore will begin the first replicate of the Staph exposure experiment the first week of May.

Captive Habitat for San Marcos Salamanders

Ms. Moore prepared and attached pond liner to the necessary tanks with the assistance of Uvalde staff Jennifer Whitt and Benjamin Thomas. Uvalde staff began running the system without salamanders to monitor for any potential problems in the system. In May Ms. Moore and Uvalde staff will randomly place salamanders in tanks and begin the first replicate of the experiment.

Additional accomplishments

Ms. Moore completed the No Fear training, received her snorkel certification, and passed the Introduction to Conservation Genetics DOI talent course. She also learned how to properly determine the sex in Texas Blind Salamander, Comal Springs Salamander, and San Marcos Salamander.

Task 4 Species Reintroduction

We performed no reintroduction work this month.

Task 5 Reporting

All staff contributed to the monthly report.

Task 6 Meetings and Presentations

No meetings or presentations were conducted this month.

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 APR Kept	UNFH APR Kept	Released	Total Collected	SMARC APR Incorporated	UNFH APR Incorporated	SMARC APR Mortalities	UNFH APR Mortalities	SMARC APR Census	UNFH APR Census
Fountain darter: San Marcos	148	147	--	295	0	0	12	9	411	415
Fountain darter: Comal	NT	NT	--	--	0	0	7	5	153	19
Comal Springs riffle beetle	78	NT	21	99	28	0	10	22	42	14
Comal Springs dryopid beetle	NT	NT	--	--	0	0	NA	NA	NA	NA
Peck's Cave amphipod	0	NT	20	20	13	0	NA	11	NA	223
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	2	174	71
San Marcos salamander	NT	NT	--	--	0	0	6	2	209	239
Comal Springs salamander	NT	NT	--	--	0	0	0	0	116	48
Texas wild rice plants	NT	22	--	22	0	5	0	0	216	141

Pictures

Figure 1. New 44 tank experimental rack built at the UNFH refugia.

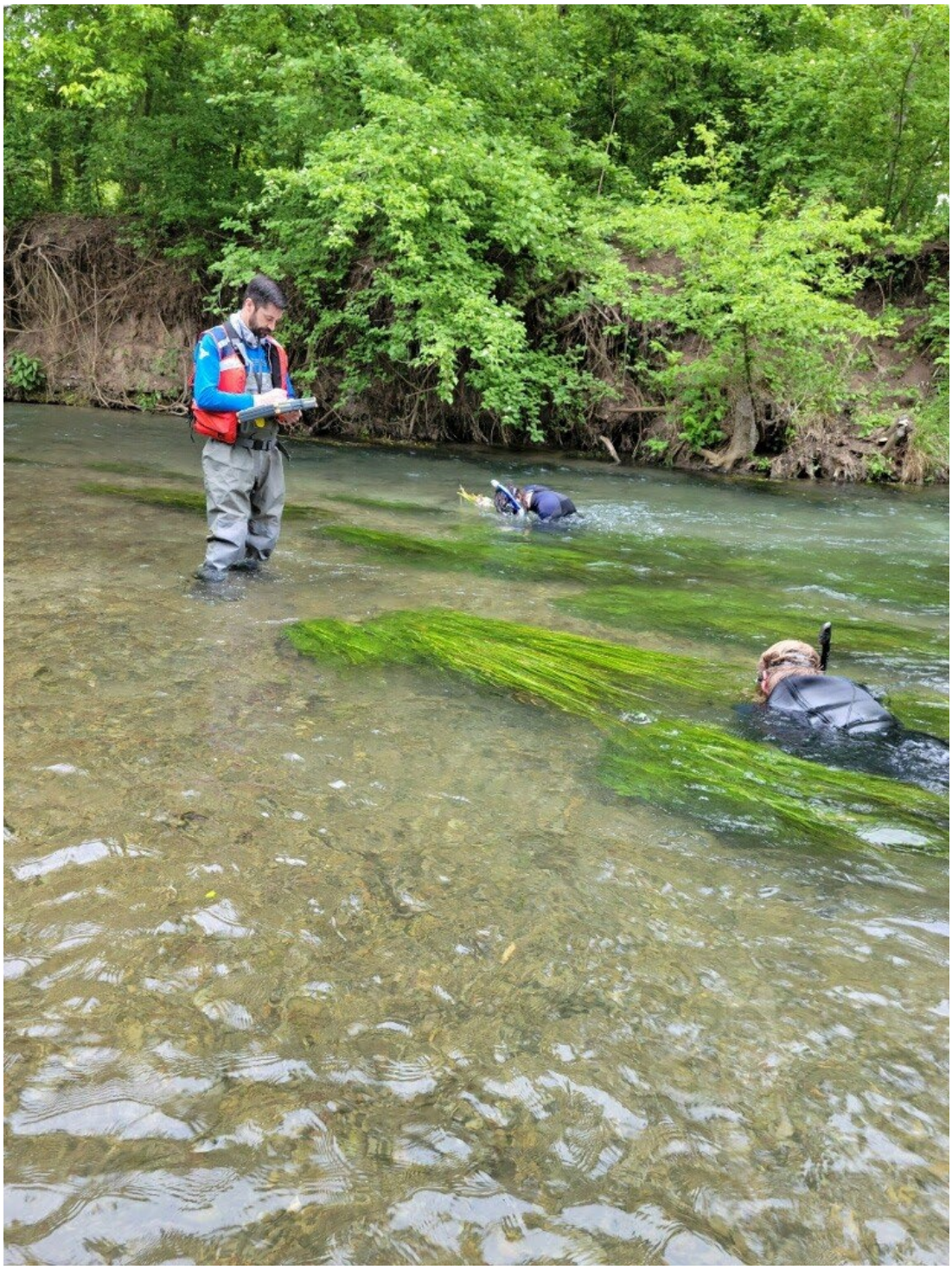

Figure 2. Adam Daw, Thomas Funk and Braden West collecting Texas wild-rice in the San Marcos River for the UNFH refuge.

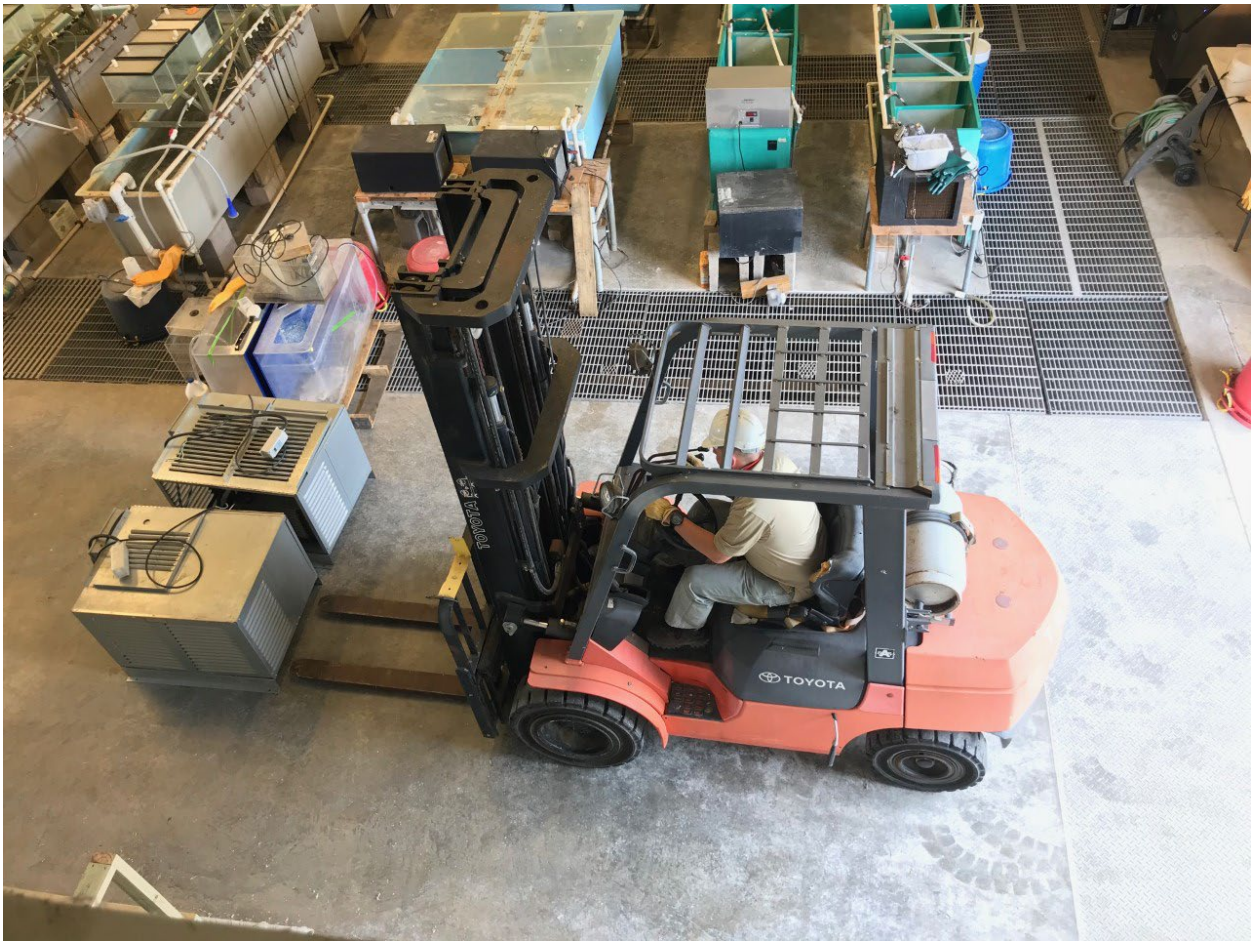

Figure 3. Benjamin Thomas switching out chillers at UNFH after heavy equipment training.

May 2021 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

David Britton, Adam Daw, Desiree Moore, and Jennifer Whitt

San Marcos Aquatic Resources Center

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Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

On May 4, 2021, the refugia acquired 258 Fountain darters from BIO-WEST biomonitoring sampling on the Comal River. These Fountain darters are currently quarantined at the SMARC and will be transferred to the UNFH after their quarantine period.

On May 9, 2021, Randy Gibson transferred 253 Peck's Cave amphipods to the SMARC from his sampling in the Comal River as part of the BIO-WEST biomonitoring event.

On May 10, 2021, SMARC staff set minnow traps for Texas blind salamanders at Primer's Fissure and Johnson's Well. The SMARC EAA staff checked the traps every 3 to 4 days, and removed the traps on May 24. Seven Texas blind salamanders were caught with three retained, two from Primer's Fissure and one from Johnson's Well, and taken to the SMARC for quarantine.

On May 20, 2021, staff from both the UNFH and the SMARC retrieved Comal springs riffle beetle lures set in Spring Run III of the Comal River. Nine adult riffle beetles and two larvae were collected from the set lures. The adult riffle beetles went to the UNFH refugia. The two larvae went to the SMARC for a research project. The team reset the previous eight lures and set five new lures for a total of 13 lures for recovery in June.

On May 26, 2021, SMARC and UNFH refugia staff collected Texas wild-rice from the San Marcos River section C. The refugia team collected a total of 147 tillers from 15 locations and transported the rice to the UNFH for quarantine.

Husbandry

Uvalde

Adam Daw set up a CO2 injection system on a refugia tank to evaluate the system's ability to maintain constant pH while minimizing calcium precipitation on the tanks, pumps, and chillers, which will reduce system maintenance.

Mr. Daw and Jennifer Whitt completed the final adjustments on one of the four rack systems in the invertebrate room at the UNFH. Changes to Rack 1 included moving the individual tank spigots to the front of the unit, facilitating greater accessibility. The redesign also incorporated moving the chiller from inside the invertebrate room to a chiller rack set up outside to reduce room temperature.

Ben Thomas and Mr. Daw finished the construction of the last rack system in the quarantine building. The addition of the new rack gives provides four isolated systems to house new collections at the UNFH.

The UNFH EAA team logged the colors and patterns of the elastomer tags on the 44 transferred Texas blind salamanders for data quality.

Twenty-two fountain darters from the refugia F1 stock were transferred to the new display tank in the reception area at the UNFH.

On May 4, 2021, staff from the FWS Southwest Fish Health Unit conducted a site visit at the UNFH. While on site they sampled 60 (wild) San Marcos River Fountain darters and 6 (F1) Comal River Fountain darters for pathogen analysis.

All UNFH refugia staff assisted with moving and grading catfish between ponds at the UNFH.

All UNFH refugia staff completed the non-motorized boat field training. Mr. Daw completed the FWS Transitioning to ArcGIC Pro class.

SMARC

Tommy Funk and Braden West have dedicated time and effort to the proper husbandry needed for the Fountain darters in quarantine. In the past, Fountain darters from the Comal River have experienced high mortality rates while in quarantine at the SMARC. After 3 weeks in quarantine the mortality of the Fountain darters from the Comal River is less than 20%.

Mr. Funk worked on data quality information on the Texas blind salamander database. The database will track the life histories of individual salamanders at the SMARC refugia.

The invertebrate racks at the SMARC acquired new aquarium heaters. With the onset of higher outdoor ambient temperatures and lower chiller temperatures, the staff can monitor and adjust these units as needed to control water temperatures in the invertebrate tanks.

Sixty San Marcos River Fountain darters and 31 Comal River Fountain darters from the Bio-West biomonitoring collection housed at the SMARC were shipped to the FWS Fish Health Unit for pathogen analysis.

All SMARC staff completed the non-motorized boat field training.

Task 2 Research

Texas Wild Rice Genetics

Justin Crow, Mr. West, Desiree Moore, and Mr. Funk collected 158 tissue samples from Texas wild-rice across 58 stands from five sections (B, C, F, G, and H) of the San Marcos River (Figure 1). Ms. Moore also assisted with the rice tiller collection for the Uvalde captive population and clipped tissue samples from those 15 new plants.

Comal Springs Riffle Beetle Pupation

Dr. Ely Kosnicki and Ms. Moore began the experiment to examine changes in pupation rates using boxes instead of flow-through tubes (Figure 2). They also conducted weekly checks of the flow rate to ensure adequate discharge is maintained.

Comal Springs Riffle Beetle Exposure to Staphylococcus

Samuel Tye (Texas State University) and Ms. Moore began the first replicate of the *Staphylococcus* exposure experiment and conducted daily checks for mortality, pupation, and proper flow rates. Dr. Camila Carlos-Shanley (Texas State University) confirmed the larvae used for the first replicate did not contain *Staphylococcus* prior to beginning the experiment. Mr. Daw, Ms. Whitt Mr. West, and Ms. Moore collected two Comal Springs riffle beetle (CSRB) larvae held at the Freeman Aquatic Building (FAB, Texas State University) until the second replicate of the experiment begins.

Captive Habitat for San Marcos Salamanders

Ms. Moore taught UNFH staff how to determine sex in San Marcos salamanders in preparation for this experiment. Mr. Thomas, Ms. Whitt, and Ms. Moore randomly placed pairs of salamanders (1 male and 1 female) in tanks and began the first replicate of the experiment. Ms. Whitt and Mr. Thomas began conducting daily checks for egg presence (Figure 3).

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

David Britton, Mr. Daw, Ms. Moore, and Ms. Whitt contributed to the monthly report.

Task 6 Meetings and Presentations

Ms. Whitt conducted educational outreach via Zoom. She met with three separate classes (7th, 11th, and 12th grades) in Idaho City to discuss the evolutionary adaptions of the Texas Blind Salamander and the other species in our care. Ms. Whitt also explained what aquifers are and how they work, highlighting the importance of the Edwards Aquifer system. She ended the presentations by talking about how students can get involved with local conservation efforts concerning water sources and the aquatic animals that live in these unique ecosystems.

SMARC EAA staff gave a tour of the SMARC refugia to the City of San Marcos Conservation Crew. All SMARC staff gave a brief description of their job duties and educated the group about the organisms.

Ms. Moore scheduled and planned a workshop to teach staff how to tag salamanders using visible implant elastomer tags. The workshop is scheduled to take place in June.

Only general discussions with EAA staff were conducted this month. We had no formal meetings or presentations.

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.

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	--	--	0	0	9	69	402	346
Fountain darter: Comal	258	0	--	--	0	0	3	0	150	19
Comal Springs riffle beetle	0	9	0	9	50	0	NA	NA	NA	NA
Comal Springs dryopid beetle	0	0	--	--	0	1	NA	0	NA	1
Peck's Cave amphipod	253	0	--	--	0	0	42	8	78	215
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	--	--	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	--	--	0	0
Texas blind salamander	3	0	4	7	0	0	0	0	174	71
San Marcos salamander	NT	NT	--	--	0	0	16	2	203	237
Comal Springs salamander	NT	NT	--	--	0	0	1	0	115	48
Texas wild rice plants	0	15	0	15	0	0	0	1	216	140

Summary of March Activities

May 4, 2021 - The refugia acquired 258 Fountain darters from BIO-WEST biomonitoring sampling on the Comal River.

May 4, 2021 - FWS Southwest Fish Health Unit conducted a fish health site visit at the UNFH. The unit analyzed 66 Fountain darters on-site,

May 4, 2021 - Sixty Fountain darters collected from the San Marcos River were shipped to the FWS Southwest Fish Health Unit for pathogen analysis.

May 9, 2021 - Randy Gibson transferred 253 Peck's Cave amphipods to the SMARC from his sampling in the Comal River as part of the BIO-WEST biomonitoring event.

May 10, 2021 - Thirty One Fountain darters collected from the Comal River were shipped to the FWS Southwest Fish Health Unit for pathogen analysis.

May 10, 2021 through May 24, 2021 - Collected Texas blind salamander from Primer's Fissure and Johnson's Well

May 13, 2021 - Collected Texas wild rice tissue samples in section B of the San Marcos River

May 18, 2021 - Collected Texas Wild Rice tissue samples in sections F, G, and H of the San Marcos River

May 20, 2021 - Collected Comal Springs riffle beetle adults and larvae from Spring Run 3, New Braunfels

May 26, 2021 - Collected Texas wild rice tillers and tissue samples in section C of the San Marcos River

Pictures

Figure 1. Tommy Funk navigating to the selected Texas wild-rice clip location in the San Marcos River.

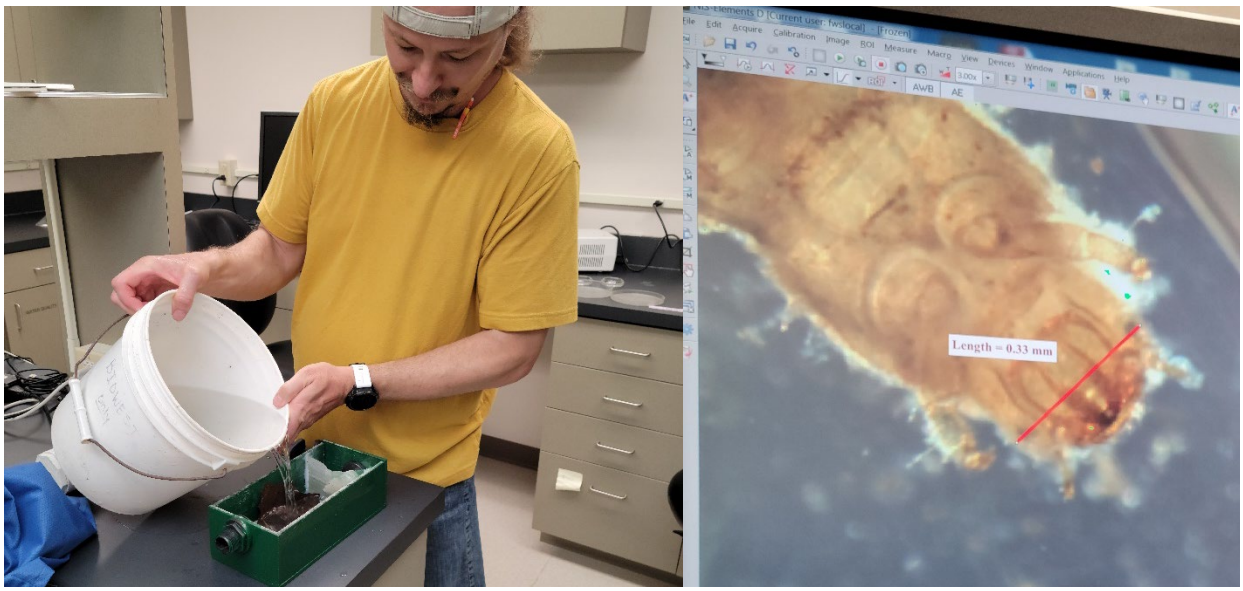

Figure 2. Dr. Ely Kosnicki preparing a box for the Comal Springs riffle beetle (CSRB) pupation experiment (left) and a CSRB larva being measured to determine current instar (right).

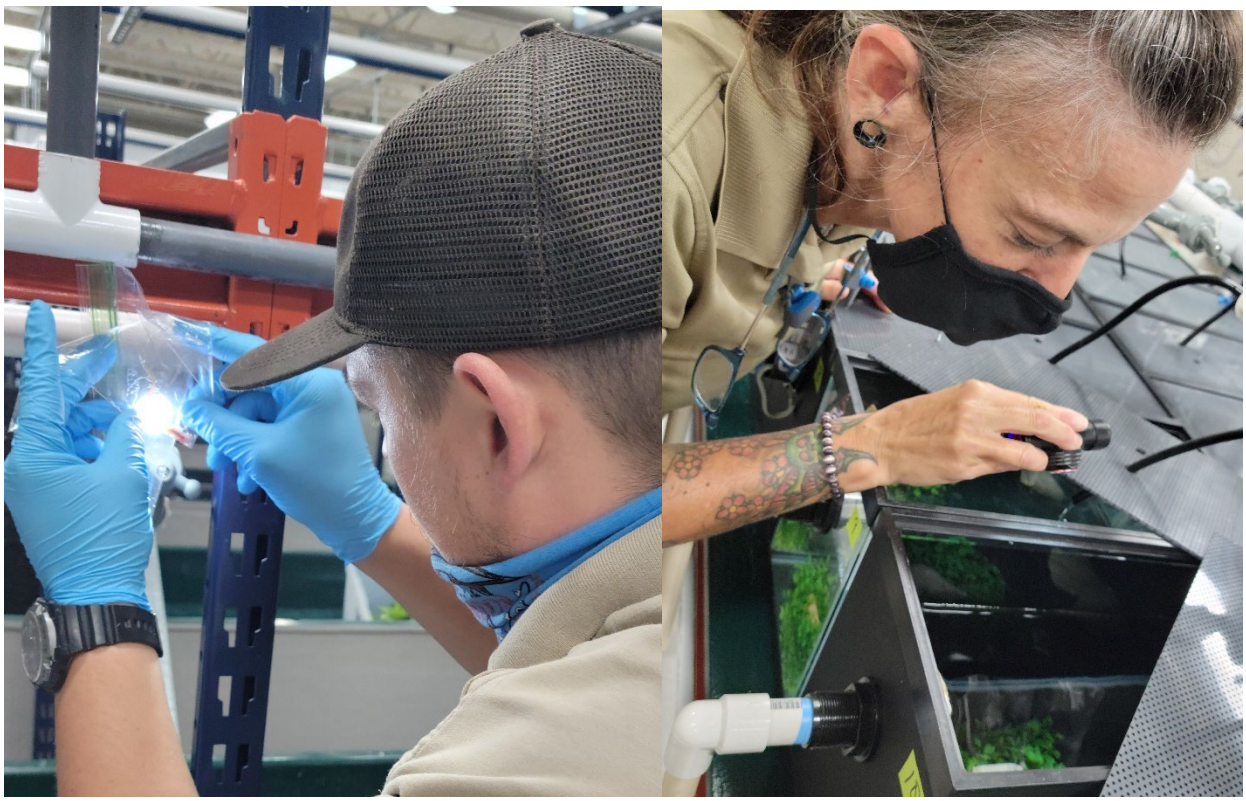

Figure 3. Ben Thomas using the candle method to determine the sex of a San Marcos salamander (left) and Jennifer Whitt checking the status of salamanders in the captive habitat for San Marcos salamanders project (right).

June 2021 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

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

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staff

The Lead Researcher position at the San Marcos Aquatic Resources Center (SMARC) was filled June 6, 2021 by Dr. Katie Bockrath. She earned her PhD in Genetics at the University of Georgia. Dr. Bockrath's research focused on assessing aquatic biodiversity using genetics, population surveys, and habitat measurements. Dr. Bockrath comes to us from the Whitney Genetics Lab at the U.S. Fish and Wildlife Service Midwest Fisheries Center in Onalaska, WI, where she used environmental DNA (eDNA) methods to test for the presence of invasive carp in water samples at the edge of their known range. Dr. Bockrath also built and ran the next-generation sequencing lab at the Midwest Fisheries Center. This sequencing lab focused on using high-throughput sequencing for early detection of invasive species and population genomics of priority native species.

Connor McMichael, a Student Conservation Association intern, started working at the Uvalde National Fish Hatchery (UNFH) at the beginning of June and will be there until mid-August. Mr. McMichael is a junior at Sam Houston University in Huntsville, Texas, and is working on his bachelor's degree in animal science with a minor in wildlife management. In pursuit of a career in wildlife conservation, Mr. McMichael would like to continue working as a technician in fish and wildlife after graduation.

A GS-05 biological technician position was advertised this month. This position will fill the GS-05 vacancy that occurred when Jennifer Whitt accepted her promotion at UNFH. Following the new employee's onboarding process, EAA staff at the UNFH will include three biological technicians.

Species Collection

On June 7, 2021, SMARC refugia staff set ten lures at the western shoreline of Landa Lake in the Comal River to collect Comal Springs riffle beetles. Thomas Funk and Braden West used their recent non-motorized boat training to set these lures via kayak.

On June 24, 2021, SMARC and UNFH staff collected 41 adult and two larvae Comal Springs riffle beetles from the 13 lures set in Spring Run 3 of the Comal River last month. The refugia team reset seven of the 13 lures in Spring Run 3. The 41 adult Comal Springs riffle beetles went to the UNFH quarantine building (Table 1) and the larvae were sent to Texas State University for the Comal Springs riffle beetle exposure to Staphylococcus research project.

Species collection wrapped up on June 29, 2021, with Comal Springs salamanders from the Spring Island collection site. Mr. West (SMARC) and Ben Thomas (UNFH) demonstrated

proper collection techniques to Mr. McMichael. The team brought 15 Comal Springs salamanders to the UNFH quarantine (Table 1).

Husbandry

Uvalde

Adam Daw and Mr. Thomas started testing the feeding of frozen mysid shrimp to the fountain darters in the refugia at the UNFH. The initial test showed positive results with the fountain darters actively consuming the frozen food. Mr. Daw and Mr. Thomas are currently assessing the best acclimation method when transitioning the fountain darters to a diet that incorporates more frozen foods. Concurrently, evaluation of the feeding of frozen mysid shrimp to the San Marcos salamanders is in process.

Mr. Thomas bleached and de-chlorinated two rack systems in the quarantine building in preparation for acid-washing. In the main refugia building, Ms. Whitt finished acid-washing five of the main holding tank systems and one rack system in the invertebrate room. Completing the acid-washing process in the refugia allowed Mr. Thomas and Mr. McMichael to scrub the tanks, fit them with new dividers (where needed), and prepare the tanks for species transfer.

An electrical failure occurred in the air conditioning (A/C) unit for the quarantine building on June 20, 2021. Individual system chillers maintained acceptable temperature ranges for the species housed in the building. Diagnosis of the problem with the A/C unit is ongoing, with the probable cause being a faulty electrical board and a shorted wire.

Ms. Whitt and Mr. McMichael scoured one of the main Texas wild rice tanks and 59 rice plants were transferred to a clean tank. Ms. Whitt and Mr. McMichael potted 37 groups of tillers from quarantine and incorporated these plants into the refugia.

SMARC

Mr. West and Mr. Funk completed the semiannual inventory of all the organisms in the care of the EAA program at the SMARC including both wild stock and F(x) generations.

Upgrades to the display tanks in the SMARC refugia are in consideration. Mr. Funk and Mr. West transferred the fountain darters from the show tank to a tank with better water quality conditions until the changes occur. To ease the stress of acclimation from their wild environment to refugia conditions, Mr. Funk salt-treated the Comal fountain darters in quarantine. To reduce rapid algae growth in rice tanks due to the increase of sunlight and heat during the summer, Mr. Funk set up additional shade cloth covers over the Texas wild rice. Mr. Funk also modified the recirculating system on the SMARC refugia invertebrate rack to improve flow and flow monitoring.

Animal Health

The UNFH received their health report from the annual site inspection by the Southwest Fish Health Unit. Six Comal (F1) and 60 San Marcos (wild) fountain darters maintained in the refugia were analyzed as part of the sampling event. All 66 fountain darters tested negative for infectious hematopoietic necrosis virus, infectious pancreatic necrosis virus, largemouth bass virus, and viral hemorrhagic septicemia virus. The UNFH maintained its "A" health classification.

The Southwest Fish Health Unit's report from the 60 San Marcos and 31 Comal fountain darters collected during the BIO-WEST biomonitoring event was received this month (Table 2). The two fish populations tested positive for two separate viruses. Aquareovirus, which has been found in the population since 2003, was detected in the San Marcos darter population, while Comal River fountain darters tested positive for largemouth bass virus, which has previously been found in the Comal population.

The Southwest Fish Health Unit staff conducted a site visit to the SMARC refugia on June 22, 2021 to perform viral and parasitic analyses on 60 fountain darters from the Comal (5) and San Marcos (55) Rivers. The fish health staff also sampled refugia Texas blind salamanders by swabbing to run tests for *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salaamandrivorans* (Bsal).

Refugia staff at the SMARC and the UNFH swabbed quarantined salamanders and the swabs were sent to Dr. Isaac Standish at the Midwest Fisheries Center Fish Health Lab for Bd/Bsal testing.

Task 2 Research

Texas Wild Rice Genetics

Desiree Moore, Mr. West, Mr. Funk, and divers Randy Gibson and Ashley Seagroves collected 84 tissue samples from Texas wild rice across 15 stands from three sections (B, C, and K) of the San Marcos River and in Spring Lake (Figure 1). Ms. Moore and Mr. West packaged and shipped the tissue samples to the Southwestern Native Aquatic Resources and Recovery Center (SNARRC) for population genetic analysis.

Comal Springs Riffle Beetle Pupation

Dr. Ely Kosnicki (BIO-WEST) and Ms. Moore continued the experiment examining changes in pupation rates using boxes instead of flow-through tubes. They conducted weekly checks of the flow rate to ensure adequate discharge is maintained.

Comal Springs Riffle Beetle Exposure to Staphylococcus

Samuel Tye (Texas State University) and Ms. Moore conducted daily checks for mortality, pupation, and proper flow rates and concluded the first replicate trial of the Staph exposure experiment. The first trial was monitored successfully, but some larvae escaped (Table 3). The research team discussed changes to the design to reduce the chances of larvae escape in future trials. Some larvae were sacrificed for *Staphylococcus* infection testing by Dr. Camila Carlos-Shanley (Texas State University). The remaining living larvae from the first trial were transferred to the SMARC for long-term monitoring (Table 3). Mr. Daw, Mr. Funk, Mr. McMichael, and Ms. Moore collected two Comal Springs riffle beetle (CSRB) larvae from Spring Run 3 of the Comal River. The larvae are currently held at the Freeman Aquatic Building (FAB, Texas State University) until the second replicate of the experiment begins.

Captive Habitat for San Marcos Salamanders

The first replicate trial of this experiment is ongoing. Ms. Whitt and Mr. Thomas continued conducting daily checks for egg presence. No oviposition has occurred thus far.

Additional Accomplishments

Dr. Bockrath worked remotely from Wisconsin as she finalized her moving arrangements. Her first week on the job, she made a trip from Wisconsin to San Marcos to meet SMARC staff and see the facility. In the process, she transported three upright, low-temperature incubators from Wisconsin to SMARC for use in husbandry research and techniques. While working remotely, Dr. Bockrath has been reading past monthly and annual reports, scientific literature and completing Financial Assistance training.

Ms. Moore completed the Financial Assistance Self-Study Course, which provides the initial training and resources for managing grants and cooperative agreements.

BIO-WEST, Inc

EAHCP long-term refugia

- BIO-WEST conducted project management and invoicing.
- BIO-WEST continued coordination with SMARC refugia director and staff.
- BIO-WEST continued internal review and development of the invertebrate components associated with the long-term refugia research plan.

Comal Springs dryopid beetle life cycle

- Continued propagation of sycamores.

- Finished moving and setting flow-through system from holding house to EEA Quarantine.
- Maintenance of new flow-through system.
- Investigated in-tank habitat options.

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

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

Task 6 Meetings and Presentations

Ms. Moore conducted a workshop to teach staff how to tag salamanders using visible implant elastomer (VIE) tags. The workshop included a presentation that taught participants when the use of VIE tags is appropriate, how to properly mix and store VIE, and how to avoid some of the most common mistakes when designing and implementing a VIE tagging scheme. The mussel program at the SMARC provided minnows for participants to practice VIE injection before tagging F1 Texas blind salamanders (Figure 2). Eight people attended the workshop: four UNFH staff, two SMARC staff from the EARP, and two SMARC staff from the mussel program.

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 June Kept	UNFH June Kept	Released	Total Collected	SMARC June Incorporated	UNFH June Incorporated	SMARC June Mortalities	UNFH June Mortalities	SMARC June Census	UNFH June Census
Fountain darter: San Marcos	NT	NT	--	--	86	143	66	6	391	485
Fountain darter: Comal	NT	NT	--	--	0	0	6	4	151	15
Comal Springs riffle beetle	0	41	1	42	0	7	35	NA	67	21
Comal Springs dryopid beetle	NT	NT	--	--	0	0	1	NA	0	NA
Peck's Cave amphipod	NT	NT	--	--	52	0	12	20	118	195
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	196	72
San Marcos salamander	NT	NT	--	--	0	0	17	3	189	234
Comal Springs salamander	0	15	12	27	0	0	0	0	115	48
Texas wild rice plants	NT	NT	--	--	0	37	2	0	214	177

Table 2. Results from the Southwest Fish Health Unit's health report on fountain darters collected from the Comal and San Marcos rivers during the BIO-WEST biomonitoring event in April and May of 2021. N is the number of fountain darters from each river collection that tested (+) positive. Multiple viruses were tested for in the viral analyses. The two populations of fountain darters tested positive (+) or negative (-) for one of two different viruses.

BIO-WEST Biomonitoring Sampling Event		
Parasite enumeration - # of individuals that tested positive for parasite		
	Comal fountain darters (N)	San Marcos fountain darters (N)
	10 fish screened	10 fish screened
Monogean-type	+(5)	+(7)
Ichthyobodo sp.	+(2)	+(1)
Centrocestus formosanus	+(9)	+(0)
Viral analysis – group sample tested positive (+) or negative (-) for virus		
	Comal fountain darters	San Marcos fountain darters
	30 fish screened	46 fish screened
Aquareovirus	(-)	(+)
Largemouth bass virus	(+)	(-)

Table 3. Survival results from the first replicate trial of the Comal Springs riffle beetle exposure to Staphylococcus research project. The total is the number of larvae included in that trial. Unknown indicates the number of larvae that were lost or escaped and cannot be included in analyses. We calculated the percent dead and alive at the end of the trial out of the total minus the number of unknown larvae. The number of larvae transferred to the SMARC accounts for the larvae sacrificed for testing by Dr. Camila Carlos-Shanley at Texas State University.

	Negative Control	Positive Control	Staph Exposed
Total	14	15	15
Unknown	2	6	6
Dead	6 (50%)	3 (33%)	4 (44%)
Alive	6 (50%)	6 (67%)	5 (56%)
Transferred	6	4	2

Summary of June Activities

June 1, 2021 – Collected Texas wild rice tissue samples in sections C and K of the San Marcos River.

June 7, 2021 – Deployed ten lures at the western shoreline of Landa Lake for future Comal Springs riffle beetle collection.

June 10, 2021 – Collected Texas wild rice tissue samples in Spring Lake and sections B and C of the San Marcos River with diver assistance.

June 14-15, 2021 – Texas wild rice tissue samples sent to and arrived at the SNARRC.

June 16, 2021 – Visible implant elastomer tagging workshop.

June 22, 2021 – FWS Fish Health Unit conducted a fish health site visit at the SMARC. The unit analyzed 60 fountain darters and swabbed a subset of Texas blind salamanders and San Marcos salamanders.

June 24, 2021 – Collected Comal Springs riffle beetle from lures in Spring Run 3 and set seven more lures for research future collection.

June 29, 2021 – Collected Comal Springs salamanders at Spring Island in New Braunfels.

Pictures

Figure 1. Ashley Seagroves holding a Texas wild rice tissue sample in the San Marcos River.

Figure 2. Staff members practicing tagging at the visible implant elastomer tagging workshop. Top panel from left to right: Connor McMichael, Jennifer Whitt, and Adam Daw. Bottom panel from left to right: Thomas Funk and Desiree Moore.

July 2021 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

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

San Marcos Aquatic Resources Center

500 East McCarty Lane

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Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

On July 1, 2021, Justin Crow and Randy Gibson from the SMARC SCUBA dive team set a collection net for Texas blind salamanders at Diversion Spring in Spring Lake. Adam Daw, Tommy Funk, and Braden West went with Mr. Crow and Mr. Gibson to observe the installation process, which entailed a stream driftnet set and secured over the spring outflow. The collection cup was checked twice a week by the SMARC staff through July for Texas blind salamanders and other organisms caught in the spring's discharge (Figure 1). Fifty-six San Marcos salamanders were collected, 53 were released, and three were transferred to the SMARC. Zero Texas blind salamanders were collected.

On July 27, 2021, juvenile and adult Comal Springs riffle beetles were collected at the western shore of Landa Lake by Dr. Katie Bockrath, Jennifer Whitt, Mr. Funk, and Mr. West. Six cotton lures were recovered and larvae were retrieved from submerged wood near Spring Island, rendering 90 larvae and 23 adult Comal Springs riffle beetles and one Peck's Cave amphipod (Figure 2). All collected larvae were transferred to Texas State University (TSU) for the Comal Springs riffle beetle *Staphylococcus* exposure experiment. The 23 adult riffle beetles and one Peck's Cave amphipod are in quarantine at the San Marcos Aquatic Resources Center (SMARC).

Husbandry

Uvalde

At the beginning of the month, Dr. Bockrath, Mr. Funk, and Mr. West visited the Uvalde National Fish Hatchery (UNFH) in Uvalde, Texas for the first time (Figure 3). Staff at the UNFH gave the SMARC team an in-depth tour of the Edwards Aquifer refugia program (EARP) section at the UNFH to better promote information exchange between the two facilities.

The SMARC staff transferred five F1 Texas blind salamanders to the UNFH for display in the visitor center (Figure 4a). In addition, Ben Thomas and Mr. McMichael transferred nine F1 San Marcos fountain darters from the UNFH refugia into the second display tank for the public to enjoy (Figure 4b).

In the invertebrate room, Mr. Daw, Mr. McMichael, and Ms. Whitt started constructing the redesign of Rack 2. Mr. Daw also installed a mechanical filter and UV sterilizer on the incoming well water line for the invertebrate room main sump. The filtration system will aid in the removal of debris and killing any heterogeneous organisms that come in with the well water.

Due to the high mortality rate that occurred in the Comal fountain darters transferred to the UNFH quarantine from the SMARC, Mr. Thomas devoted time and effort to the fountain darters' husbandry needs.

Deep cleaning of the UNFH facility continued in July. The UNFH EARP team continued to clean and acid wash tanks in the refugia and quarantine buildings. Near the end of the month, all UNFH staff assisted with cleaning the area housing the refugia chillers.

SMARC

A sudden increase in total dissolved gas (TDG) was recorded in the Edwards Aquifer Refugia Program (EARP) incoming chilled well water supply on July 14, 2021. TDG levels remained elevated through the evening of July 15, 2021. The EARP quarantine system's monitoring probe ensured a rapid response from the SMARC staff. Due to the quick response and actions of the EARP team, no salamander or fountain darter mortalities occurred. Peck's cave amphipods showed no signs of gasification at the time of the incident. However, a slight elevation in mortality rates was observed a week later during inventory. TDG levels slowly decreased and returned to normal on the morning of July 17, 2021.

SMARC staff continued to clean and organize areas in the refugia and quarantine building to maintain a sanitary and easily navigable facility.

To reduce unneeded stress and algae growth on the Texas wild rice in the greenhouse, Mr. Funk initiated a new shortened flow bar design in the rice tanks. The shortened flow bar is designed to increase water flow to a smaller area before adding a pump to provide additional flow to the remaining area of each tank.

Mr. West replaced and plumbed the pump on rice tank 5 in the greenhouse to ensure that an open tank is always available for the rotation of rice plants and tank cleaning. After acid washing the wild rice tanks, Mr. Funk and Mr. West started a rotation plan to decrease long-term algae growth.

Mr. Daw, Mr. Funk, and Mr. West started replacing the refugia chillers at the SMARC that were damaged during the winter freeze.

Animal Health

Due to the high morality of the Comal fountain darters from the most recent collection, three fish were sent to the Southwest Fish Health Unit for analysis.

The SMARC and the UNFH received Dr. Isaac Standish's salamander *Batrachochytrium dendrobatidis* (Bd) and *Bactrachochytrium salamandrivorans* (Bsal) test results from the swab samples sent last month to the Midwest Fisheries Center Fish Health Lab. Numerous

salamanders tested positive for Bd, a fungus commonly found in the wild populations of salamanders in the Edwards Aquifer region. However, all of the salamanders tested negative for Bsal. The salamanders in quarantine at both stations were cleared to enter the standing stock populations.

Task 2 Research

Texas Wild Rice Genetics

Staff at the Southwestern Native Aquatic Resources and Recovery Center (SNARRC) purchased plant DNA extraction kits needed to begin genetic analysis of Texas wild rice samples.

Comal Springs Riffle Beetle Pupation

Dr. Ely Kosnicki (BIO-WEST) and Desiree Moore continued the experiment examining changes in pupation rates using boxes instead of flow-through tubes. They conducted weekly checks of the flow rate to ensure adequate discharge is maintained.

Comal Springs Riffle Beetle Exposure to Staphylococcus

Dr. Bockrath, Mr. Funk, Mr. West, and Ms. Whitt collected 90 larvae for the second replicate trial of the Staph exposure experiment. The larvae are being held at the Freeman Aquatic Building at TSU until the second replicate begins.

Captive Habitat for San Marcos Salamanders

The first replicate trial of this experiment is ongoing. Mr. McMichael, Mr. Thomas, and Ms. Whitt continued conducting daily checks for egg presence. No oviposition has occurred thus far.

Additional Accomplishments

Dr. Bockrath moved to Texas and worked to become more familiar with the SMARC and our partners. She began reading historical reports, research proposals, and work plans. She also read literature applicable to Edward's Aquifer species. She participated in sampling events (i.e., Diversion net check and Comal Springs riffle beetle collection) to become more familiar with sites and sampling procedures (Figure 2). She continued her training for Fisheries Information System reporting and GrantSoluntions. She visited TSU to tour the Freeman Aquatic Building, where the Staph exposure project is held, and toured the UNFH. Dr. Bockrath met with Dr. Kosnicki, Dr. Steven Mussmann, Dr. Weston Nowlin, and Ruben Tovar to discuss research. She also met with Mr. Daw and Ms. Moore to discuss future research needs.

Dr. Bockrath also created a database and began cataloging tissue samples from all specimens at the SMARC and UNFH. She began developing an SOP for tissue preservation, metadata collection, and archiving.

Ms. Moore completed the Applied Conservation Genetics Course to prepare for future research projects involving genetics data. She also sorted old SMARC data sheets and created an organized filing system to make future data entry easier. She also created a schedule for conditioning food items (wood, cloth, and leaves) for Comal Springs riffle beetle to ensure food is always available for all projects. Ms. Moore began a chemical inventory/SDS update and waste removal at the SMARC to meet proper safety precautions. She also updated signs and safety maps for the SMARC refugia and quarantine buildings.

Mr. Thomas and Ms. Whitt were trained in operations of the UNFH backhoe and are now Tier One Heavy Equipment certified (Figure 5).

The following is the monthly report received from BIO-WEST:

BIO-WEST, Inc F21AC02194

EAHCP LONG-TERM REFUGIA

BIO-WEST conducted project management and invoicing (17 hrs).

- what was done,
    - Created new project with accounting, approved invoice, created project detail report, created cumulative billing report, tried to set up new VPN with server (for creating new project), discussions with company president regarding new administrative format.
- how the work has progressed from last month,
    - NA.
- what are the products for this month, and
    - An Invoice was produced and delivered.
- what are you planning for next month
    - Increased tracking and recording of time spent on sub-tasks.

BIO-WEST continued coordination with SMARC refugia director and staff (5 hrs).

- what was done,
    - Open discussions with SMARC staff regarding chemical SDS, update of SDS's related to materials kept by BIO-WEST at SMARC, research, flow refuge operations, Covid protocol updates.
- how the work has progressed from last month,
    - Not identifiable.
- what are the products for this month, and
    - Not identifiable.
- what are you planning for next month

    - Continued dialog

BIO-WEST continued internal review and development of the invertebrate components associated with the long-term refugia research plan (6 hrs).

- what was done,
    - Read reports and papers related to species of concern.
- how the work has progressed from last month,
    - Research is better informed.
- what are the products for this month, and
    - None.
- what are you planning for next month
    - Continue to stay up-to-date with information and studies related to species life-histories.

Comal Springs riffle beetle research

Maintenance of new flow-through system (8 hrs).

- what was done,
    - Monitoring of system flow, inspection for leaks, temperature download and aggregation for dd calculations, rearrangement of drains, reorganization of supplies, record discharge of flow-through pupation tanks.
- how the work has progressed from last month,
    - Hopeful that our set-up does not have to be moved again this year.
- what are the products for this month, and
    - None.
- what are you planning for next month
    - Continued monitoring of system flow, inspection for leaks, temperature download and aggregation for dd calculations, rearrangement of drains, reorganization of supplies.

Look through BIO-WEST standing stock of subjects for future experimentation (10 hrs).

- what was done,
    - Looked through three tubes of potential test subjects of F1 subjects generated from previous studies, curated dead specimens, placed adults and pupae in other flow-through apparatuses, identified a tube that has many potential test subjects for density studies in September.
- how the work has progressed from last month,
    - many adjustments have been made to the flow of the flow-through boxes for the pupation study.
- what are the products for this month, and
    - None.
- what are you planning for next month

- Complete the test run of three pupation boxes.

BIO-WEST, Inc F20AC11672

EAHCP LONG-TERM REFUGIA

BIO-WEST conducted project management and invoicing (8 hrs).

- what was done,
  - Approving invoice, creating project detail report, updating cumulative billing report,
- how the work has progressed from last month,
  - This sub-task has become more cumbersome and time consuming.
- what are the products for this month, and
  - An Invoice was produced and delivered, some but not all hours were tracked.
- what are you planning for next month
  - Increase time and energy tracking and recording of time spent on sub-tasks.

BIO-WEST continued coordination with SMARC refugia director and staff (hours not recorded).

- what was done,
  - Open discussions with SMARC staff.
- how the work has progressed from last month,
  - Not identifiable.
- what are the products for this month, and
  - Not identifiable.
- what are you planning for next month
  - Continued dialogues and discussions with regard to operations, research logistics.

BIO-WEST continued internal review and development of the invertebrate components associated with the long-term refugia research plan (hours not recorded).

- what was done,
  - Read reports and papers related to species of concern.
- how the work has progressed from last month,
  - Research is better informed.
- what are the products for this month, and
  - None.
- what are you planning for next month
  - Continue to stay up-to-date with information and studies related to species life-histories.

Comal Springs dryopid beetle life cycle

Continued propagation of sycamores (1 hrs).

- what was done,
  - Transporting saplings, pruning roots and stem, evaluating growing medium and discarding dead sticks.
- how the work has progressed from last month,
  - It has not.

- what are the products for this month, and
    - None.
- what are you planning for next month
    - Continued monitoring.

Implementation and maintenance of new flow-through tank (3 hrs).

- what was done,
    - Monitoring of tank flow, inspection for leaks, rearrangement of drains, had overall flow increased by SMARC staff.
- how the work has progressed from last month,
    - Water appears to be flowing at an adequate level for experimentation of new tank system.
- what are the products for this month, and
    - An operational tank.
- what are you planning for next month
    - Continued monitoring of tank flow, inspection for leaks, etc…

Subject collection and deployment (5 hrs).

- what was done,
    - Collected and transported five female and two male adult subjects for reproduction in new tank system.
- how the work has progressed from last month,
    - The first trial was launched.
- what are the products for this month, and
    - None.
- what are you planning for next month
    - None.

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

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

Task 6 Meetings and Presentations

Only general discussions with EAA staff were conducted this month. We had no formal meetings or presentations.

Table 1. New collections and total census in July 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 July Kept	UNFH July Kept	Released	Total Collected	SMARC July Incorporated	UNFH July Incorporated	SMARC July Mortalities	UNFH July Mortalities	SMARC July Census	UNFH July Census
Fountain darter: San Marcos	NT	NT	--	--	0	0	4	4	387	483
Fountain darter: Comal	NT	NT	--	--	0	0	0	1	139	14
Comal Springs riffle beetle	23	0	0	23	0	40	NA	6	NA	55
Comal Springs dryopid beetle	NT	NT	--	--	0	0	--	--	0	1
Peck's Cave amphipod	1	0	2	1	0	0	26	5	92	190
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	2	0	194	72
San Marcos salamander	3	0	53	56	0	0	6	1	180	233
Comal Springs salamander	NT	NT	--	--	0	21	0	0	NA	69
Texas wild rice plants	NT	NT	--	--	0	0	NA	1	NA	176

Summary of July Activities

July 1, 2021 – Deployed the net to sample from Diversion Spring in Spring Lake.

July 5-29, 2021 – Collected salamanders from the Diversion net in Spring Lake twice weekly.

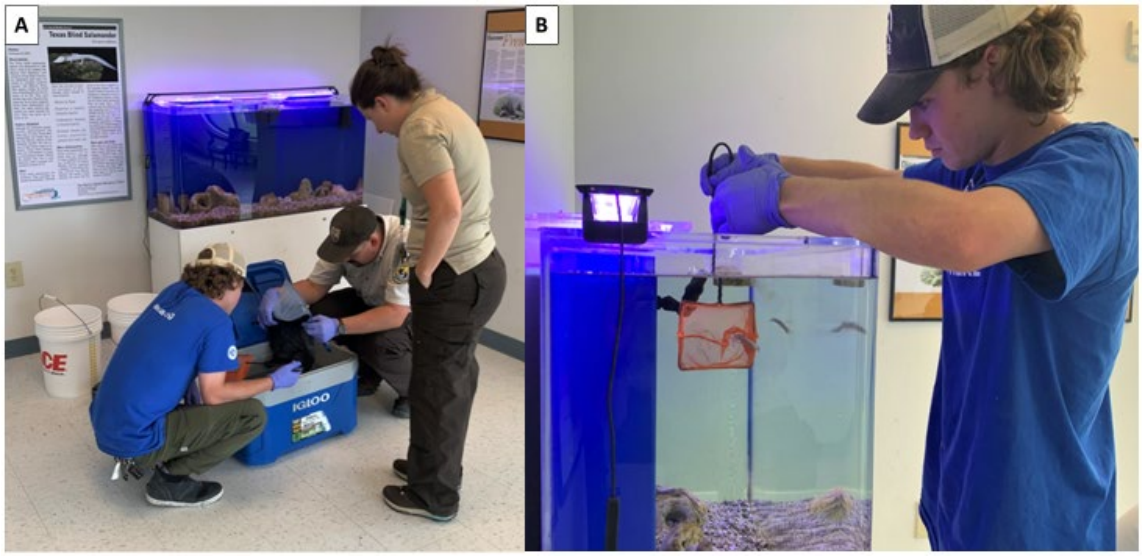
July 27, 2021 – Collected Comal Springs riffle beetle from the lures set at the western shoreline of Landa Lake and in situ wood at Spring Island.

July 29, 2021 – Removed collection cup from Diversion Spring in Spring Lake drift net.

Pictures


RIVAL

Figure 3. Adam Daw, Dr. Katie Bockrath, Jennifer Whitt, Ben Thomas, Tommy Funk, and Braden West gather around the Texas blind salamander display tank at the Uvalde National Fish Hatchery.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B
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Figure 5. Jennifer Whitt (left) and Ben Thomas (right) practice using the backhoe to complete their Tier 1 Heavy Equipment certifications.

August 2021 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

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

San Marcos Aquatic Resources Center

500 East McCarty Lane

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Task 1 Refugia Operations

Species Collection

On August 12, 2021, Tommy Funk and Desiree Moore collected 10 San Marcos fountain darters from Spring Lake in San Marcos, TX, which were sent to the Southwestern Fish Health Unit for parasite enumeration (Figure 1).

On August 16, 2021, Braden West, Adam Daw and Mr. Funk collected 12 Comal Springs fountain darters from Landa Lake in New Braunfels, TX, which were sent to the Southwestern Fish Health Unit for parasite enumeration.

From August 18 - 25, 2021, minnow traps where deployed at Primer's fissure and Johnson's well in San Marcos, TX to capture Texas blind salamanders. Thomas Funk and Braden West checked the traps three times a week, capturing six salamanders in total. Two of the four salamanders at Primer's fissure and one of the two salamanders at Johnson's well were retained for the refugia at the San Marcos Aquatic Resource Center (SMARC).

On August 24, 2021, Mr. Daw joined Dr. Andy Gluesenkamp and staff members from the San Antonio Zoo's Center for Conservation and Research at Rattlesnake Cave in San Marcos, TX to remove the debris that accumulated in front of the cave's gate during the pandemic (Figure 3). The last time the Edward's Aquifer Refugia Program (EARP) had set traps for Texas blind salamanders in the cave was January of 2020.

On August 27, 2021, the top half of the drift net at Diversion Springs in Spring Lake was removed. Mr. Funk and Mr. West provided surface support for the SMARC dive team, which included Justin Crow and Ashley Seagroves (Figure 2).

Husbandry

Uvalde

Jennifer Whitt completed the acid washing process of two large tanks and a sump system in the Uvalde National Fish Hatchery (UNFH) refugia. Ben Thomas then completed a detailed cleaning of the tanks in preparation to house EARP species.

Mr. Thomas devised a way to construct adhesive-free habitat structures for the darters and salamanders. The structure incorporates artificial vegetation onto the PVC tiles without the use of glue to provide vertical habitat space (Figure 4).

Mr. Daw finished the restoration of an old acrylic tank to be used as the third display tank in the visitor's center at the UNFH. To keep salamanders in the exhibition side of the display tanks, Mr. Thomas designed and fabricated curved screens from perforated PVC sheeting to cover the outflow vents.

Mr. Daw and Mrs. Whitt continued work on the redesigned invertebrate rack system.

Mr. Daw completed the DOIU Building and Leading Effective Teams training.

SMARC

SMARC staff started the tagging process of salamanders incorporated in 2021 with visible implant elastomer (VIE) tags. VIE tagging will help biologists and technicians identify individual salamander sexes and collection years. This tagging system will aid in future data collection and research.

Mr. Funk and Mr. West continued to implement the planned improvements to the Texas wild rice tanks. Mr. Funk acid washed empty rice tanks and added an additional pump to the clean systems. Mr. West rotated rice plants from algae overgrown tanks to the clean systems. Biological and chemical algae control methods were administered to EARP Texas wild rice tanks at the SMARC as needed.

Mr. Funk started the revision of the standard operating procedures (SOP) for daily checks and weekend walkthroughs for EARP organisms at the SMARC. The SOP document is near completion.

The SMARC staff continued the clean-up and reorganizing process at their facility.

Animal Health

Fountain darters from the headwaters of the Comal (12) and San Marcos (10) rivers were collected and shipped to the Southwestern Fish Health Unit in Dexter, NM for parasite enumeration.

Task 2 Research

Texas Wild Rice Genetics

Staff at the Southwestern Native Aquatic Resources and Recovery Center (SNARRC) worked on the genetic analysis of Texas wild rice tissue samples for the Texas wild rice genetics assessment project. First, all the samples were arranged in order and cross checked against the spreadsheet inventory. There were discrepancies between the samples and spreadsheet that Melody Saltzgiver identified and worked with Ms. Moore and Ms. Whitt to reconcile. Then, DNA was extracted from all the samples with a Macherey-Nagel nucleospin plant kit. The microsatellite PCRs for those samples are being processed on the 3500xl now and the genotypes are beginning to be scored.

Comal Springs Riffle Beetle Pupation

Dr. Ely Kosnicki (BIO-WEST) and Desiree Moore continued the experiment examining changes in pupation rates using boxes instead of flow-through tubes. They checked the three boxes for evidence of pupation and retained any healthy individuals for future examination. One larva pupated and eclosed, 25 larvae were missing, and 21 living larvae were housed for future examination (Table 2). Missing larvae were presumed to have escaped or been pushed out of their box due to erratic flow conditions (i.e., overflow, low flow). The living larvae from Box 1 and Box 2 (Table 2) were moved to flow-through tubes for long-term housing. The living larvae from Box 3 were reset in a clean flow-through box to be checked for pupation in one month. Ms. Moore placed leaves and cloth in a conditioning box according to the conditioning schedule.

Dr. Bockrath, Dr. Kosnicki, and Ms. Moore discussed potential changes to the next experiment in this project, which is to examine the pupation of larvae held at different densities.

Dr. Kosnicki deployed wood at Spring Run 2 for biofilm conditioning. This wood will be used in the experiment examining wild and captive developed biofilm as food sources for Comal Springs riffle beetle.

Dr. Kosnicki began the inventory of his Comal Springs riffle beetles in his holding systems. His upper general holding system contained 43 small, 85 medium, and 71 large larvae and seven adults.

Dryopid Life History and Housing

Dr. Kosnicki collected five female and one male Comal Springs dryopid beetle from the Spring Island area of New Braunfels, TX. He also deployed the second sycamore black box at the SMARC and identified potential habitat for future examination. He continued the propagation of sycamores, documenting growth of five indoor saplings.

Comal Springs Riffle Beetle Exposure to Staphylococcus

Dr. Bockrath and Ms. Moore met with Dr. Camila Carlos-Shanley (Texas State University) to assess the progress and modify the methods of the *Staphylococcus* exposure experiment. Dr. Carlos-Shanley and Samuel Tye (Texas State University) grew, washed, and diluted bacterial cultures to the appropriate optical density and added them to their respective agarose flasks. The agarose was aseptically distributed to each jar for the experiment and stored at 4°C overnight before the second experimental trial. To begin the second trial, Dr. Bockrath, Ms. Moore, and Mr. Tye outfitted each jar with cloth, water, and one riffle beetle larva. The three researchers have checked the jars daily for appropriate flow and presence of burrowed larvae. Mr. Tye assessed all larvae for pupations, lethargy, and mortality weekly. Thus far, no larvae have burrowed into the agarose and adequate flow was maintained. Two larvae from the negative control treatment (i.e., no bacteria added) were found dead on August 25, 2021.

Captive Habitat for San Marcos Salamanders

Dr. Bockrath, Mr. Daw, Ms. Moore, Mr. Thomas, and Ms. Whitt randomly assigned pairs of salamanders (one male, one female) to aquaria and began the second trial of the San Marcos salamander captive habitat project (Figure 5). The second replicate trial of this experiment is ongoing. Mr. Thomas and Ms. Whitt continued conducting daily checks for egg presence. No oviposition has occurred thus far.

Additional Accomplishments

Dr. Bockrath and Ms. Moore sorted all preserved specimens at the SMARC (Figure 6). Specimens were separated into groups for donation, disposal, and retention for genetic analyses based on their condition and species status. Dr. Bockrath developed a tissue archiving system for genetic samples from these specimens and samples in the future.

Dr. Bockrath finished reading all 2017-2022 project plans and continued to read annual, interim, and monthly reports. She also read literature related to refugia species to become more familiar with gaps in knowledge concerning life history, reproduction, and genetic diversity. Dr. Bockrath began summarizing past research and used that information to begin updating the refugia priority and knowledge matrices.

Dr. Bockrath and Ms. Moore continued their training for GrantSolutions. Dr. Bockrath completed the DOIU Building and Leading Effective Teams training. Because Dr. Bockrath could not be on station for the visible implant elastomer (VIE) tagging workshop in June, Ms. Moore taught her to tag using VIE during salamander tagging for the refugia.

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

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

Task 6 Meetings and Presentations

Dr. David Britton, Dr. Bockrath, Mr. Daw, and Ms. Moore met with the EAA to discuss current research schedules and potential research projects for the 2022 work plan on August 31, 2021.

Mr. Daw attended the Aquaculture America 2021 conference in San Antonio as a board member of the U.S. Aquaculture Society and a member of the society's Diversity and Inclusion and Student Activities committees. While at the conference, he met with scientists and product vendors to discuss ideas to improve refugia operations. As a result of discussions initiated at the conference, the UNFH will beta test a new water filter designed by Aquaculture Systems Technologies LLC. The filter will arrive at the beginning of September and undergo trials to evaluate its potential use on the refugia systems.

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.

Species	SMARC August Kept	UNFH August Kept	Released	Total Collected	SMARC August Incorporated	UNFH August Incorporated	SMARC August Mortalities	UNFH August Mortalities	SMARC August Census	UNFH August Census
Fountain darter: San Marcos	NT	NT	--	--	0	0	27	12	357	471
Fountain darter: Comal	NT	NT	--	--	0	0	2	0	136	14
Comal Springs riffle beetle	NT	NT	--	--	19	0	34	5	54	50
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	NA	0	NA
Peck's Cave amphipod	NT	NT	--	--	1	0	NA	5	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	3	NT	3	6	6	0	3	2	NA	70
San Marcos salamander	NT	NT	--	--	48	0	12	4	201	229
Comal Springs salamander	NT	NT	--	--	0	0	0	0	114	69
Texas wild rice plants	NT	NT	--	--	0	0	3	1	NA	175

Table 2. Survival and pupation results from the box vs tube experiment of the Comal Springs riffle beetle pupation research project. Initial # is the number of larvae placed in that box at the start of the experiment. The numbers of dead and living larvae and adults found at the conclusion of the experiment are reported. Unaccounted indicates the number of larvae that were lost or escaped.

Replicate	Initial #	Dead larvae	Living larvae	Living adults	Unaccounted
Box 1	20	11	1	0	8
Box 2	20	2	3	0	15
Box 3	20	0	17	1	2
Total	60	13	21	1	25

Summary of August Activities

August 9-27, 2021 – Checked all boxes for pupation to conclude the box vs. tube experiment of the Comal Springs riffle beetle pupation research project.

August 12, 2021 – Collected San Marcos fountain darters from Spring Lake (San Marcos, TX), which were sent to the Southwestern Fish Health Unit for parasite enumeration.

On August 16, 2021 – Collected Comal Springs fountain darters from Landa Lake (New Braunfels, TX), which were sent to the Southwestern Fish Health Unit for parasite enumeration.

August 18, 2021 – Began the second trial of the San Marcos salamander captive habitat research project.

August 18 - 27, 2021 – Collected Texas blind salamanders from Johnson's well and Primer's fissure in San Marcos, TX.

August 19, 2021 – Began the second trial of the Comal Springs riffle beetle exposure to *Staphylococcus* research project.

August 27, 2021 – Top half of drift net removed from Diversion Springs in Spring Lake (San Marcos, TX)

August 31, 2021 – Met with the EAA to discuss the 2022 research work plan.

Pictures

Figure 1. Tommy Funk using a dip net to collect fountain darters from Spring Lake in San Marcos, TX.

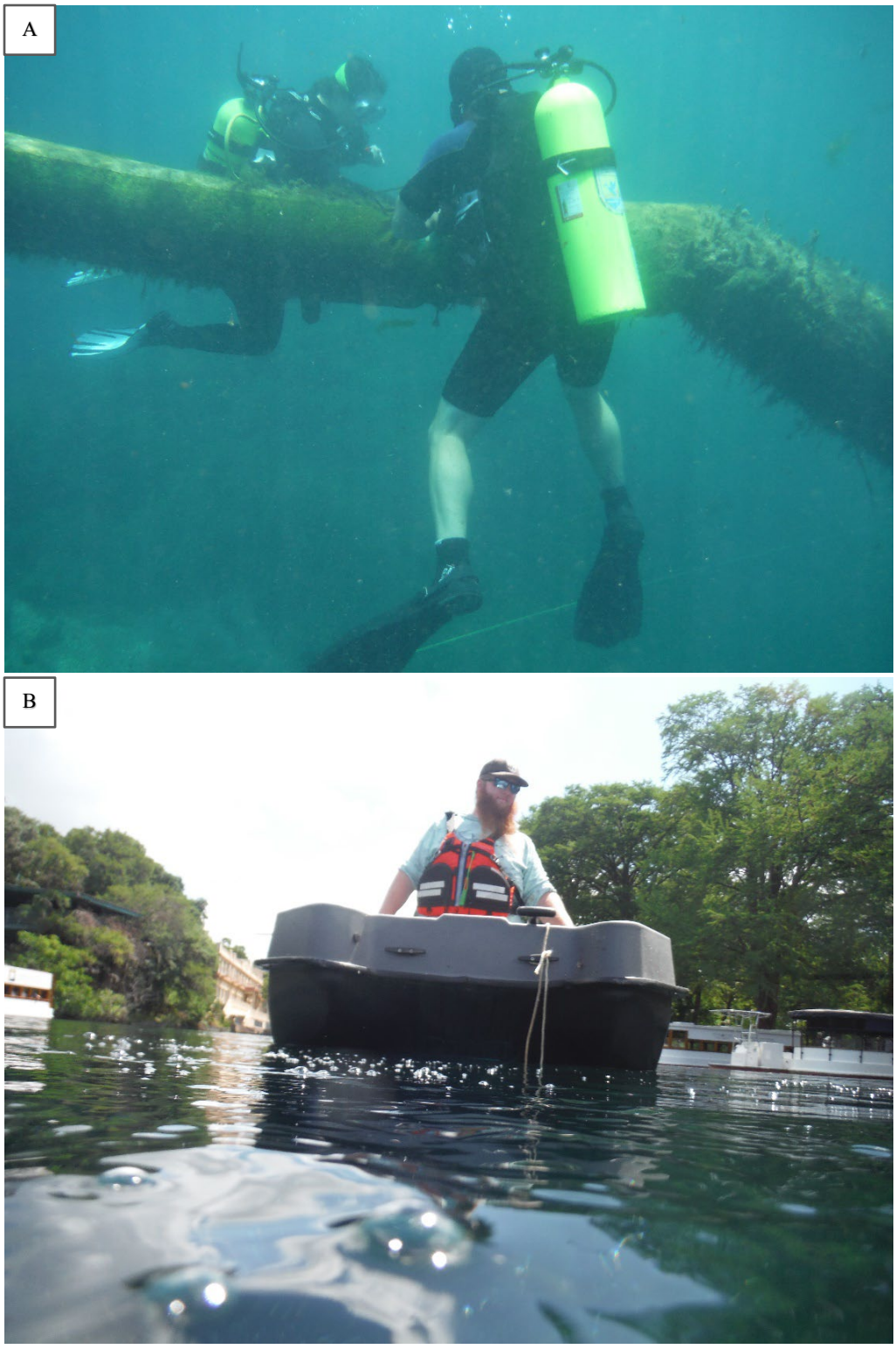

Figure 2. A) Justin Crow and Ashley Seagroves removing the drift net from Diversion Springs in San Marcos, TX. B) Braden West providing surface support for divers while they remove the drift net.

Figure 3. A) A porcupine guarding the entrance to Rattlesnake Cave in San Marcos, TX. B) Dr. Andy Gluesenkamp from the San Antonio Zoo removing debris from the cave opening.

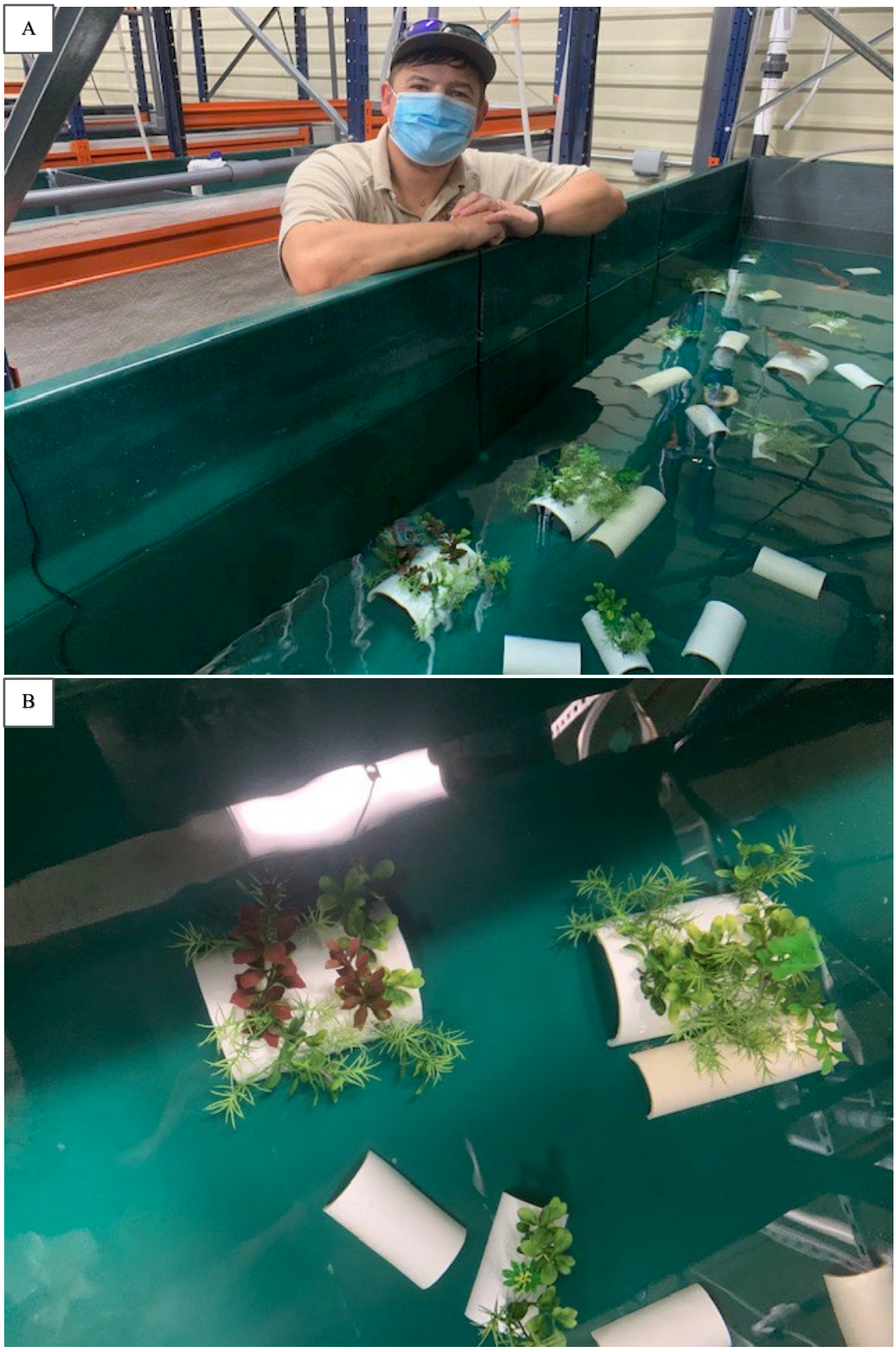

Figure 4. A) Ben Thomas with his newly designed habitat structures at the UNFH. B) Close-up of the habitat structures.

Figure 5. Adam Daw, Dr. Katie Bockrath, Desiree Moore, and Jennifer Whitt assigning pairs of San Marcos salamanders to aquaria for the captive habitat research project at the UNFH.

Figure 6. Dr. Katie Bockrath and Desiree Moore sorting preserved specimens at the SMARC.

September 2021 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

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

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Task 1 Refugia Operations

Species Collection

On September 14, 2021, Adam Daw, Tommy Funk, Alex Klingele, Desiree Moore, Ben Thomas, Braden West, and Jennifer Whitt collected 20 Comal Springs salamanders and 64 Peck's cave amphipods from Spring Island in New Braunfels, TX (Figure 1). The Comal Springs salamanders were retained for the refugia at the Uvalde National Fish Hatchery (UNFH), and the Peck's cave amphipods went to the San Marcos Aquatic Resource Center (SMARC).

Ms. Moore and Ms. Whitt collaborated to update the invertebrate lure collection datasheet and create an in situ invertebrate datasheet to improve data collection efficiency. The datasheets were finalized and implemented at the Comal Springs riffle beetle collection.

On September 30, 2021, Dr. Katie Bockrath, Dr. Ely Kosnicki (BIO-WEST), Mr. Daw, Mr. West, and Ms. Whitt collected 71 adult Comal Springs riffle beetles at Spring Island (New Braunfels, TX) for the Comal Springs riffle beetle pupation research project. John Boggess and Kristy Kollaus joined the Edwards Aquifer Refugia Program (EARP) team at Spring Run 3. Mr. Boggess shot pictures of Dr. Bockrath and Mr. Daw to accompany the article in the EAHCP Steward Newsletter that will highlight their work for the EARP (Figure 2).

Husbandry

Uvalde

To strengthen the biosecurity measures for effluent leaving the refugia buildings, Mr. Thomas created new data logs to track the chlorine concentration-time index for each system's outflow in the EARP.

Mr. Daw taught Ms. Whitt how to heat weld the PVC pipes and fitting to strengthen the seals and prevent water loss, allowing them to complete the construction of the second invertebrate rack system (Figure 3).

Mr. Thomas placed the finishing touches on the third aquarium display tank in the UNFH visitor's center, completing the set of displays that highlight the threatened and endangered species housed at the hatchery.

Mr. Daw installed a pre-production water filter loaned to the UNFH by Aquaculture Systems Technologies LLC. Beta testing done at the UNFH refugia will be conducted to test the potential use of the filter on refugia systems in return for feedback on its design and effectiveness. Mr. Daw started testing the CO_2 injection system on a tank without animals in the refugia to evaluate if modifications made to the previous test version improved its efficiency.

Mr. Thomas moved 22 Comal Springs fountain darters from quarantine, incorporating them into the refugia population.

SMARC

Mr. Funk completed a standard operating procedure draft for the SMARC weekend walkthroughs and system checks to ensure that the EARP standards are clearly communicated to any staff, volunteers, and visitors. Mr. Funk rerouted chilled well water to the Texas wild rice after damage occurred to a non-chilled well water pipeline outside of the greenhouse where the rice is housed.

Mr. Funk coordinated with Drew Berdo, a volunteer at the SMARC. Mr. Berdo took a tour of the SMARC and submitted his initial volunteer paperwork.

All SMARC and UNFH staff provided input for a biosecurity standard operating procedure document for visiting researchers. Dr. Bockrath and Ms. Moore finalized the document to prevent biosecurity breaches from visiting researchers at the SMARC.

Mr. Funk and Mr. West prepared for the new show tanks at the SMARC. They moved all show tank organisms to other tanks, removed and cleaned the previous show tanks, and moved the racks elsewhere to make room for the new show tank racks.

The SMARC staff continued the clean-up and reorganizing process at their facility.

Animal Health

No work was done this month for animal health reporting.

Task 2 Research

Texas Wild Rice Genetics

Staff at the Southwestern Native Aquatic Resources and Recovery Center (SNARRC) worked on the genetic analysis of Texas wild rice tissue samples for the Texas wild rice genetics assessment project. After scoring two panel sets, Melody Saltzgiver (SNARRC) discovered that one of the markers used in the past amplifies two loci. She ordered four new primers to try amplifying new loci. Two of the new primers were monomorphic and were removed from analysis. Ms. Saltzgiver included the remaining two new primers into the full dataset. Ms. Saltzgiver is analyzing the genetic data and writing the first draft of the report.

Comal Springs Riffle Beetle Pupation

Dr. Bockrath, Dr. Kosnicki , and Ms. Moore modified the project plan for the Comal Springs riffle beetle pupation project. The modifications include adult riffle beetle collection for F1 larval production, construction of a new flow-through system to hold the broodstock and experiments and modifying the project timeline to account for delays in larvae production. After the EAA approved these modifications, Ms. Moore and Mr. West set up the flow-through system. Dr. Bockrath, Mr. Daw, Dr. Kosnicki, Mr. West, and Ms. Whitt collected 71 adult riffle beetles for larvae production. Dr. Bockrath, Dr. Kosnicki and Ms. Moore placed the riffle beetles in three flow-through tubes (n = 20, 22, and 29) in the SMARC quarantine.

Dr. Kosnicki completed the inventory of his holdings at the SMARC (Table 2).

Dr. Kosnicki examined the three groups of larvae retained from Phase I of the experiment examining changes in pupation rates using boxes instead of flow-through tubes (Table 3). There were no changes in Group 1. The three larvae in the Group 2 tube were not found and the tube was removed. One larva in the Group 3 box pupated but looked weak and was recorded as a mortality the following week. In Group 3, one larva was found dead, nine larvae were found alive and reset in the box, and six larvae were not found (Table 3).

Dryopid Life History and Housing

Dr. Kosnicki performed maintenance and adjustments to the flow-through system and tanks in response to well switches, pressure changes, temperature adjustments, a slight overflow of Tank 1, and the addition of a second flow line. He also transferred materials and individuals from Tank 2 to Tank 3. He conducted field expeditions and collections resulting in two adults added to Tank 2 on September 17, 2021.

Dr. Kosnicki continued sycamore propagation, documenting growth of five indoor saplings (Figure 4).

Comal Springs Riffle Beetle Exposure to Staphylococcus

Dr. Bockrath and Ms. Moore met with Dr. Camila Carlos-Shanley (Texas State University) and Samuel Tye (Texas State University) to assess the status of the *Staphylococcus* exposure experiment and discuss plans for analyzing the data. Dr. Bockrath, Ms. Moore, and Mr. Tye checked the containers daily for appropriate flow and the presence of burrowed larvae. Mr. Tye assessed all larvae for pupations, lethargy, and mortality weekly. Significant calcium deposits were found on and cleaned from the screens of containers in Tank 2 on September 1, 2021. These deposits could have flowed into the containers from the pipes. The deposits might have decreased flow to some containers and notes were made to account for those differences.

As of September 27, 2021, a total of 11 mortalities were observed in the experiment: eight mortalities in the negative control treatment (i.e., no bacteria added), one mortality in the positive control treatment (i.e., *Bacillus* bacteria added), and two mortalities in the staph treatment (i.e., *Staphylococcus* bacteria added).

Ms. Moore recorded the monthly inventory of Comal Springs riffle beetles at the SMARC from Trial 1 (Table 4). The mortalities recorded could be due to low flow events from clogging problems in this system. Dr. Bockrath and Ms. Moore developed a plan to build a new system for improved flow.

Captive Habitat for San Marcos Salamanders

The second trial of this experiment is ongoing. Mr. Thomas and Ms. Whitt continued conducting daily checks for egg presence. No oviposition has occurred thus far.

Additional Accomplishments

Dr. Bockrath and Ms. Moore developed 2022 research plans and a budget for Task 2 research and added them to the 2022 workplan. They met with Chad Furl and Kristy Kollaus to finalize 2022 research plans. Dr. Bockrath also met with the EAA Science Committee and introduced herself to the EAA and partners.

Dr. Bockrath, Mr. Daw, and Mr. Furl interviewed with John Bogges and Oliva Ybarra for the EAHCP Steward Newsletter and podcast.

Dr. Bockrath and Ms. Moore began drafting 2021 end-of-year research reports and 2022 research proposals.

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

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

Task 6 Meetings and Presentations

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

Dr. David Britton, Dr. Bockrath, and Ms. Moore met with the EAA to discuss and finalize research projects for the 2022 work plan on September 24, 2021.

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.

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	--	--	0	0	7	18	350	453
Fountain darter: Comal	NT	NT	--	--	0	22	2	0	134	36
Comal Springs riffle beetle	NT	NT	--	--	0	0	NA	NA	NA	NA
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	1	0	0
Peck's cave amphipod	64	NT	4	68	0	0	14	10	79	175
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	198	70
San Marcos salamander	NT	NT	--	--	0	0	11	4	200	225
Comal Springs salamander	NT	20	43	63	0	0	0	0	114	69
Texas wild rice plants	NT	NT	--	--	0	0	0	4	211	171

Table 2. Total census in September of Comal Springs riffle beetles in Dr. Ely Kosnicki's (BIO-WEST) holdings at the SMARC.

Tank	Generation	Living larvae	Languid larvae	Dead larvae	Living pupae	Dead pupae	Living adults	Dead adults
General holdings	F1 with some F2	473	0	19	0	0	10	30
F2 larvae	F2	0	16	2	0	0	0	0
F1 larvae tube 1	F1 with some F2	117	0	59	4	5	30	12
F1 larvae tube 2	F1	9	0	6	0	1	7	4
F1 + F2 larvae tube	F1 and F2	24	0	22	0	2	7	10

Table 3. Survival and pupation results from the box vs tube experiment of the Comal Springs riffle beetle pupation research project. These larvae were reset for further examination because they did not pupate during the designated experimental period. Initial # is the number of larvae placed in that box or tube at the conclusion of the designated experimental period. The numbers of dead and living larvae and adults found at the first post-experiment check are reported. Unaccounted indicates the number of larvae that were lost or escaped.

Replicate	Initial #	Dead larvae	Living larvae	Living adults	Unaccounted
Tube 1	1	0	1	0	0
Tube 2	3	0	0	0	3
Box 3	17	1	9	1	6
Total	21	1	10	1	9

Table 4. Survival results from the first trial of the Comal Springs riffle beetle exposure to* Staphylococcus *research project. The total is the number of larvae included in that trial. Unknown indicates the number of larvae that were lost or escaped and cannot be included in analyses. We calculated the percent dead and alive at the end of the trial out of the total minus the number of unknown larvae. The number of larvae transferred to the SMARC accounts for the larvae sacrificed for testing by Dr. Camila Carlos-Shanley at Texas State University. The number of larvae alive in each treatment 1- and 2-months post transfer is reported.

	Negative control	Positive control	Staph exposed
Total	14	15	15
Unknown	2	6	6
Dead	6	3	4
Alive	6	6	5
Transferred	6	4	2
Alive 1-month	3	2	2
Alive 2-month	1	2	1

Summary of September Activities

September 14, 2021 – Collected Comal Springs salamanders and Peck's cave amphipods from Spring Island (New Braunfels, TX).

September 24, 2021 – Met with the EAA to discuss the 2022 research work plan.

September 30, 2021 – Collected lures from Spring Run 3 and Comal Springs riffle beetles from Spring Island (New Braunfels, TX).

Pictures

Figure 1. Braden West transferring Peck's cave amphipods to Jennifer Whitt for close examination and enumeration.

Figure 2. EAHCP Steward Newsletter writer and photographer John Boggess taking a photo of Adam Daw and Dr. Katie Bockrath that will accompany an article spotlighting their work for the EARP.

Figure 3. Jennifer Whitt heat welding the corner seams on the filter box that was added to the redesigned invertebrate rack with Adam Daw's supervision.

Figure 4. New root growth of a sycamore sapling on September 21, 2021.

October 2021 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

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

San Marcos Aquatic Resources Center

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Task 1 Refugia Operations

Species Collection

On October 12, 2021, Tommy Funk, Ben Thomas, and Braden West collected tillers from 19 different stands of Texas wild rice in section B of the San Marcos River. Mr. Thomas brought the tillers to the Uvalde National Fish Hatchery (UNFH).

On October 12, 2021, Ashley Seagroves and Randy Gibson (San Marcos Aquatic Resources Center; SMARC) collected one Texas blind salamander and two San Marcos salamanders in a drift net set at Diversion Springs, San Marcos River. They donated these salamanders to the SMARC refugia.

On October 18 and 19, 2021, Mr. Funk received fountain darters captured from the middle section of the San Marcos River via drop net during the BIO-WEST biannual biomonitoring event. There were 67 fountain darters collected the first day and 115 fountain darters collected the second day, which were transferred to the SMARC.

On October 20, 2021, Adam Daw received 62 fountain darters captured during the BIO-WEST biannual biomonitoring event and transferred to the UNFH. This drop net collection of fountain darters took place in the lower section of the San Marcos River.

On October 26, 2021, Mr. Funk received 60 Comal fountain darters captured during the BIO-WEST biannual biomonitoring event and transferred them to the SMARC. These darters were retained for parasite analysis.

On October 28, 2021, Mr. Funk and Mr. West set Comal Springs riffle beetle lures at Spring Run 3 (Comal River, New Braunfels). After the lures were set, the team looked to collect Comal Springs dryopid beetles from woody debris. Mr. Daw and Jennifer Whitt searched woody debris around Spring Island (Comal River, New Braunfels) for Comal Springs dryopid beetles (Figure 1). There were no dryopid beetles found at either location during this sampling event.

On October 28, Mr. Gibson donated 20 Peck's Cave amphipods to the SMARC refugia captured via drift net at Spring Run 3 as part of the BIO-WEST biannual biomonitoring event.

Husbandry

Uvalde

Mr. Daw and Mr. Thomas designed and cut Plexiglass tops and PVC clamps to cover refugia tank water-outflow containers to minimize the chlorine vapor emitted into the air in the refugia, invertebrate, and quarantine buildings.

Mr. Thomas and UNFH Lead Biologist, Valentin Cantu, cleaned and prepped the area around the quarantine building outflow lift station for easier access. Mr. Daw installed a new chlorine injection system onto the lift station to streamline the efficiency of disinfecting the water leaving the refugia quarantine building.

Mr. Thomas and Ms. Whitt swabbed seven Comal Springs salamanders in quarantine for *Batrachochytrium salamandrivorans* (Bsal) and *B. dendrobatidis* (Bd) analysis. (Figure 2).

Ms. Whitt etched out patterns on the smooth surfaces of wooden dowels to give them texture. The wooden dowels are conditioned and added to the Comal Springs riffle beetle and Comal Springs dryopid beetle boxes to simulate woody debris found in the beetles' natural environment (Figure 3).

Following Edwards Aquifer Refugia Program (EARP) quarantine protocols, Ms. Whitt proactively treated the 62 new fountain darters from the Comal River for ectoparasites and acclimated them to the quarantine tanks.

In one of the Texas wild rice tanks, Ms. Whitt changed out the 90° elbows on the pumps with a 45° to increase the radius of the flow mid-tank.

SMARC

Mr. Funk followed EARP quarantine protocols, prepped tanks, and acclimated the new EARP species that came into quarantine in October. Mr. Funk proactively treated the San Marcos fountain darters for ectoparasites.

Mr. Funk shortened the in-line flow bars in Texas wild rice Tanks 2 and 4 to condense the flow of incoming water into a smaller area of the tanks and create more space. Mr. Funk added sump pumps to the newly created spaces to increase the overall flow in these two tanks. Mr. Funk increased the height of the drainpipe in Tank 4 to reduce the likelihood of cavitation in the event of a pump intake screen clog.

Dr. Katie Bockrath and Desiree Moore gave Dr. Chad Furl and Kristy Kollaus of the EAA a tour of the SMARC EARP facilities.

Mr. Funk, Ms. Moore, and Mr. West started training a new volunteer, Drew Berdo, on the protocols at the refugia and greenhouse at the SMARC. Mr. Funk focused on husbandry duties, Mr. West focused on water quality, and Ms. Moore focused on biosecurity, safety, and cleaning protocols.

The SMARC staff continued the clean-up and reorganizing process at their facility.

Animal Health

Of the 182 fish received during the BIO-WEST biomonitoring event on October 19, 2021, 60 were reserved for parasite analysis. Three of the darters died due to handling stress; the remaining 57 fish were shipped to the Southwestern Native Aquatic Resources and Recovery Center (SNARRC) Fish Health Unit for parasite analysis.

Mr. Funk retrieved 60 fountain darters from the Comal River at Spring Run 3 that were collected during the BIO-WEST biomonitoring event on October 26, 2021. One darter died due to handling stress while the remaining 59 fish were shipped to the SNARRC Fish Health Unit for parasite analysis.

Task 2 Research

Texas Wild Rice Genetics

Staff at the SNARRC worked on the genetic analysis of Texas wild rice tissue samples for the Texas wild rice genetics assessment project. After starting the report, Melody Saltzgiver (SNARRC) discovered it was not easy to compare these new data to the previous study (Wilson et al. 2017) with only three shared markers and some genotyping errors in the previous data. She re-ran the samples from Wilson et al. (2017) with the new markers and re-ran the new samples with the old markers so all samples were scored in the same manner and run on the same sequencer. Then, she reanalyzed those data and began incorporating the results in the report for this project.

Comal Springs Riffle Beetle Pupation

Ms. Moore added a second flow bar to the flow-through research system to make space for the new tubes for the Phase II experiment examining the pupation of Comal Springs riffle beetle larvae at different densities. Dr. Ely Kosnicki and Israel Prewitt (BIO-WEST) prepared the tubes and mesh for Phase II and Ms. Moore added resources for the larvae and set up the tubes for the experiment.

Dr. Kosnicki inventoried one of three breeding chambers to assess F1 larvae production. Eighty-one F1 larvae were in the breeding chamber at the time of survey. Eight of the 29 adult beetles in the brooding chambers were not Comal Springs riffle beetles and two Comal Springs riffle beetle mortalities had occurred. Because some adult beetles were not Comal Springs rifle beetle, a portion of the F1 larvae are suspected to be non-target species. All confirmed Comal Springs riffle beetle adults and all larvae were put back into the breeding chamber to allow for additional reproduction and for the F1 larvae to develop into late-instar larvae to confirm species

identification. The other two breeding chambers were checked for flow and internal conditions. Larvae and adults were not surveyed in the other two breeding chambers.

Dryopid Life History and Housing

Dr. Kosnicki maintained the housings and adjusted the flow-through system and tanks. Dr. Kosnicki inspected Tank 1 and found four live adults (3 females and one male) and eggs on spongy wood (Figure 5). One dead adult female was also found in Tank 1. The mortality was removed and preserved in ethanol. The live adults were transferred to Tank 2 and the eggs on wood were transferred back to Tank 1.

Dr. Kosnicki inspected suspected habitat in the field but did not observe any dryopid beetles.

Comal Springs Riffle Beetle Exposure to **Staphylococcus**

Dr. Bockrath, Ms. Moore, and Samuel Tye (Texas State University) concluded the second replicate trial of the Staph exposure experiment. They monitored the second trial successfully, with no escaped or missing larvae (Table 2). Some larvae were sacrificed for *Staphylococcus* infection testing by Dr. Camila Carlos-Shanley (Texas State University). Dr. Carlos-Shanley extracted DNA from 37 larvae and has prepared the samples for sequencing. The remaining living larvae from the second trial were transferred to the SMARC for long-term monitoring. No pupation has occurred thus far.

Ms. Moore recorded the monthly inventory of Comal Springs riffle beetles at the SMARC from Trial 1 (Table 2). The Trial 1 tubes were retired when no living larvae were located. No pupation occurred in Trial 1 larvae.

Ms. Moore created survival curves for each treatment in the experiment (Figure 6) and completed analyses comparing the survival of larvae among treatments. Ms. Moore began drafting the final report for this project.

Captive Habitat for San Marcos Salamanders

The second trial of this experiment is ongoing. Mr. Thomas and Ms. Whitt continued conducting daily checks for egg presence. Three of the female salamanders in the trial were replaced. Two of these salamanders were removed because of health concerns, and the third was found deceased in the tank. No oviposition has occurred thus far.

Additional Accomplishments

Dr. Bockrath and Ms. Moore continued drafting 2021 end-of-year research reports and 2022 research proposals.

Dr. Bockrath reviewed 2021 report drafts and 2022 proposal drafts. She also worked on the 2022 work plan and budget and started the process of recruiting a Student Conservation Associated (SCA) intern for the 2022 fountain darter project. She also discussed project progress and deadlines with all research partners and discussed 2022 research projects with Dr. Kosnicki and Mr. Gibson.

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

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

Task 6 Meetings and Presentations

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

EARP staff met with Austin Ecological Services staff to discuss how our offices can work together to share data pertinent to building a complete refugia.

References

Wilson, W.D., J.T. Hutchinson, and K.G. Ostrand. 2015. Genetic diversity assessment of *in situ* and *ex situ* Texas wild rice (*Zizania texana*) populations, and endangered plant. Aquatic Botany 136:212-219.

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

Species	SMARC 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	182	62	0	244	0	0	12	11	336	442
Fountain darter: Comal	60	0	0	60	0	0	2	0	131	36
Comal Springs riffle beetle	NT	NT	--	--	0	0	20	6	34	44
Comal Springs dryopid beetle	0	0	--	--	0	0	0	0	0	0
Peck's Cave amphipod	20	NT	0	20	50	0	NA	9	NA	166
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	1	NT	0	1	0	0	2	0	196	70
San Marcos salamander	2	NT	0	2	0	0	10	6	177	219
Comal Springs salamander	NT	NT	--	--	0	0	0	0	113	69
Texas wild rice plants	NT	19	0	19	0	0	1	0	211	171

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

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

Summary of October Activities

October 4, 2021 – Concluded Trial 2 of the Comal Springs riffle beetle exposure to *Staphylococcus* experiment and transferred living larvae to the SMARC.

October 12, 2021 – Dr. Chad Furl and Kristy Kollaus visited the SMARC for a tour.

October 12, 2021 – Collected Texas wild rice tillers from section B of the San Marcos River.

October 12, 2021 – Ms. Seagroves and Mr. Gibson donated one Texas blind salamander and two San Marcos salamanders collected from their sampling event at Diversion Springs at Spring Lake, San Marcos, TX.

October 13, 2021 – EARP staff met with Austin Ecological Services staff.

October 18 and 19, 2021 – BIO-WEST donated fountain darters from the middle section of the San Marcos River collected during their biomonitoring event.

October 20, 2021 - BIO-WEST donated fountain darters from the lower section of the San Marcos River collected during their biomonitoring event.

October 26, 2021 – BIO-WEST donated fountain darters from the Comal River collected during their biomonitoring event.

October 28, 2021 – Set Comal Springs riffle beetle lures at Spring Run 3 (Comal River, New Braunfels, TX).

October 28, 2021 – Mr. Gibson donated Pecks Cave amphipods captured at Spring Run 3 (Comal River, New Braunfels) to the SMARC.

Pictures

Figure 1. Adam Daw inspects a piece of woody debris for Comal Springs dryopid beetles. Photo credit: Jennifer Whitt, USFWS

Figure 2. Ben Thomas retrieves a Comal Springs salamander from a tank in the quarantine building at the UNFH for swabbing. Photo credit: Jennifer Whitt, USFWS

Figure 3. A) Conditioned wooden dowels etched with patterns and dated with the first conditioning month and year (10/21). B) Jennifer Whitt holding a wooden dowel with etches following the natural patterns in the wood. Photo credit: Jennifer Whitt, USFWS

Figure 4. Traveling at a snail's pace, this Texas blind salamander caught a ride this month in the display tank at the UNFH Visitor's Center. Photo credit: Adam Daw, USFWS

Figure 5. "Panel A. Male dryopid found on sycamore roots. Panel B. Dryopid egg found embedded within spongy wood" (BIO-WEST October 2021 Invoice). Photo credit: Dr. Ely Kosnicki

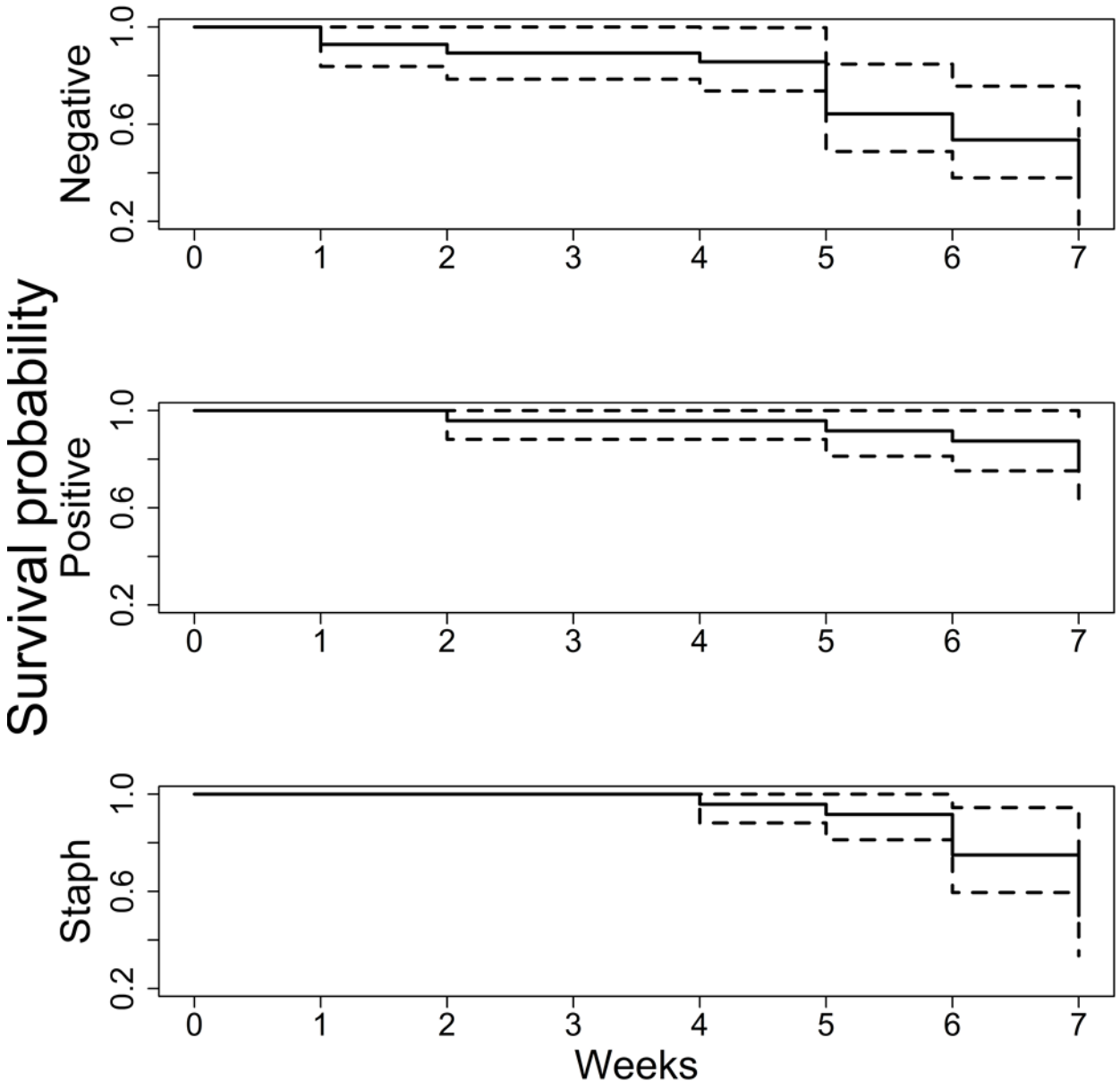

Figure 6. Kaplan–Meier survival curves developed for Comal Springs riffle beetle larvae exposed to* Staphylococcus *sp. (staph),* Bacillus *sp. (positive), and no bacteria (negative). All groups were held in the same conditions except agarose in their containers contained the bacteria for their respective treatments. We show the survival probability with 95% confidence intervals (dashed lines) over time (weeks) where mortality occurred 1–7 weeks post exposure.

November 2021 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

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

San Marcos Aquatic Resources Center

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Task 1 Refugia Operations

Species Collection

On November 8 - 19, 2021, Tommy Funk and Braden West deployed baited minnow traps at Primer's fissure and Johnson's well in San Marcos, TX to capture Texas blind salamanders. Mr. Funk, Desiree Moore, and Mr. West checked the traps three times a week (Figure 1), capturing five Texas blind salamanders at Primer's fissure and three at Johnson's well. Two of the five salamanders at Primer's fissure were retained for the refugia at the San Marcos Aquatic Resource Center (SMARC). The traps were removed on the final salamander check on November 19, 2021.

On November 19, 2021, Mr. West facilitated the transfer of two Texas blind salamanders donated to the SMARC refugia. The salamanders were captured via driftnet in Sessom Creek during a sampling event conducted by Victor Castillo at Texas State University.

On November 30, 2021, Adam Daw, Mr. Funk, Mr. West, and Jennifer Whitt collected 4 Comal Springs salamanders and 78 Peck's cave amphipods from Spring Island in New Braunfels, TX (Figure 2). Four Comal Springs salamanders and 73 Peck's cave amphipods were retained for the refugia at the Uvalde National Fish Hatchery (UNFH).

Husbandry

Uvalde

Ms. Whitt started training new volunteers on the protocols at the UNFH refugia. Ms. Whitt and volunteer Wayne Whitt scoured and prepped the empty rice tank and transferred 61 Texas wild rice plants to the clean system. Caliborn Whitt (volunteer) cleaned the three display tanks in the reception room at the UNFH.

Ms. Whitt monitored the weekly changes in float time and biofilm development in the etched and nonetched wooden dowels for the Comal Springs riffle beetle and dryopid beetle boxes in the refugia (Figure 3).

Mr. Daw and Ben Thomas cleaned and rearranged the lab room to accommodate the new lab equipment ordered for the refugia to reinforce the program’s research and husbandry capabilities.

After installing the new rack system in the quarantine building storage room, Mr. Thomas cleaned and reorganized the items stored there, increasing ease and accessibility to collection gear needed for field work.

SMARC

Dr. Katie Bockrath, Kevin Rubio (SCA intern), Mr. Daw, Mr. Funk, Mr. West, and Ms. Whitt deep cleaned the areas around the Texas wild rice in the SMARC greenhouse. This coordinated effort included pressure washing the outside of the tanks and the floor and the removal of sediment and debris from the outflow drainage areas (Figure 4).

After observing the start of anoxic conditions in the soil, Mr. Funk and Mr. West repotted Texas wild rice plants housed in Tank 4.

Mr. West compiled an updated, simplified, and more efficient list of gear and equipment needed for the Edwards Aquifer Refugia Program (EARP) field collection trips.

Animal Health

The health reports on the San Marcos River and Comal River fountain darters collected during the Bio-West biannual survey in October were completed by the South Western Fish Health Unit. A total of 57 fish collected from the San Marcos River were sent for analysis. Two of the ten fish screened for *Centrocestus formosanus* were positive. Additionally, five or the ten fish screened for Monogenean parasites were positive. No viruses were detected in the San Marcos River fish. A total of 59 fish collected from the Comal River were sent for analysis, seven of the ten fish analyzed for *Centrocestus formosanus* were positive for the parasite and two of the ten fish analyzed for Monogenean parasites were positive. The Comal River fountain darters were positive for Largemouth bass virus.

Additional Accomplishments

On November 3, 2021, Mr. Daw and Randy Gibson participated in a field trip organized by the National Cave and Karst Management Symposium in San Marcos, TX. They met the group at Spring Run 3 in Landa Park, New Braunfels, TX, discussed the EARP, and demonstrated the methods to capture Comal Springs riffle beetles and Peck's cave amphipods.

Task 2 Research

Texas Wild Rice Genetics

Staff at the Southwestern Native Aquatic Resource and Recovery Center (SNARRC) finalized the data analysis of Texas wild rice genetics and worked on drafting the final report for the Texas wild rice genetics assessment project. Ms. Moore drafted a PowerPoint to present the results of this project to the EAA.

Comal Springs Riffle Beetle Pupation

The well water was scheduled to be turned off at the SMARC for repairs on November 22, 2021. Ms. Moore and Mr. West prepared and tested the backup recirculation system for the flow-through system holding Comal Spring riffle beetles for the pupation project to continue providing the riffle beetles water during the shut off. Mr. West coordinated the system switch to and from recirculation the day of repairs. Ms. Moore and Dr. Bockrath drafted the interim report and a PowerPoint to present the results of this project thus far to the EAA.

Dr. Bockrath, Ms. Moore, and Israel Prewitt (BIO-WEST) confirmed flow was within the adequate range for all tubes at least weekly. Mr. Prewitt inventoried the three breeding chambers to remove any suspected non-target beetles. After non-target beetles were removed, 37 adult Comal Springs riffle beetles were confirmed across the tubes. Because some adult beetles were not Comal Springs riffle beetle, a portion of the F1 larvae are suspected to be non-target species. All larvae were put back into the breeding chambers to allow the larvae to develop further to confirm species identification.

Dryopid Life History and Housing

Dr. Ely Kosnicki (BIO-WEST) performed maintenance and adjustment of flow-through system and tanks, including inspection of tank 1 subjects and production. He also performed data management in the forms of entry, quality assurance, and preliminary calculations.

Comal Springs Riffle Beetle Exposure to **Staphylococcus**

The package with DNA extracted from experimental larvae was lost during shipping to the sequencing facility. The package could not be recovered, and sequencing was not completed. Dr. Camila Carlos-Shanley's lab (Texas State University) enumerated preserved larvae still in their care in hopes to extract replacement DNA for sequencing. The lab has five larvae from the negative control group, six larvae from the positive control group, and five larvae from the staph group. All larvae are from Trial 1.

Ms. Moore recorded the monthly inventory of Comal Springs riffle beetles at the SMARC from Trial 2 (Table 2). Several mortalities occurred, but the state of several dead larvae (crushed against the outflow screen) indicated a high-flow event that occurred due to an error might have contributed to some of those mortalities. One adult Comal Springs riffle beetle was found in each of the negative control and staph tubes after their first month at the SMARC.

Ms. Moore and Dr. Bockrath drafted the report for this project from the SMARC. Dr. Carlos-Shanley drafted the report for this project from Texas State University.

Captive Habitat for San Marcos Salamanders

Mr. Thomas and Ms. Whitt continued conducting daily checks for egg presence until they concluded the second trial. They placed the salamanders back in their sex-segregated Refugia tanks. Mr. Thomas and Ms. Whitt separated 45 San Marcos salamanders in the refugia by sex in preparation for the third trial. Mr. Daw bleached the research system to prepare for the third trial. No oviposition occurred during the second trial. Ms. Moore and Dr. Bockrath drafted the interim report for this project.

Additional Accomplishments

Dr. Bockrath and Ms. Moore continued drafting 2022 research proposals and presentations. Dr. Bockrath reviewed all 2021 report and 2022 proposal drafts. She also worked on the 2022 work plan and budget and continued the process of recruiting a Student Conservation Association (SCA) intern for the 2022 fountain darter project.

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

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

Dr. Bockrath submitted annual report drafts for all 2021 research projects.

Task 6 Meetings and Presentations

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

EARP staff went to the EAHCP staff appreciation dinner.

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

Species	SMARC 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	--	--	0	0	9	7	327	435
Fountain darter: Comal	NT	NT	--	--	0	0	1	3	130	33
Comal Springs riffle beetle	NT	NT	--	--	0	0	NA	NA	NA	NA
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	0	0	0
Peck's cave amphipod	NT	73	5	78	18	0	26	10	121	156
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	4	NT	4	8	0	0	3	0	193	70
San Marcos salamander	NT	NT	--	--	0	0	5	8	172	211
Comal Springs salamander	NT	4	12	16	0	0	0	0	113	69
Texas wild rice plants	NT	NT	--	--	0	0	6	2	204	169

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

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

Summary of November Activities

On November 3, 2021 – Field trip with the National Cave and Karst Management Symposium at Spring Run 3 in Landa Park, New Braunfels, TX.

November 8 - 19, 2021 – Collected Texas blind salamanders from Johnson's well and Primer's fissure in San Marcos, TX.

November 17, 2021 – Concluded Trial 2 of the captive habitat for San Marcos salamander research project at the UNFH.

On November 19, 2021 – Accepted Texas blind salamanders donated by Texas State University to the SMARC.

On November 30, 2021 – Collected Comal Springs salamanders and Peck's Cave amphipods from Spring Island in New Braunfels, TX.

Pictures

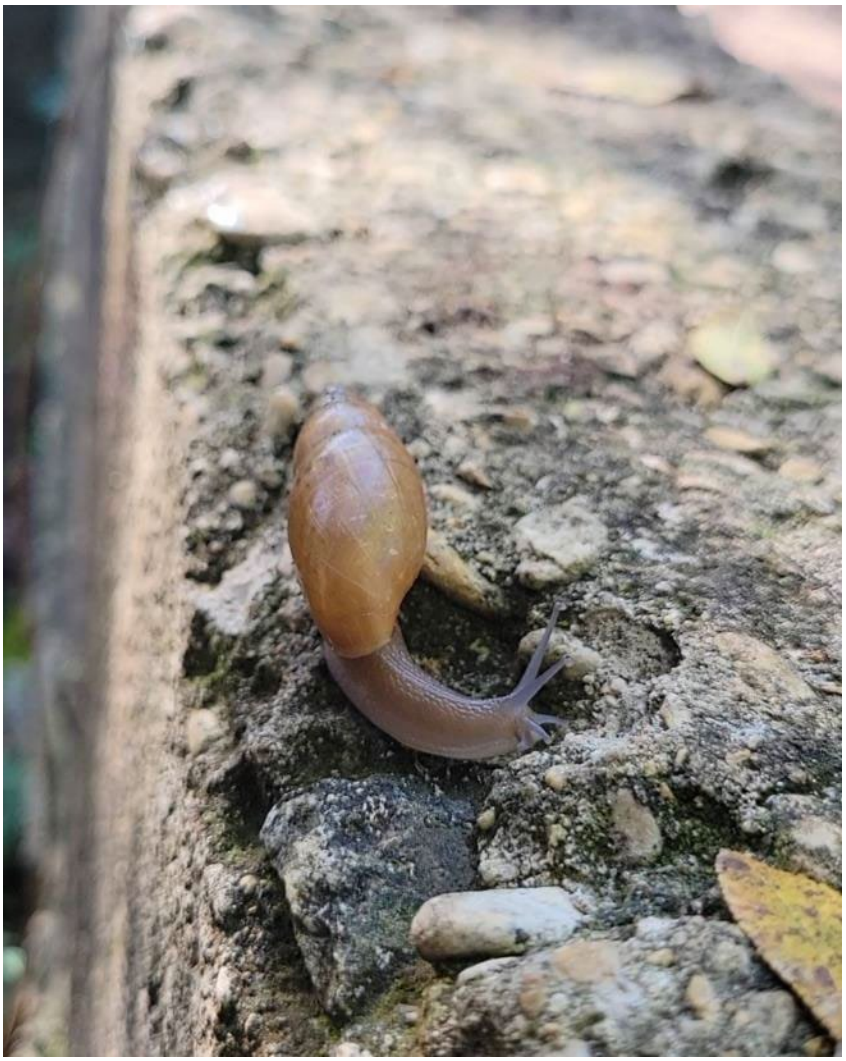

Figure 1. Terrestrial snail found at Primer's fissure while staff were checking traps in the well. Photo credit: Desiree Moore, USFWS

Figure 2. Adam Daw, Braden West, and Jennifer Whitt collecting Peck's cave amphipods at Spring Island, New Braunfels, Texas. Photo credit: Tommy Funk, USFWS

Figure 3. Etched and nonetched dowels showing biofilm progression in time from oldest (placed in conditioning box October 5, 2021) in front to newest in back (November 17, 2021). Photo credit: JL Whitt, USFWS

Figure 4. A) Jennifer Whitt power washing the floors and tanks in the San Marcos Aquatic Resources Center greenhouse. B) Tommy Funk removing sediment and debris from a tank in the greenhouse. Photo credit: Dr. Katie Bockrath, USFWS

December 2021 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

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

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Task 1 Refugia Operations

Species Collection

On December 14, 2021, Tommy Funk, Braden West, and Jennifer Whitt retrieved the cotton lures set in October at Spring Run 3 in Landa Park in New Braunfels, TX (Figure 1). The six adult Comal Springs riffle beetles collected were retained for the refugia at the Uvalde National Fish Hatchery (UNFH).

On December 21, 2021, Mr. Funk, Ben Thomas, and Mr. West collected 129 Texas wild rice tillers from 13 stands in Segment B of the San Marcos River in San Marcos, TX. The rice was retained for the refugia population at the UNFH.

Husbandry

Uvalde

Mr. Thomas and Ms. Whitt conducted year-end inventories for all Edward Aquifer Refugia Program (EARP) species at the UNFH (Figure 2). Tanks and systems were disinfected and cleaned when organisms were transferred to new tanks during the inventory.

Mr. Thomas transferred Texas blind salamander eggs laid in the main refugia to an incubation tank he set up in the quarantine building (Figure 3). This is the first spawning of Texas blind salamanders at the UNFH.

SMARC

Mr. Daw and Mr. Funk removed a large tank system from the quarantine area to prepare the space for a new quarantine rack system.

Mr. Funk and Mr. West conducted year-end inventories for all Edward Aquifer Refugia Program (EARP) species at the SMARC (Figure 4).

To make the field collection data-recording process for the Texas blind salamanders more concise, Mr. West created surveys in ArcGIS Survey123 that will track sampling effort, location, salamander genetic sampling, and catch/release data into one form.

Mr. Funk set up several tank systems to incubate the 152 San Marcos and 5 Texas blind salamander eggs in the SMARC quarantine.

Juan Martinez (SMARC) assisted Desiree Moore and Mr. West in relocating broken heater/chiller units to make more room in the refugia area.

Ms. Moore and Mr. West winterized the heater/chillers and pumps that might be affected by winter weather.

Animal Health

No work was done this month for animal health.

Task 2 Research

Texas Wild Rice Genetics

Dr. Katie Bockrath presented the results of the Texas wild rice genetic assessment to the Research Work Group for the Edwards Aquifer Habitat Conservation Plan (EAHCP) and again at the EAHCP Year-End Joint Meeting.

Comal Springs Riffle Beetle Pupation

Ms. Moore presented an update on the CSRB pupation project to the Research Work Group for the EAHCP.

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

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

Dryopid Life History and Housing

Mr. Prewitt performed maintenance and adjustment of flow-through system and tanks, including inspection of tank 1 subjects and production. Dr. Ely Kosnicki (BIO-WEST) performed data entry, quality assurance, analysis, and reporting.

Comal Springs Riffle Beetle Exposure to Staphylococcus

Dr. Bockrath requested a no-cost extension for this project to allow time for extracting and sequencing the DNA from back-up Trial 1 larvae.

Ms. Moore recorded the monthly inventory of Comal Springs riffle beetles at the SMARC from Trial 2 (Table 2). Two adult Comal Springs riffle beetles were found in the positive control tube and no living larvae remained in any treatment. The living adults were placed back in their tubes for monitoring.

Captive Habitat for San Marcos Salamanders

Dr. Bockrath, Mr. Daw, Mr. Thomas, and Ms. Whitt randomly assigned pairs of salamanders (one male, one female) to aquaria and began the third and last trial of the San Marcos salamander captive habitat project (Figure 5). The third replicate trial of this experiment is ongoing. Mr. Thomas and Ms. Whitt continued conducting daily checks for egg presence. No oviposition has occurred thus far.

Additional Accomplishments

Dr. Bockrath submitted paperwork to move the Student Conservation Association (SCA) interns at the SMARC and UNFH forward. Dr. Bockrath, Mr. Daw, and Scott Walker reviewed applications for the SCA intern at the UNFH.

Dr. Bockrath progressed in setting up the genetics lab by moving the furniture purchasing process forward. Ms. Moore and Mr. West began moving office furniture to make way for the genetics lab furniture.

Task 4 Species Reintroduction

No work was done this month for reintroduction.

Task 5 Reporting

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

Dr. Bockrath submitted 2021 research reports, 2022 research proposals, 2022 project plan, 2022 budget, and year-end presentations to Dr. Chad Furl and Kristy Kollaus for review.

Dr. Bockrath and Mr. Daw reviewed and addressed EAA comments for end-of-year reports, proposals, and project plan.

Task 6 Meetings and Presentations

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

Dr. Bockrath, Mr. Daw, and Ms. Moore attended the EAHCP Refugia Research Work Group meeting. Dr. Bockrath presented an update on the Texas wild rice genetic assessment and 2022-2023 planned CSRB genetic assessment research. Ms. Moore presented an update on the CSRB pupation work and 2022 planned CSRB propagation handbook development.

Dr. Bockrath, Mr. Daw, and Ms. Moore attended the EAHCP CSRB Work Group Meeting.

Dr. Bockrath, Mr. Daw, and Ms. Moore attended the EAHCP Year-End Joint meeting. Dr. Bockrath presented an update on the 2021 Texas wild rice genetic assessment.

Dr. Bockrath and Mr. Daw interviewed with the KSAT-12 Sara Spivey Meteorology news crew and gave a tour of the Refugia. Mr. Funk and Mr. West assisted the tour and set up tanks for close-up photos and videos of the Texas blind salamanders (Figure 6).

Ms. Moore and Mr. West led two separate tours for prospective SCA interns at the SMARC.

Ms. Whitt conducted educational outreach virtually. She met with 7th grade students at Boise Online Secondary School to discuss the evolutionary adaptions of the Texas blind salamander and the other species in our care.

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

Species	SMARC 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	--	--	116	62	28	14	415	483
Fountain darter: Comal	NT	NT	--	--	0	0	5	1	125	35
Comal Springs riffle beetle	NT	6	0	6	0	0	11	12	23	32
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	0	0	0
Peck's cave amphipod	NT	NT	--	--	0	0	NA	3	NA	153
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	0	0	1	0	192	70
San Marcos salamander	NT	NT	--	--	0	0	11	12	161	199
Comal Springs salamander	NT	NT	--	--	0	0	0	4	114	65
Texas wild rice plants	NT	13	0	--	0	0	13	0	191	169

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

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

Summary of December Activities

On December 6, 2021 – Research Work Group for the Edwards Aquifer Habitat Conservation Plan, San Marcos, TX.

December 7, 2021 – Comal Springs Riffle Beetle Work Group for the Edwards Aquifer Habitat Conservation Plan, San Marcos, TX.

December 14, 2021 – Collected Comal Springs riffle beetles from Spring Run 3 in Landa Park, New Braunfels, TX.

On December 15, 2021 – KSAT-12 Sara Spivey Meteorology news crew toured the SMARC and interviewed staff.

December 16, 2021 – Edwards Aquifer Habitat Conservation Plan Year-End Joint Committee Meeting, San Marcos, TX.

December 21, 2021 – Collected Texas wild rice from section B of the San Marcos River in San Marcos, TX.

Pictures

Figure 1. Braden West and Tommy Funk collecting cotton lures from Spring Run 3 at Landa Park, New Braunfels, Texas. Photo credit: JL Whitt, USFWS

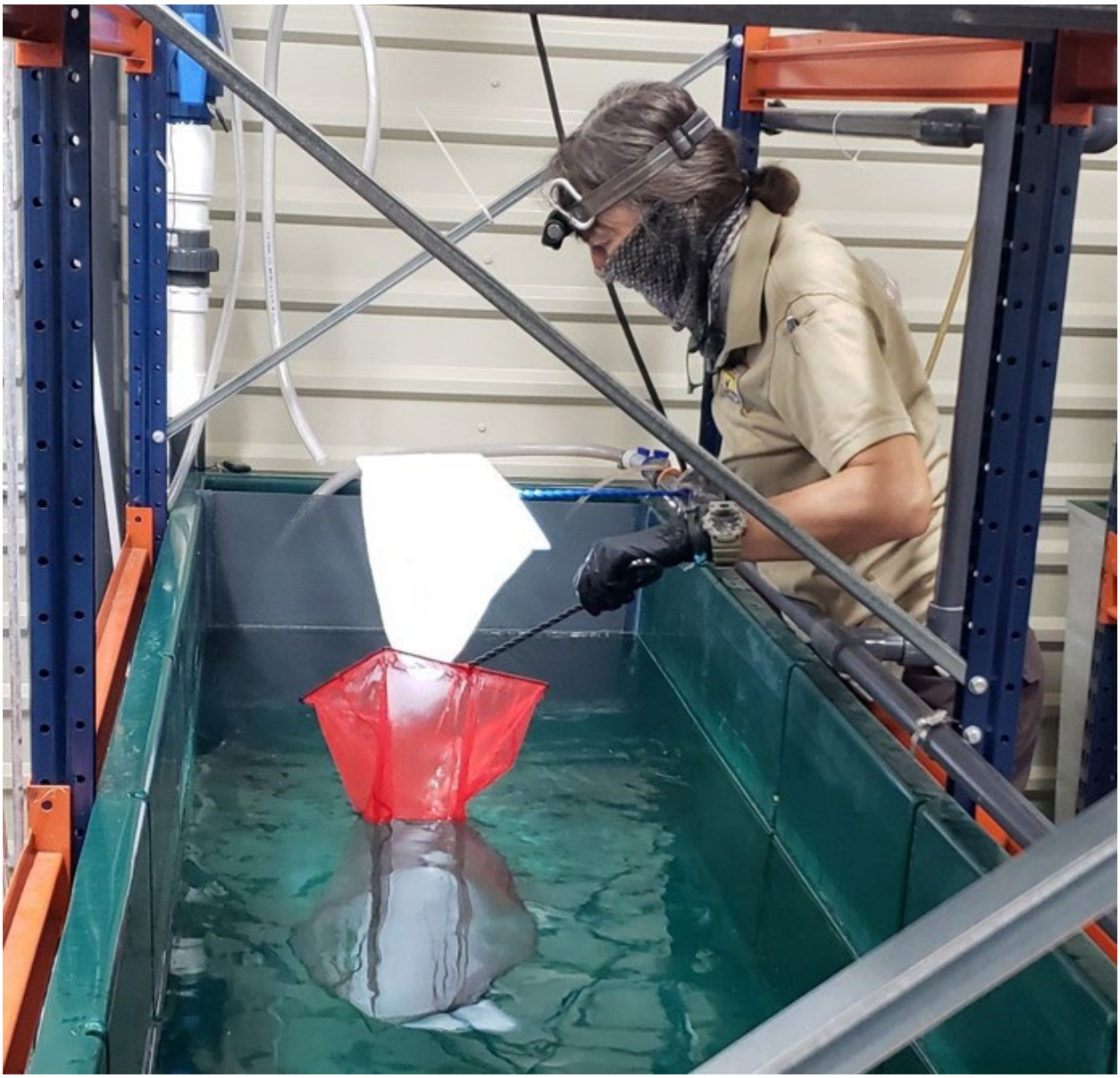

Figure 2. Jennifer Whitt transferring San Marcos fountain darters during year-end inventory. Photo credit: Ben Thomas, USFWS

Figure 3. Texas blind salamander eggs at the UNFH. Eggs are circled in red. Photo credit: Adam Daw, USFWS

Figure 4. Tommy Funk conducting the year-end inventory for San Marcos fountain darters at the SMARC. Photo credit: Desiree Moore, USFWS

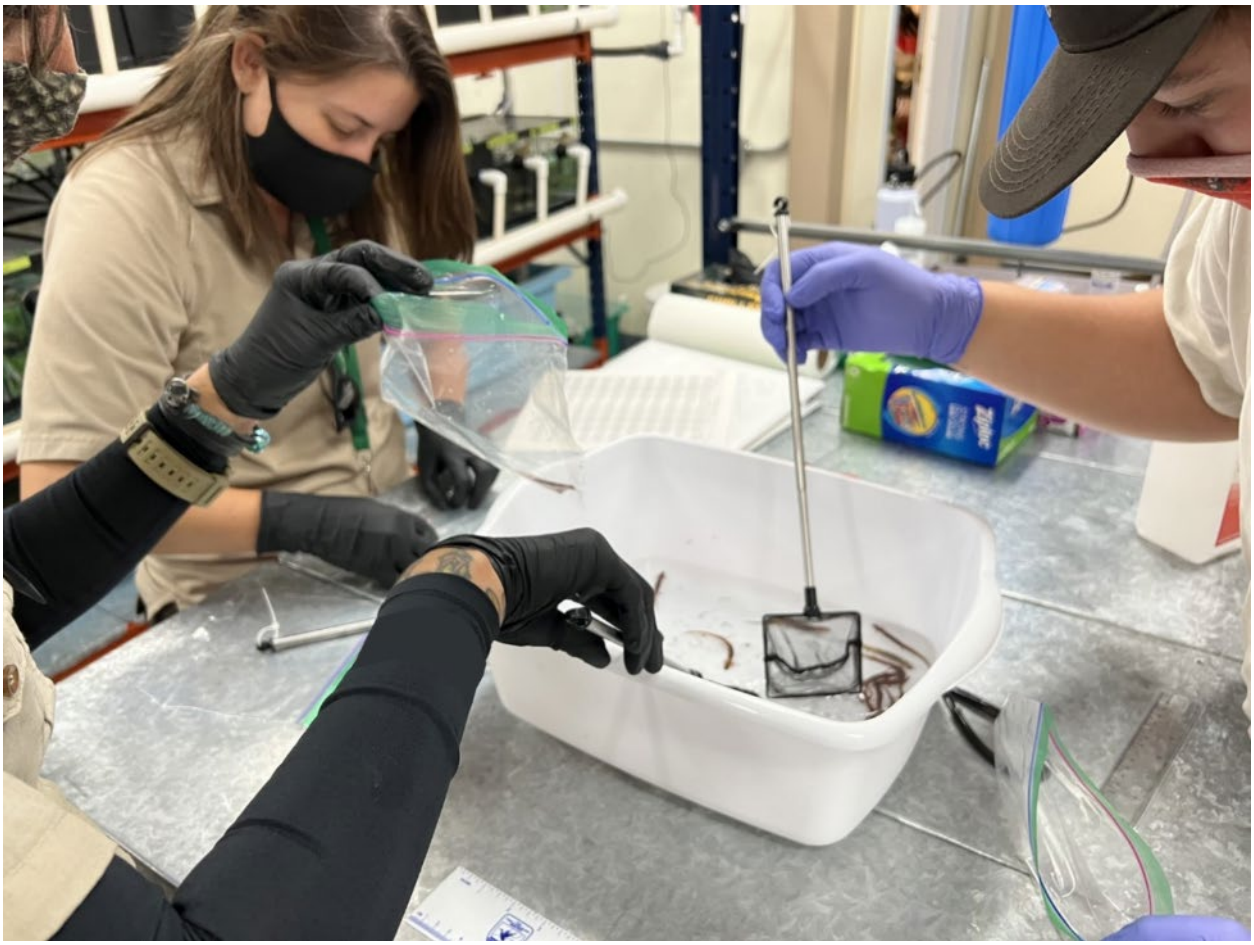

Figure 5. Jennifer Whitt, Dr. Katie Bockrath, and Ben Thomas sexing and measuring pairs of salamanders (one male, one female) for the third trial of the San Marcos salamander captive habitat project. Photo credit: Scott Walker, USFWS

Figure 6. Dr. Katie Bockrath, Adam Daw, Sarah Spivey, Tommy Funk, and Braden West after the KSAT-12 tour and interviews. Photo credit: KSAT-12

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/991 March 23, 2021

Memorandum

To: Adam Daw, San Marcos Aquatic Resource Center

From: Huseyin Kucuktas, Southwestern Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 21-38).

On March 10, 2021, Southwestern Fish Health Unit (SFHU) staff received 9 fountain darters (4 UNFH, and 5 SMARC collections) from a captive population captured originally from the San Marcos River, Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory.

Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC for the Edwards Aquifer Refugia Program. External examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of all left side gills for 9 fish. Testing was performed per the standard SFHU protocol for this study and additional diagnostic methods were used based on communications with hatchery staff.

Results:
Only a single immature *Centrocestus formosanus* cyst and one Monogenean parasite were observed in 1 of 5 fountain darters from SMARC collection. No *C. formosanus* cysts were observed on any of 4 fountain darters from UNFH collection. A single *Myxobolus* spp. from gill imprints, and *Hexamita* sp. from intestinal microscopic evaluations were noted in the UNFH collection fish examined. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo.

If you have any questions about test methodology or results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 21-38 for all follow up correspondence.

cc: Trista Becker, Southwestern Fish Health Unit
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 21-38

Date examined: 3/10/2021 Date Collected: 3/9/2021

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)	716	625	448	490		674	302	99	544	185
Total Length (mm)	42	39	36	40		41	37	22	39	30

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

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

Note: A total of four fountain darters from UNFH were received

Examiner signature *MB* *HK*

Revised on 9/20/2017

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

In Reply Refer To:
FWS/R2/FR-SFHU/992 March 23, 2021

Memorandum

To: Adam Daw, San Marcos Aquatic Resource Center

From: Huseyin Kucuktas, Southwestern Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 21-39).

On March 10, 2021, Southwestern Fish Health Unit (SFHU) staff received 10 fountain darters from a captive population captured originally from the Comal River, Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory.

Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC for the Edwards Aquifer Refugia Program. Examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of all left side gills for 10 fish. Testing was performed per the standard SFHU protocol for this study and additional diagnostic methods were used based on communications with hatchery staff.

Results:

Numerous *Centrocestus formosanus* cysts, both mature and immature, were observed on the gill arches of 2 out of 10 fountain darters from the Comal 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.

Other parasites observed during this evaluation included a significant number of *Ichthyobodo* sp. (formerly *Costia*) on most of the gill arches on 4 out of 10 fountain darters from this collection.

If you have any questions about test methodology and results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 21-39 for all follow up correspondence.

cc: Trista Becker, Southwestern Fish Health Unit
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 21-39

Date examined: 3/10/2021 Date Collected: 3/9/2021

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)	646	551	404	583	393	391	500	442	555	411
Total Length (mm)	43	40	38	40	37	37	36	37	40	38

***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	0,1,0,0	4,6,9,3	0,1,1,0
Immature (left gills only)	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,2,0,0	8,14,24,5	0,2,1,0
Monogenea	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
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	Costia, all gills, *(tntc)**	Costia, all gills, *(tntc)**	Costia, 1st, 3rd gills *(tntc)**	0,0,0,0	Costia, all gills, *(tntc)**	0,0,0,0	Costia, 2nd, 4th gills *(tntc)**

* *tntc,* too numerous to count

Examiner signature MB HK

Revised on 9/20/2017

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	
			Last Sample Date	Classification
Uvalde NFH 754 County Road 203 Uvalde, TX 78801 (830) 278-2419	☑ Hatchery	☐ Unsecured: Open Spring, Stream	*5/4/2021	A
			05/07/19	A
			05/08/18	A
Scott Walker: Acting Hatchery Manager	☐ Wild	☑ Secured: Well, Sterilized	05/02/17	A
			05/03/16	A

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
CCF	FY 2017 & 2018	39-60	1,250	(E): Uvalde NFH	30	30	30			60	60		60			60		30
					-	-	-			-	-		-			-		-
CCF	FY 2018-2020	4-15	2,400	(E): Uvalde NFH	30	30	30			60	60		60			60		30
					-	-	-			-	-		-			-		-
CSP	2016-2017	varies	1,000	(E): Phantom Springs, TX		30	30			60	60		60			60	60	
						-	-			-	-		-			-	-	
DEV	2015-2016	60-72	251	(E): San Felipe Springs, TX		30	30			30	30		30			30	30	
						-	-			-	-		-			-	-	
FOD	unknown/mixed	>6	~447	(E): San Marcos River, TX						60	60		60			60		
										-	-		-			-		
FOD	unknown/mixed	>6	~34	(E): Comal River, TX						6	6		6			6		
										-	-		-			-		
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Remarks[6]: * Aquatic Animal Health Inspection testing was not conducted in 2020 due to COVID travel restrictions. A = Asian Tapeworm, B = Edwardsiella tarda

Inspecting Biologist Signature	Concurred (signature and title)	
Print: Jason Woodland **Date:** Fish Biologist	**Print:** Trista Becker, MS, DVM **Date:** Fish Health Unit Leader	**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): 21-53

Fountain darters originating from the Comal River in Texas and the Devils River minnow fish lots do not meet 95% confidence testing level due to low stock numbers.

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

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/999 June 12, 2021

Memorandum

To: Adam Daw, San Marcos Aquatic Resource Center

From: Huseyin Kucuktas, Southwestern Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the Comal River, Texas (Case Number 21-55).

On May 11, 2021, Southwestern Fish Health Unit (SFHU) staff received 31 fountain darters collected from the Comal River (GNIS ID: 1372140). These fish were collected using dip net by staff from the San Marcos ARC and shipped live to the SFHU laboratory. The location was recorded at latitude 29.7106° and longitude -98.1276° Comal County, Texas. River water temperature at the time of collection was recorded at 23°C.

Assays and examinations for the sampled fish included virology and parasitology. Viruses screened for included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected in standard cell culture. A total of thirty fish were screened for viruses. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC for the Edwards Aquifer Refugia Program. Examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of all left side gills for 10 fish. Testing was performed per the standard SFHU protocol for this study.

Results:
Largemouth bass virus was isolated in cell culture and confirmed by PCR, results were negative for other tested viruses. *Centrocestus formosanus* cysts, mostly mature, were observed on the gill arches from 9 out of 10 fountain darters. Other parasites observed during this evaluation included a significant number of *Ichthyobodo* sp. (formerly *Costia*) on most of the gill arches of two fountain darters. Additionally, Monogenean parasites were present on one or more gill arches of half of the darters. The parasite data sheet indicating 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 and results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 21-55 for all follow up correspondence.

cc: Trista Becker, Southwestern Fish Health Unit
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 21-55

Date examined: 5/11/2021 Date Collected: 4/27/2021

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)	165	277	175	173	212	69	159	156	101	92
Total Length (mm)	27	31	28	25	30	20	26	28	24	22

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

Mature (left gills only)	L	0,0,0,1	0,0,0,1	0,3,0,0	1,0,2,0	0,0,6,0	0,1,0,1	1,0,0,0	0,0,1,0	0,0,2,0	0,0,0,0
Immature (left gills only)	L	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	0,0,0,0	0,0,0,0
Monogenea	L	1,0,0,0	0,1,0,0	0,0,1,0	0,0,0,0	0,2,1,1	1,0,1,0	1,0,0,0	1,1,0,0	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	0,1,1,1*	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	1,1,1,1*	0,0,0,0

*tntc, Costia *tntc, Costia

Examiner signature HK

Revised on 9/20/2017

San Diego Zoo Wildlife Alliance

Molecular Diagnostics Report
Amphibian Disease Laboratory
Beckman Center for Conservation Research

Set ID: 6072 Friday, July 16, 2021

SOUTHWESTERN FISH HEALTH UNIT USFWS

Amphibian ID	Common Name	Species	Chytrid PCR [1]	Bsal PCR [2]	Date Collected
7377	Houston Toad	Bufo houstonensis	Negative	Negative	6/22/2021
7653	Houston Toad	Bufo houstonensis	Negative	Negative	6/22/2021
9458	Houston Toad	Bufo houstonensis	Negative	Negative	6/22/2021
9489	Houston Toad	Bufo houstonensis	Negative	Negative	6/22/2021
EN1	San Marcos Salamander	Eurycea nana	Positive	Negative	6/22/2021
EN2	San Marcos Salamander	Eurycea nana	Positive	Negative	6/22/2021
EN4	San Marcos Salamander	Eurycea nana	Positive	Negative	6/22/2021
ER1	Texas Blind Salamander	Eurycea rathbuni	Positive	Negative	6/22/2021
ER4	Texas Blind Salamander	Eurycea rathbuni	Positive	Negative	6/22/2021
ER8	Texas Blind Salamander	Eurycea rathbuni	Positive	Negative	6/22/2021
ES2	Barton Springs Salamander	Eurycea sosorum	Positive	Negative	6/22/2021
ES3	Barton Springs Salamander	Eurycea sosorum	Positive	Negative	6/22/2021
ESW5	Barton Springs Salamander	Eurycea sosorum	Positive	Negative	6/22/2021
R3T1	Houston Toad	Bufo houstonensis	Negative	Negative	6/22/2021
R3T2	Houston Toad	Bufo houstonensis	Negative	Negative	6/22/2021
TIT4M	Houston Toad	Bufo houstonensis	Negative	Negative	6/22/2021

Positive chytrid skin swab samples indicate the presence of DNA from the amphibian chytrid fungus. Antifungal treatment with follow up PCR is suggested before animals are introduced into the zoo collection. Occasionally, multiple treatment cycles are required to clear animals from infection. Equivocal results indicate the presence of small amounts of fungal DNA. Re-testing of aniamsl and follow up with the laboratory are recommended.

[1] Taqman PCR for Chytrid Fungus (Batrachochytrium dendrobatides)

[2] Taqman PCR for Chytrid Fungus (Batrachochytrium salamandrivorans)

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/998 June 12, 2021
Memorandum

To: Adam Daw, San Marcos Aquatic Resource Center

From: Huseyin Kucuktas, Southwestern Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 21-54).

On May 5, 2021 Southwestern Fish Health Unit (SFHU) staff received 47 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 the collection location at latitude 29.8900° and longitude -97.9340° in Hays County, Texas. River water temperature at the time of collection was recorded at 20.5°C

Assays and examinations for the sampled fish included virology and parasitology. Virus screening included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected in standard cell lines. A total of forty-six darters were screened for viruses. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC for the Edwards Aquifer Refugia Program. 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 and standard SFHU protocols for this study.

Results:
Zero gill arches from the 10 fish examined had *Centrocestus formosanus*. A total of 7 out of 10 darters had Monogenean-type parasites, and only a single darter had *Ichthyobodo* sp. (formerly *Costia*) infestation. Aquareovirus was isolated in cell culture and confirmed by PCR testing and results were negative for other tested viruses. Fountain darters from San Marcos River have historically tested positive for Aquareovirus since 2003. Although the pathogenicity of these viruses has been experimentally demonstrated in other fish species, their pathogenicity has yet to be determined for the San Marcos fountain darters. Given the endangered species status of this species, biosecurity measures are essential to prevent the spread of this virus. 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 21-54 for all follow up correspondence.

cc: Trista Becker, Southwestern Fish Health Unit
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 21-54

Date examined: 5/4/2021 Date Collected: 4/27/2021

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)	255	305	244	103	199	222	254	114	83	163
Total Length (mm)	31	34	30	23	30	30	31	25	21	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	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	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	0,0,0,0	1,1,1,0	1,0,1,0	0,0,0,0	0,2,1,1	1,0,1,0	0,1,1,0	0,1,1,1	0,0,0,0	0,0,1,0
Myxobolus sp.	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Other	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	1,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0

Costia

Examiner signature HK DH

Revised on 9/20/2017

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
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In Reply Refer To:
FWS/R2/FR-SFHU/1006 September 13, 2021
Memorandum

To: Adam Daw, San Marcos Aquatic Resource Center

From: Huseyin Kucuktas, Southwestern Fish Health Unit/SNARRC

Subject: Comal River fountain darter diagnostic case 21-68

Diagnosis: Environmental gill disease, trauma

Case history:
Staff at Uvalde NFH noticed a consistent slight elevation in mortality from a group of fountain darters collected this spring from the Comal River, TX. Symptoms observed at the beginning of May 2021 included some fish having lesions and exhibiting erratic behavior and lethargy as well as inappetence. After transportation to San Marcos ARC, darters were treated with formalin on the day of collection. Fish were then transferred to Uvalde NFH at the beginning of June where a 3-day salt bath treatment was applied in early June followed by a second round of formalin and salt treatments through mid-June. The water quality parameters on the day of sample collection for diagnostics were reported as a DO of 4.5 mg/ml, Temperature 20 ºC, pH 7.3, Ammonia TAN 0.0 mg/l, Nitrite 0.0 mg/l, Alkalinity 191 mg/l $CaCO_3$, and Nitrate 3.0 mg/l. A total of 4 fountain darters (2 alive, 2 dead) were shipped overnight to the Fish Health Laboratory for diagnostic evaluation.

Recent parasite enumeration study results indicated heavy *Ichthyobodo* sp. (formerly *Costia*) infestation as well as notable numbers of *Centrocestus sp.* cysts from darters collected at the Comal River (Case# 39 – report 992). Additionally, this population is consistently positive on BF2 cell culture for largemouth bass virus (LMBV). Histopathology has often described splenic inflammation and hepatic necrosis which may be attributed to LMBV, but Koch's postulates for this virus have not been pursued in the Fountain darter or any related species. For example, an exposure trial with characterization of gross and histopathologic lesions attributable to known dose concentrations of viral load exposure would provide the type of clinical information required to make such a diagnosis beyond detection of the presence of viral particles in wild collection fish.

Examination and test results:
The 2 live darters were euthanized with standard protocols. Mucus swabs were collected from the body surface of all 4 fish and a water sample also was collected from the shipping container for LMBV testing by PCR. Note that this is not a standard procedure for determining LMBV status in

fish. Due to the low availability of target animals available for sampling, standard procedures could not be utilized.

Gross examination of darters noted bilateral ocular hemorrhage with unilateral exophthalmia in one of the fish (see Figure 1), which was also noted by hatchery staff prior to shipping. The gills had swelling and rod-shaped bacteria consistent with environmental gill disease.

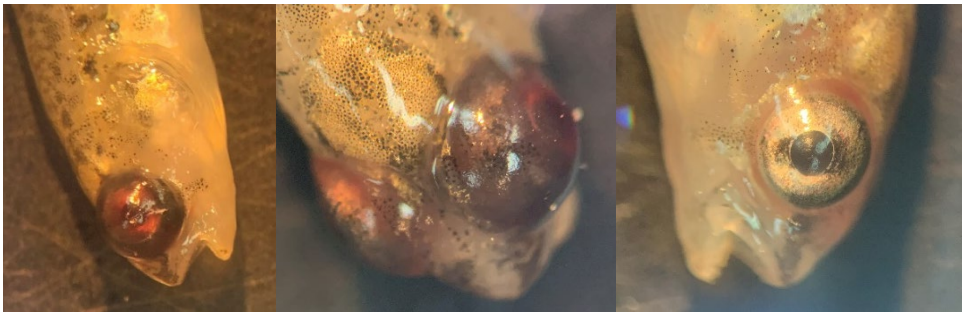

Figure 1: Gross images under the dissecting microscope of the ocular trauma noted in one Fountain darter.

All four fish were fixed in formalin (z-fix) and submitted to Washington Animal Disease and Diagnostic Laboratory (WADDL) for histopathologic evaluation. Both body mucus and water samples were tested negative for LMBV by PCR. It is worth noting that the lack of histologic lesions does not rule out the possibility of infection with LMBV just that lesions typical of LMBV disease are not observed. The determination of LMBV by clinical signs in Fountain darters is complicated by their lack of a swim bladder, which is the main organ affected in cases of clinical disease in bass. Histologic lesions indicated the presence of hemorrhagic and inflammatory lesions around the eye of the two submitted darters, which is likely due to trauma.

Several diagnostic submissions of fountain darters both from San Marcos River and Comal River collections over a 10-year time-frame indicated a variety of disease conditions, with or without pathogenic organisms. Parasite enumeration studies typically find at least low levels and often moderate loads of Centrocestus parasites encysted in the gills of this population of fish, which may be impacting their survival in refugia.

High loads of *Ichthyobodo* (formerly *Costia)* were noted on gill exams from the Comal River fish submitted to the Fish Health Unit in March and May. Acute signs of trauma could be related to handling when transferred or intraspecific aggression within the tank but in this case is most likely related to irritation due to heavy parasite infestation. Sometimes in cases of high parasite loads (such as with heavy Costia infection), fish can injure themselves flashing and scratching the sides of tanks and any objects within the habitat due to intense pruritis (itchiness). The presence of *Ichthyobodo* would've been cleared with the formalin treatments conducted prior to submission. The gill disease was treated with a 3-day Chloramine-t immersion treatment at 15ppm. Afterward, the mortality appeared to have resolved.

cc: Trista Becker, Southwestern Fish Health Unit/SNARRC
Scott Walker, Uvalde NFH

United States Department of the Interior
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In Reply Refer To:
FWS/R2/FR-SFHU/1007 September 15, 2021

Memorandum

To: Adam Daw, San Marcos Aquatic Resource Center

From: Huseyin Kucuktas, Southwestern Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 21-71)

On August 18, 2021, Southwestern Fish Health Unit (SFHU) staff received 10 fountain darters from a captive population collected originally from the San Marcos River, Texas. As part of a routine parasite analysis of the wild population, used for the Edwards Aquifer Refugia, fish were sampled by staff at the San Marcos ARC and held at the San Marcos Aquatic Resource Center's Edwards Aquifer Refugia quarantine building before shipping.

Examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of all left-side gills for 10 fish. Testing was performed per the standard SFHU protocol for this study and additional diagnostic methods were used based on communications with hatchery staff.

Results:

The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo. No *Centrocestus formosanus* cysts, mature or immature, were observed on any of 10 fountain darters from the San Marcos River.

The only other parasite observed during this evaluation included a low number of *Monogeneans* on some of the gill arches on 9 out of 10 fountain darters from this collection.

If you have any questions about test methodology and results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 21-71 for all follow up correspondence.

cc: Trista Becker, Southwestern Fish Health Unit
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 21-71

Date examined: 8/18/2021 Date Collected: 8/12/2021

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)	201	132	137	281	191	92	183	139	122	200
Total Length (mm)	30	27	25	32	30	24	30	26	27	30

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

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

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

Examiner signature MB

United States Department of the Interior
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In Reply Refer To:
FWS/R2/FR-SFHU/1008 September 15, 2021

Memorandum

To: Adam Daw, San Marcos Aquatic Resource Center

From: Huseyin Kucuktas, Southwestern Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 21-72)

On August 18, 2021, Southwestern Fish Health Unit (SFHU) staff received 10 fountain darters from a captive population collected originally from the Comal River, Texas. As part of a routine parasite analysis of the wild population, used for the Edwards Aquifer Refugia, fish were sampled by staff at the San Marcos ARC and held at the San Marcos Aquatic Resource Center's Edwards Aquifer Refugia quarantine building before shipping.

Examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* as well as other parasites was conducted by examination of all left-side gills for 10 fish. Testing was performed per the standard SFHU protocol for this study and additional diagnostic methods were used based on communications with hatchery staff.

Results:

The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo. No mature *Centrocestus formosanus* cysts, were observed on any gill arches. However, immature cysts were observed on single gill arches of 3 darters.

Low number of *Monogeneans* were observed on some of the gill arches on 6 out of 10 fountain darters from this collection.

If you have any questions about test methodology and results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 21-72 for all follow up correspondence.

cc: Trista Becker, Southwestern Fish Health Unit
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 21-72

Date examined: 8/18/2021 Date Collected: 8/16/2021

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)	325	241	155	290	228	184	210	183	214	156
Total Length (mm)	33	30	27	24	23	22	30	27	30	26

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

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

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

Examiner signature MB

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
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In Reply Refer To:
FWS/R2/FR-SFHU/1012 November 30, 2021

Memorandum
To: Adam Daw, San Marcos Aquatic Resource Center

From: Huseyin Kucuktas, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 22-02).

On October 28, 2021, Southwestern Fish Health Unit (SFHU) staff received 60 fountain darters (*Etheostoma fonticola*) collected from the San Marcos River (GNIS ID: 1375972), Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for fish health testing. San Marcos ARC staff recorded collection of fountain darters at latitude 29.885912° and longitude -97.935818° in Hayes County, Texas, and the river water temperature was 23°C.

Assays and examinations for the sampled fish included virology and parasitology. Viral screening of 60 fish included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected using standard cell lines. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC. External examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of the left side set of gills for 10 fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2016 edition) and standard SFHU protocols.

Results:
Although no adult *Centrocestus formosanus* was observed in any of 10 fish examined, immature *C. formosanus* were detected in 2/10 fish. Additionally, 5/10 fish examined had Monogenean parasites on gills. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo. No viruses were detected by cell culture.

If you have any questions about test methodology or results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 22-02 for all follow up correspondence.

cc: Trista Becker, Southwestern Fish Health Unit/ Southwestern Native ARRC
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 22-02

Date examined: 10-28-2021 Date Collected: 10/19/2021

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)	410	360	350	270	290	400	360	250	240	210
Total Length (mm)	29	29	27	25	26	28	27	26	24	23

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

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

United States Department of the Interior
Fish and Wildlife Service#
Southwestern Native Aquatic Resources and Recovery Center
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In Reply Refer To:
FWS/R2/FR-SFHU/1013 December 9, 2021

Memorandum

To: Adam Daw, San Marcos Aquatic Resource Center

From: Huseyin Kucuktas, Southwestern Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 22-03). This is a corrected report from the report dated November 30, 2021.

On October 28, 2021, Southwestern Fish Health Unit (SFHU) staff received 60 fountain darters (*Etheostoma fonticola*) collected from the Comal River (GNIS ID: 1372140). These fish were collected using dip net by staff from the San Marcos ARC and shipped live to the SFHU laboratory. The location was recorded at latitude 29.714504° and longitude -98.135654° Comal County, Texas, and river water temperature at the time of collection was 23°C.

Assays and examinations for the sampled fish included virology and parasitology. Viral screening of 60 fish included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected in the standard cell lines used. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC for the Edwards Aquifer Refugia Program. Screening for *Centrocestus formosanus* was conducted by examination of all left side gills from 10 fish. Testing was performed per the standard SFHU protocol for this study

Results:
Largemouth bass virus was isolated in cell culture and confirmed by PCR. No other viruses were detected. Numerous immature *Centrocestus formosanus* cysts were observed on the gill arches from 7/10 fountain darters from the Comal River. Additionally, Monogenean parasites were observed on a single gill arch from 2/10 fish. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo.

If you have any questions about test methodology and results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 22-03 for all follow up correspondence.

cc: Trista Becker, Southwestern Fish Health Unit
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 22-03

Date examined: 10-28-2021 Date Collected: 10/26/2021

Collection site: Comal River

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	268	185	175	102	120	272	304	292	308	330
Total Length (mm)	25	24	23	21	20	25	27	26	27	28

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

Examiner signature DH, JW

Revised on 9/20/2017