Appendix A | **2024 Edwards Aquifer Refugia Work Plan**

Edwards Aquifer Authority
2024 Work Plan

Amendment #2; Approved by the EAA Board of Directors on June 11, 2024

2024 Edwards Aquifer Authority Work Plan Budget

EAHCP Section	Conservation Measure	Table 7.1	Estimated 2024 Budget[a]
5.1.1	Refugia	$1,678,597	$1,884,343
5.1.2	VISPO	$4,172,000[b]	$9,253,167[c]
5.1.3	RWCP	$493,250	$0
5.1.4	Stage V	NA	NA
5.5.1	ASR Leasing & Forbearance	$4,759,000	$5,765,190
	ASR O&M	$2,194,000	$0
5.7.2	Water Quality Monitoring	$200,000	$65,000
6.3.1	Biological Monitoring	$400,000	$755,774[d]
6.3.3	Ecological Model	$25,000	$0
6.3.4	Applied Research	$0	$250,000
FMA §2.2	Program Management	$750,000	$1,743,757
Total		$14,671,847	$19,717,231

a. Estimated annual work plan cost per Funding and Management Agreement § 4.4.

b. Dollars in Table 7.1 of the EAHCP were calculated from a volume goal of 40,000 acre-feet (ac-ft). The volume goal was amended to 41,795 ac-ft in 2019 and Table 7.1 dollars are no longer applicable.

c. On October 1, 2023, the VISPO program was triggered, resulting in suspension payments totaling $9,253,167.

d. Includes Critical Period Monitoring if required.

2024 Edwards Aquifer Authority (EAA) Work Plan and Funding Application Amendments

Amendment #	Date EAHCP Committee Approved	Conservation Measure Amended	Y/N Funding Application Change	Funding Application Change ($)	Date EAA Board Approved	Comments
0	5/3/2023	Original Work Plan	NA	NA	NA	Original Work Plan
1	10/5/23	VISPO and Program Management	N	NA	11/14/2023	Updated Work Plan with updated costs for VISPO and Program Management
2	5/23/2024	Refugia	Y	$614,993	6/11/2024	Updated Refugia with known activities and revised 2024 costs

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 2024, 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 in the wild), plus research conducted on the Covered Species.

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

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

* US Fish and Wildlife Service determined the Texas troglobitic water slater is not warranted for listed as threatened or endangered under the Endangered Species Act (Federal Register Document Number 88 FR 83368.

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 populations in the wild 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.

2024 Assumptions
As work plans are developed almost a year prior to implementation, it is possible that methods described herein will be contingent on the status of the current year's activities or authorization from the HCP process. If conditions change, this work plan may need to be amended to accommodate realized outcomes.

The following potential situations could necessitate methodology adjustments.

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

Target for 2024 (Deliverables and Methods by Task):

Task 1. Refugia Operations

Standing Stocks: USFWS staff will take all appropriate steps to collect and maintain standing/refugia stocks at their respective target captive population size to provide refugia for all the Covered Species. Table 2 contains the target species numbers.

Table 2. Target refugia numbers and census by species.

Species	Standing Stock	Refugia Stock	Salvage Stock	Anticipated SMARC census (Jan 2024)	Anticipated SMARC census (Dec 2024)	Anticipated UNFH census (Jan 2024)	Anticipated UNFH census (Dec 2024
Fountain darter (Comal)	1000	1000†	2000	250	500	250	500
Fountain darter (San Marcos)	1000	1000†	2500	500	500	500	500
Texas wild rice	430	430†	1500	215	215	215	215
Texas Blind Salamander	500	500†	500	250	250	60	80
San Marcos salamander	500	500†	500	250	250	250	250
Comal Springs salamander	500	500†	500	150	150	135	135
Peck's cave amphipod	500	500†	500	250	250	250	250
Comal Springs riffle beetle	500	500†	500	75	75	75	75
Comal Springs dryopid beetle	500	500†	500	*	20	*	20
Edwards Aquifer diving beetle	500	500†	500	*	*	*	*
Texas troglobitic water slater	500	500†	500	*	*	*	*

† Includes specimens within standing stock
*Catch rates and hatchery survival are uncertain given the rarity of the species.

Collection: In 2024, the USFWS will collect Covered Species as required to reach and maintain target standing and refugia stock numbers as shown in Table 2. The USFWS will coordinate species collections with other ongoing HCP activities (e.g., Biological Monitoring Program) so that collections for refugia do not adversely impact other efforts. The USFWS will carry out species collections through a variety of passive and active collection methods and will minimize aquatic invasive species transfer by conducting collections in accordance with a Hazard Analysis Critical-Control Point Plan. The USFWS will document and report collection efforts to the EAA. The USFWS will distribute captured organisms between the SMARC and UNFH facilities to ensure redundancy and to expedite the obligation to establish and maintain two refugia populations at separate locations. The USFWS will hold all species in respective quarantine areas until their health has been assessed. Staff will incorporate quarantined organisms into the general refugia population once they have determined that such specimens are healthy and free from invasive species. The USFWS will share reports, including test results, produced as part of the quarantine process.

The following sections briefly describe planned 2024 collection, maintenance, and propagation efforts for each species.

Fountain Darters:

Collection: In 2024, the USFWS will collect fountain darters from the San Marcos River in four seasonal sampling events. This will reduce habitat disturbance and allow EARP staff to track survival and disease occurrence on a seasonal basis. For refugia purposes, USFWS staff will retain fountain darters collected by biomonitoring staff via drop nets. Staff will collect fish proportionally from the three sections of the San Marcos River: 1) Upper = Spring Lake, 2) Middle = Spring Lake dam to Rio Vista dam, and 3) Lower = below Rio Vista dam to Cape's Dam. The USFWS will thoroughly investigate unusual mortality events. The USFWS will include summary reports to the EAA as part of the monthly reports. Collections will target sufficient fish so to account for regular, expected mortality, such that the captive population should remain at or above the target.

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

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

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

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

Texas wild rice:

Collection: USFWS staff will collect Texas wild rice tillers from San Marcos River segments (Figure 1), with a break during summer months when collected wild rice does not survive well due to heat stress. In 2024, staff will target stands and genetic variants that are not already part of the refugia population or require supplementation in collections for SMARC and UNFH. The refugia populations will reflect the wild populations in both their respective proportion, based on the most recent Texas wild rice survey data, and historical genetic diversity (2021 genetic assessment and Wilson et al. 2016). During tiller collection, the USFWS will record the geographic coordinates, area coverage, and depth of the stand or individual plant. USFWS staff will collect tillers by wading and SCUBA diving. The USFWS will consider georeferenced aerial imagery to help identify distinct TWR stands used for tiller collection.

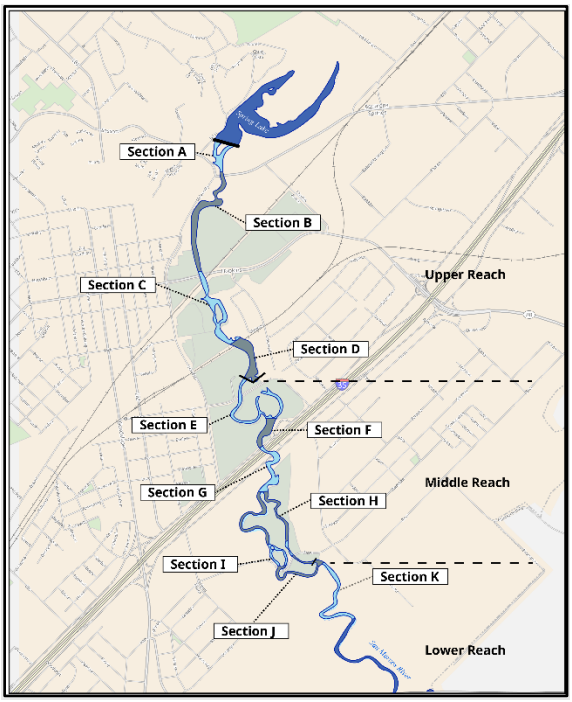

Figure 1. Letters define designated San Marcos River reaches where Texas wild rice is collected for refugia populations.

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

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

Texas blind salamanders:

Collection: USFWS will collect Texas blind salamanders using nets and traps. Staff will deploy traps quarterly for approximately 14 consecutive days with traps checked every 2-4 days to collect Texas blind salamander individuals from Primers Fissure, Johnson's well, Rattlesnake cave, and Rattlesnake well (Table 5). To avoid oversampling these habitats, staff will only collect 1/3 of salamanders observed from each of these locations during quarterly sampling events. Staff will also collect salamanders from a driftnet on Diversion Springs in Spring Lake throughout the year. We will retain all specimens from this site, under the assumption that any Texas blind salamander leaving a spring orifice that enters a stream or lake environment will ultimately succumb to predation. We will check these sites up to three times per week when applicable. Staff will transport all specimens alive and maintain them in the SMARC or UNFH refugia. Texas State University staff generally check drift nets on Sessom Creek and Texas State University Artesian Well; Texas State University transfers live Texas blind salamanders to SMARC according to their permits, when appropriate. USFWS staff may periodically check nets on these sites when they are not being checked by Texas State University staff.

Health Testing: Texas blind salamanders are known to carry *Batrachochytrium dendrobatidis* (Bd), a fungal disease listed by Animal and Plant Health Inspection Service (APHIS) as a reportable exotic disease under the United States National List of Reportable Animal Diseases (NLRAD) as prescribed Title 9 of the Code of Federal Regulations (CFR) part 57. The NLRAD regulation means that the USFWS has a legal obligation to report detections of this disease. We also have a professional obligation to follow the USFWS Fish Health Policy, which includes an Exotic Disease Eradication Plan (713 FW 3). Project leaders at UNFH and SMARC have the responsibility to assist in the development, and comply with, site-specific aquatic animal cultural sanitation and decontamination plans covering the provision of the Fish Health Policy, including the exotic disease eradication plan.

As part of quarantine procedures, USFWS staff will swab all large Texas blind salamanders. If they are too small to be swabbed, then we will do a representative batch swab of group-housed salamanders once they are large enough to be safely swabbed. USFWS staff will process these samples at SMARC or other facility to screen for *Batrachochytrium dendrobatidis* (Bd,

commonly referred to as chytrid fungus) and *Batrachochytrium salamandrivorans* (Bsal) prior to specimen incorporation into the general refugia population. Staff will retain duplicate swabs in case further testing is warranted. Staff will hold all salamanders in quarantine for at least 30 days and until test results have returned. Previous tests of wild caught salamanders at SMARC (both Texas Blind and San Marcos salamanders) have regularly tested positive for Bd. Positive testing for Bsal will be treated more cautiously as it has not yet been documented in North America. Staff would retain such salamanders in quarantine until further study and recommendations from FWS Fish Health.

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

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

San Marcos salamanders:

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

As part of quarantine procedures, USFWS staff will swab San Marcos Salamanders for disease testing. If they are too small to be swabbed, then we will do a representative batch swab of group housed salamanders once they are large enough to be safely swabbed. USFWS staff will process these samples at SMARC or other facility to screen for Bd and Bsal prior to specimen incorporation into the general refugia population. Staff will retain duplicate swabs in case further testing is warranted. Chytrid testing will occur in batches where groups of five swabs will be pooled for analysis. Staff will hold all salamanders in quarantine for at least 30 days and until test results have returned. Positive testing for Bsal will be treated more cautiously as it has not yet been documented in North America.

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

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

Comal Springs salamanders:

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

As part of quarantine procedures, USFWS staff will swab all large Comal Springs salamanders. If they are too small to be swabbed, then we will do a representative batch swab of group housed salamanders once they are large enough to be safely swabbed. USFWS staff will process these samples at SMARC or other facility to screen for Bd and Bsal prior to specimen incorporation into the general refugia population. Staff will retain duplicate swabs in case further testing is warranted. Chytrid testing will occur in batches where groups of five swabs will be pooled for analysis. Staff will hold all salamanders in quarantine for at least 30 days and until test results have returned. Clinically, the salamanders appear normal and do not have any lesions or signs of disease. Positive testing for Bsal will be treated more cautiously as it has not yet been documented in North America. Staff would retain such salamanders in quarantine until further study and recommendations from FWS Fish Health.

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

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

Comal Springs riffle beetle:

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

1. Staff will not sample the same spring orifice two times in a row.
2. Staff will collect all riffle beetle adults and larvae from lures.

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

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

Propagation: Propagation methods for this species are being developed.

Peck's cave amphipod:

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

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

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

Comal Springs dryopid beetle:

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

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

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

Edwards Aquifer diving beetle:

Collection: Edwards Aquifer diving beetles have been collected in the past at the Texas State University Artesian Well and Diversion Springs. USFWS staff will accept Edwards Aquifer

diving beetles during drift net checks at the Artesian Well when as Texas State University encounters them.

Maintenance: USFWS will combine collected Edwards Aquifer diving beetles, regardless of collection location. Staff will transfer captured specimens to the SMARC or UNFH and house them in custom-made aquatic holding systems. Edwards Aquifer diving beetles are predators; staff will feed them small invertebrates (e.g., ostracods).

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

Texas troglobitic water slater:

Collection: Texas troglobitic water slaters are primarily found in Artesian Well on Texas State Campus. Recent research by Will Coleman (Texas State University) suggests that this is a deep aquifer species, rarely found at the surface. Mr. Coleman was unable to keep any alive, as all specimens he collected were injured. USFWS will continue to work with invertebrate experts to determine what might be the optimum way to collect this species. USFWS staff will deploy and check drift nets in the Artesian Well as Texas State University allows.

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

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

Table 5. A tentative schedule for all species sampling during 2024. Collections listed here are subject to change with extenuating circumstances such as weather and coordination with external partners. USFWS will notify EAA and partners of sampling dates as they become known or changed.

Edward's Aquifer Species Collection Plan 2024			
Date (month)	**Interval**	**Location**	**Target Species**
January	14 Consecutive days with traps checked 2-3 times a week	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
January	1 day sampling event, hand pick from downed wood	Landa Lake	Comal Springs dryopid beetle

Edward's Aquifer Species Collection Plan 2024			
Date (month)	**Interval**	**Location**	**Target Species**
February	14 Consecutive days with traps checked 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
February	Set lures	Spring Run, Landa Lake	Comal Springs dryopid beetle, Comal Springs riffle beetle, Peck's cave amphipod
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 collection event	Spring Run, Landa Lake	Comal Springs dryopid beetle, Peck's cave amphipod
February	3 day sampling event, retrieve BIO-WEST lures	Comal Springs	Comal Springs riffle beetle
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	Comal Springs dryopid beetle
March	4-day sampling event	Landa Lake, Comal River, and San Marcos River	Fountain darters
April	Set lures	Comal Springs	Comal Springs riffle beetles
April	Check 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander

Edward's Aquifer Species Collection Plan 2024			
Date (month)	**Interval**	**Location**	**Target Species**
April	1 day sampling event	San Marcos River	Texas wild rice
April	Drift net, donated from bio-monitoring	Comal Springs	Peck's cave amphipod
May	1-2 day sampling event	Spring Lake and Eastern Spillway	San Marcos Salamanders
May	Retrieve lures	Comal Springs	Comal Springs riffle beetle
May	14 Consecutive days with traps check 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
May	1-day sampling event	San Marcos River	Texas wild rice
June	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
June	1 day sampling event	Comal Springs	Comal Springs salamander
June	Set lures	Western Shore	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod
July	14 Consecutive days with traps check 2-3 times a week	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
July	Collect lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod

Edward's Aquifer Species Collection Plan 2024			
Date (month)	**Interval**	**Location**	**Target Species**
July	4-day sampling event	Comal River, San Marcos River, Landa Lake	Fountain darters
August	Set lures	Western Shore	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod, Texas troglobitic water slater
August	14 Consecutive days with traps check 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
August	1-2 day sampling event	Spring Lake and below dam	San Marcos salamander
September	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
September	1 day sampling event, hand pick	Landa Lake	Peck's Cave amphipod
September	1 day sampling event	Comal Springs	Comal Springs salamander
September	Collect lures	Western Shore	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod
October	14 Consecutive days with traps checked 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	Peck's cave amphipod
October	1 day sampling event	San Marcos River	Texas wild rice

Edward's Aquifer Species Collection Plan 2024			
Date (month)	**Interval**	**Location**	**Target Species**
October	1 day sampling event, hand pick from downed wood	Spring Runs, Landa Lake	Comal Springs dryopid beetle
November	14 Consecutive days with traps checked 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
November	1 day sampling event, hand pick	Landa Lake	Peck's cave amphipod
November	1 day sampling event	Comal Springs	Comal Springs salamander
November	Set lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod
December	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
December	1 day sampling event	San Marcos River	Texas wild rice
December	Collect lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod
December	4-day sampling event	Comal River, San Marcos River, Landa Lake	Fountain darters

Refugia Stocks:

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

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

Propagation: Propagation for stocking is not anticipated during 2024.

Salvage Stocks:

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

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

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

Construction/Renovation/Infrastructure/Facility:

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

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

Staffing/Labor/Personnel:

The two Program Leads (Research and Husbandry/Collections) will mentor and train lower-graded employees, oversee facility maintenance and repair, develop, and implement budgets, and organize activities that relate to all contract activities. The program leads will manage and coordinate research, propagation, culture, and field activities related to the refugia. The leads are

expected to provide proper and efficient use of facilities and staff resources. These leads will work with the Center Director and the Deputy Director to ensure that contractual obligations are met in a timely manner. In coordination with the Deputy Center Director, the EARP team will prepare all the written materials required for the reimbursable agreement reporting. Likewise, the EARP team will prepare oral presentations to be used as briefing statements, outreach presentations, internal reports, work summaries, and technical presentations at professional meetings. The two leads will continue to work and communicate regularly with partners, USFWS personnel and other researchers to meet USFWS and contract goals.

Under the direction of the Program Leads, biologists and biological science technicians, split between SMARC and UNFH, will assist with the collection, daily upkeep, maintenance, propagation, and research efforts for the ten species at the SMARC and UNFH. This includes maintaining culture and experimental production systems, keeping records along with entering, filing, and collating data. The biologists and 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 SMARC and UNFH operate under the USFWS Southwest Region's Federal Fish and Wildlife Permit for Native, Endangered, and Threatened Species Recovery (number TE676811-0) and the Texas Parks and Wildlife Scientific Research Permits (UNFH SPR-0822-106, SMARC SPR-0622-090).

Biosecurity:

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

Husbandry Pilot Studies:

Mark/Recapture of Texas blind salamanders – Between 2021 - 2023, Texas blind salamanders marked via tail clips were recaptured in the same sampling year. Tail clipping provides information on if a salamander has been previously observed in the wild, but without unique tags, it is impossible to determine if a single salamander is continuously being recaptured or if the refugia recaptures multiple different individuals. A portion of salamanders are collected for the refugia at any one collected event so that refugia collections do not detrimentally harm the wild population. Better understanding how often the Refugia encounters the same individuals during collection events informs refugia collections by assessing the potential impacts of removing individuals from specific locations. The refugia plans to continue to uniquely mark wild caught Texas blind salamanders collected at Primer's and Johnson's Wells using p-Chips. The tagged salamanders will be released and scanned when recaptured during routine sampling

events. In 2023, p-chipped salamanders were recaptures in the same location in sequential collection events. Ultimately, this information will allow the Refugia to reassess take limits and impacts of take at Primer's and Johnson's Well.

Offspring separation strategies for Peck's cave amphipod – Cannibalism is common in Peck's cave amphipods. Maternal cannibalism of offspring remains the largest limitation for reliable captive propagation of Peck's cave amphipods. In 2023, the Refugia conducted a pilot study testing separation housing that allowed offspring to be physically separate from adults. The Refugia will continue to experiment with different offspring exclusion strategies that separate offspring from brooding females and allow for brooding females to be transferred from general housing to a brooking chamber without harm and with minimal stress.

Seasonal collections of San Marcos and Comal Springs fountain darters – Survival rates of collected fountain darts have ranged from 0% to 100% with anecdotal evidence suggesting a seasonal impact on survival. Necropsy of fountain darter mortalities have revealed parasites and varying parasite loads previously unreported in fish health reports. The EARP will collect Comal Springs and San Marcos fountain darters in four collections on a seasonal basis and observe survival rates. Mortalities will be necropsied to investigate parasite load and to see if there is a correlation to parasite load and seasonal collections. This Collections study may inform when fountain darters should be collected while maintaining high survivorship. Additionally, parasite load information can inform potential treatment options while in quarantine to increase survivorship.

Task 2. Research

The Research Plan for 2024 will be a continuation of 2023 research projects. Partnered research projects started in 2023 were planned as two-year projects. Due to the supersaturation event in 2023, research was delayed to prioritize refugia standing stock collections. Final analysis and reporting of FWS lead research will continue into 2024. Planned research is a series of projects designed to improve propagation of captive populations, genetic assessment of wild populations, and improvements to reintroduction plans. To inform refugia collections and reintroduction plans, the EARP will continue 2023 research on a population genetic analysis of Comal Springs riffle beetle and Peck's cave amphipod. Building on 2023 mark recapture research on the San Marcos salamander, a genetic assessment of Texas blind and San Marcos salamanders will be conducted. Collaborative research will focus efforts on further improving dryopid beetle propagation, and the continuation of evaluating tagging techniques for EAHCP covered invertebrate species (i.e., PCAs and CSRBs) for the purpose of tracking individual survival and propagation in the refugia.

The total cost for proposed 2024 research is approximately $882,779. The following section describes the basic components of each of these proposed 2024 activities.

Table 6. Updated table showing the level of knowledge for each covered species. Knowledge score is a gradient from 0 to 5, where 0 is complete lack of knowledge and 5 indicates the

existence of documented procedures for that species. Species with knowledge scores of 5 in each category indicate the species is in complete refugia.

Species	Collection	Husbandry	Propagation	Genetics	Reintroduction
Fountain darter	5	4	5	4	4
Texas wild rice	5	5	5	5	5
Texas blind salamander	4	5	4	3	1
Peck's cave amphipod	4	4	3	2	1
San Marcos salamander	5	4	3	3	2
Comal Springs salamander	5	4	3	3	1
Comal Springs riffle beetle	5	4	4	4	3
Comal Springs dryopid beetle	3	2	2	0	1
Texas troglobitic water slater	1	1	0	1	1
Edwards Aquifer diving beetle	1	0	0	0	1

Project 1:

Title: Dryopid Beetle Captive Propagation
Species: *Stygoparnus comalensis*
Principal: BIO-WEST
Overview: Comal Springs dryopid beetles have long-life stages with long durations between hatching to pupation and pupation to eclosion. Previous research investigated the number of instar stages of dryopid larvae, oviposit location, and pupation success in captive holding. This proposed research builds on the previous, more exploratory, research to precisely identify instar stages and pupation rates. Environmental measurements and observations of locations with dryopid beetles will be collected and assessed to inform required refugia conditions for successfully holding and propagating dryopid beetles.
Budget: Two-year study

- BIO-WEST support: Year 1 rollover used in year 2: 52,800, **Year 2:** $72,200, Total $125,000
- FWS support: $10,000
- Total year 2: $135,000

Benefit to the Refugia: Successful captive holding and propagation is key for a functional captive assurance population. This research will gather additional knowledge on preferred wild habitat conditions to inform refugia conditions and encourage propagation in a captive setting.
Expected Results: A final report will be presented to the EAA and a peer-reviewed publication will be generated, if appropriate.

Project 2:

Title: Genetic Assessment of Wild Peck's Cave Amphipod
Species: *Stygobromus pecki*
Principal/Co-PI: Texas State University / USFWS
Overview: The refugia can reliably collect, house, and propagate Peck's cave amphipod, but little is known about their genetic diversity or population structure. This study will

assess the genetic diversity of Peck's cave amphipod in the wild and the refugia populations. This will be a two-year project where tissues are collected, DNA extracted, and methods optimized the first year. The second year will be sequencing and data analysis.

Budget: Two-year study

- Texas State Support: Year 1 rollover used in year 2: $31,074, **Year 2: $**96,380, Total: $127,454
- FWS Support: $10,000
- Total: $137,454

Benefit to the Refugia: This study will assess the population structure and genetic diversity of wild Peck's cave amphipod. This study will also determine how well the captive refugia population reflects the wild population and will inform the reintroduction plan.

Expected Results: A final report will be presented to the EAA and a peer-reviewed publication will be generated, if appropriate.

Project 3:

Title: Reproductive Triggers of San Marcos Salamander Using Gene Expression Profiles

Species: *Eurycea nana*

Principal/Co-PI: University of Texas

Overview: Successful reproduction is contingent on a number of environmental cues (e.g., circadian rhythm, change in seasonal temperature, etc.) perceived by an organism's sensory organs (eyes—phototransduction; olfactory bulb—chemosensory; skin—temperature), and are part of the initial signaling that indicates the ideal reproduction periods. The consistent conditions of the Edwards-Trinity Aquifer (e.g., temperature, pH, and ambient light), and the aquifer's associated outflows, make determining breeding cues for the *Eurycea* species difficult, which makes consistent and reliable captive breeding difficult. Despite previous Refugia research attempting to trigger courtship and reproduction in *Eurycea* species, reproduction is still not reliable or predictable. This proposed research will use gene expression profiles to identify biological mechanisms associated with reproductive state and susceptibility. The goal is to identify when salamanders are ready to reproduce and identify potential conditions required to trigger reproductive events.

Budget: Two-year study

- **University of Texas Support:** Year 1 Rollover used in year 2: $43,745, Year 2: $112,720, Total $156,465
- **FWS Support:** $10,000
- **Total:** $166,465

Benefit to the Refugia: Assess the optimal timing for captive propagation of San Marcos salamanders and identify potential reproduction triggers to inform further research.

Expected Results: A final report will be presented to the EAA and a peer-reviewed publication will be generated, if appropriate.

Project 4:

Title: Tagging Aquatic Invertebrates
Species: *Microcylloepus pusillus or Heterelmis vulnerata* (surrogate for *Heterelmis comalensis*) and Peck's cave amphipod
Principle/Co PI: Auburn University / USFWS
Overview: The Refugia uses tags to individually identify the salamanders collected from different locations or dates so they can be housed in the same tank while retaining their specific collection information. Maximizing Refugia space through this approach guarantees sufficient refugia space is available for the minimum Refugia Stand and Salvage Stock numbers of all covered Refugia species. Tagging is straightforward for larger species, such as the salamanders and fountain darters, but tagging the aquatic invertebrates is challenging. They are significantly smaller than most available tags (e.g., PIT), making these tags unsuitable. The recent p-Chip tagging study was very successful in salamanders, and the p-Chip's very small size makes it a promising tagging strategy for aquatic invertebrates. This study aims to assess p-Chip tagging efficacy in Peck's cave amphipod and Comal Springs riffle beetle through internal implantation and external attachment, respectively.
Budget: Two-year study

- Auburn University Support: Year 1 rollover used in year 2: $37,590, Year 2: $52,080, Total: $89,670
- FWS Support: $10,000
- Total: $99,670

Benefit to the Refugia: Individually tracking aquatic invertebrates would allow specific survival data to be collected and correlated to collection date, location, method, etc. Additionally, individuals collected at different times and locations could be pooled together in the same housing, maximizing Refugia space available for Refugia and Salvage stock. For PCA, specifically, once tagged, individuals of the same size can be housed together to reduce cannibalism.
Expected Results: A final report will be presented to the EAA and a peer-reviewed publication will be presented to the EAA and a peer review publication

Project 5:

Title: Continuation of Mark Recapture of wild San Marcos Salamanders
Species: *Eurycea nana*
Principal/Co-PI: USFWS
Overview: A successful reintroduction requires individuals to survive after reintroduction. To determine if individuals survive reintroduction events, the same individuals need to be recaptured through repeated surveys. To fully assess reintroduction success, a mark recapture study must occur first to determine baseline expectation for recapture rates of uniquely identified individuals occurring in the wild. Once this baseline expectation is determined, future reintroduction success rates can be more accurately measured. This research will inform the future reintroduction strategies by assessing how

often individuals are recaptured after being marked. Additionally, this research will inform how often salamanders stay in the same location or move between locations, helping the Refugia determine key locations that will increase successful reintroduction of San Marcos salamanders, in the event reintroduction is necessary.

Budget:

- USFWS salary and materials:$42,500
- Student Conservation Association Intern: $15,000
- Total: $57,500

Benefit to the Refugia: Inform reintroduction plans and add to the knowledge matrix

Expected Results: A final report will be presented to the EAA and a peer-reviewed publication will be generated, if appropriate

Project 6:

Title: Continuation of Genetic Assessment of Comal Springs Riffle Beetle

Species: *Heterelmis comalensis*

Principal: USFWS

Overview: A population wide assessment through fine sampling can provide population metrics to inform future conservation and refugia needs. FWS will work to collect Comal Springs riffle beetles across their range. FWS staff will use high-throughput sequencing to make population measurements at the genetic level.

Budget: $42,500

Benefit to the Refugia: A genetic assessment of the Comal Springs riffle beetle population in the Comal Springs System will provide valuable information on genetic variation and distribution of that variation in the wild. We do not yet know the extent individuals move between spring openings, thus genetic exchange (migration). Unique variation at specific spring openings would require different levels of representation in the refugia to reflect wild populations. Better understanding the variation in the wild would inform the minimum number of individuals needed in refugia to maintain wild variation in captivity.

Expected Results: A final report will be presented to the EAA and a peer-reviewed publication will be generated, if appropriate.

Project 7:

Title: Genetic Assessment of Texas Blind Salamanders

Species: *Eurycea rathbuni*

Principal: USFWS

Overview: A fully functioning captive assurance population is representative of the wild population and reflects the genetic diversity and unique genotypes found in the wild. Additionally, captive propagation efforts should take into account the genetics of captive held individuals to maintain genetic diversity in the refugia to ensure captive propagation efforts do not result in a reduction in diversity of Fx progeny. This work builds on a genetic assessment of wild Texas blind salamanders (Chippendale 2009) Tail clips will be collected from standing stock and captive propagated salamanders in the refugia. All refugia salamanders will be uniquely tagged with p-chips so that individual genetic profiles can be generated and tracked. High-throughput sequencing will be used to assess

genetic variation of wild caught and Fx captive breed Texas blind salamanders.
Budget: $42,500
Benefit to the Refugia: A genetic assessment of Texas blind salamanders will determine if the the Refugia individuals are reflective of the wild population, provide individual genetic profiles (genotypes) to current Refugia standing stock, and inform captive breeding strategies if reintroduction of Fx were needed.
Expected Results: A report will be presented to the EAA and a peer-reviewed publication will be generated, if appropriate.

Project 8:

Title: Genetic Assessment of San Marcos Salamanders
Species: *Eurycea nana*
Principal: USFWS
Overview: A fully functioning captive assurance population is representative of the wild population and reflects the genetic diversity and unique genotypes found in the wild. Additionally, captive propagation efforts should take into account the genetics of captive held individuals to maintain genetic diversity in the refugia to ensure captive propagation efforts do not result in a reduction in diversity of Fx progeny. Tail clips were collected from wild San Marcos salamanders during the 2023 Mark Recapture tagging study. These tail clips will be used to assess wild genetic diversity. Tail clips will be collected from standing stock and captive propagated salamanders in the refugia. All refugia salamanders will be uniquely tagged with p-chips so that individual genetic profiles can be generated and tracked. High-throughput sequencing will be used to assess genetic variation of wild caught and Fx captive breed Texas blind salamanders.
Budget: $42,500
Benefit to the Refugia: A genetic assessment of San Marcos salamanders will determine if the standing stock in the Refugia are reflective of the wild population, provide individual genetic IDs (genotypes) to current Refugia standing stock, and inform captive breeding strategies if reintroduction of Fx were needed.
Expected Results: A report will be presented to the EAA and a peer-reviewed publication will be generated, if appropriate.

Task 3. Species Propagation and Husbandry

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

Task 4. Species Reintroduction

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

Reintroduction Plan for 2024: None

Any anticipated triggers being prepared for: Given current weather predictions, spring flows, and the Edwards Aquifer water level, no anticipated triggers are anticipated during the 2024 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, Texas blind salamander, San Marcos salamander, Peck's cave amphipod; contingent on when genetic study results are finished.
5.3 Species specific reintroduction plans: Refine as needed
5.4 2024 EAHCP Annual Program reporting– A year-end report of 2024 activities will be provided to the EAA no later than 1/31/2025.
5.5 Program reporting as required by ITP and TPWD. TPWD Scientific Research Permit Report will be filed July 31, 2024.
5.6 Descriptions and photographs of procedures from collections to restocking – Photographs and documentation of collection and restocking will be included in the monthly report to the EAA CSO along with the year-end report.
5.7 Summaries of any data analyses, research, or genetic analyses – Research projects and results of collection efforts will be provided to the EAA in the monthly reports, year-end documentation, and stand-alone documents (agreed upon by Center Director and HCP CSO).
5.8 Description of terms and conditions of any permits received – As permits are received, their contents will be conveyed to the EAA.
5.9 Monthly electronic reports to HCP CSO: A monthly report of all activities will be provided to the HCP CSO. We anticipate providing the report by the 10th of each month for the previous month's activities.

Task 6. Meetings and Presentations

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

- Professional Scientific Meetings

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

Budget:

U.S. Fish and Wildlife Service 2024		Task Budget Amount	Total Task Budget Amount
TASK 1	**Refugia Operations**		**$893,213.16**
	SMARC Refugia & Quarantine Bldgs.		
	Equipment & Building Maintenance	$20,000	
	Utilities	$8,000	
	UNFH Refugia & Quarantine Bldgs.		
	Equipment & Building Maintenance	$20,000	
	Utilities	$30,000	
	SMARC Species Husbandry and Collection Salaries	$190,000	
	UNFH Species Husbandry and Collection Salaries	$320,000	
	Water Quality System	$5,000	
	Divers Salaries	$5,000	
	2017 Rollover Funds	-$23,858.84	
	Fish Health	$8,000	
	SMARC Reimbursable	$100,000	
	UNFH Reimbursable	$50,000	
	Subtotal	*$732,141.16*	
	Admin Cost Subtotal	*$$161,072.00*	
TASK 2	**Research**		**$882,779**
	BIO-WEST: Dryopid	$125,000	
	Texas State University: PCA Genetics	$127,454	
	University of Texas: Salamander Gene Expression	$156,465	
	Auburn University: Invertebrate Tagging	$89,670	
	Student Conservation Association Intern (Salamander Tagging Study)	$15,000	

U.S. Fish and Wildlife Service 2024		Task Budget Amount	Total Task Budget Amount
	FWS Salary	$180,000	
	FWS Materials	$30,000	
	Subtotal	*$723,589*	
	Admin costs for Task 2	*$159,190*	
TASK 3	**Species Propagation and Husbandry**		$0
	Subtotal		
TASK 4	**Species Reintroduction**		$0
	Subtotal		
TASK 5	**Reporting**		$86,230
	SMARC Staff	$53,775	
	UNFH Staff	$$16,955	
	Subtotal	*$70,730*	
	Admin costs for Task 5	*$15,500*	
TASK 6	**Meetings and Presentations**		$22,120
	SMARC Staff	$11,000	
	UNFH Staff	$7,131	
	Subtotal	*$18,131*	
	Admin costs for Task 6	*$3,989*	
	TOTAL	**$1,884,342.16**	

*Agreement with Texas State is pending.

Projected (2024) Budget Summarized by Task:

Task 1: $893,213.16
Task 2: $882,779
Task 3: $0
Task 4: $0
Task 5: $86,230
Task 6: $22,120

Projected (2024) Subcontractor Expenses Summarized by Task

Task 1: $0
Task 2: BIO-WEST ($125,000)
Task 2: Texas State ($127,454)
Task 2: University of Texas ($156,465)
Task 2: USGS Auburn University Co-op ($89,670)
Task 2: Student Conservation Association Intern ($15,000)
Task 3: $0
Task 4: $0
Task 5: $0
Task 6: $0

Timeline of 2024 Milestones

January	Subcontracted research awards executed
	2024 Specific Research Study Plans finalized
July	Submit and renew TPWD permit
November	Draft Research Reports
December	Draft Annual report

Literature Cited

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Appendix B | **Mark and recapture of San Marcos salamanders** (Final Report)

Mark Recapture of Wild San Marcos Salamanders (*Eurycea nana*)

2024 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Desiree M. Moore and Dr. Katie Bockrath

San Marcos Aquatic Resources Center

U.S. Fish and Wildlife Service

Table of Contents

Table of Contents **1**
Background **2**
Objectives **2**
Benefits to the Refugia **3**
Methods **3**

Pilot Study 3
Field Study 3
Modeling 7

Results **8**
Discussion **14**
References **17**

Background

A fully functioning refugia program must be able to successfully reintroduce individuals produced from the captive-assurance population to the wild in the case of a catastrophic event. A successful reintroduction requires the reintroduced individuals to survive in the wild after release. Mark-recapture studies are commonly used to determine if individuals are still present in the wild after reintroduction (Canessa et al. 2016). However, the number of recaptures that should be expected is unknown without a baseline study to show recapture rates of tagged salamanders.

Mark-recapture studies can be used to assess how long reintroduced salamanders persist in the wild after reintroduction and determine the best size or stage at which salamanders should be released in the wild. Repeated sampling following reintroduction is important to confirm survival of reintroduced individuals. If all released individuals are tagged, the duration of their presence can be determined. Additionally, if the survivorship of released individuals is determined prior to a catastrophic event, the minimum number of salamanders needed for reintroduction may be estimated.

Additionally, a fully functioning refugia program needs to have a well-informed estimate of minimum number of individuals to maintain in captivity to represent the wild population. Having an estimate of wild population size informs minimum captive holding numbers. Mark-recapture studies provide can provide good estimates of population size (Koivuniemi et al. 2019).

This research will inform the San Marcos salamander captive holding goals and reintroduction plan by estimating wild population sizes and assessing how often individuals are recaptured after being tagged in the wild. Additionally, this research will inform movement patterns of San Marcos salamanders, which can inform reintroduction location and strategies. This study can provide the first step to examine the success of San Marcos salamander reintroduction in case it becomes necessary.

Objectives

Our objective is to 1) examine the recapture rates associated with wild San Marcos salamanders tagged with p-Chip microtransponder tags, 2) assess how collection methods and conditions impact the number of salamanders captured, and 3) estimate population size.

Benefits to the Refugia

This research will inform the San Marcos salamander captive holding goals and reintroduction plan by estimating wild population size, determining key locations to release San Marcos salamanders back into the wild and expected recapture rates to potentially examine reintroduction success in the future.

Methods

Pilot Study

A pilot study in the laboratory was conducted to ensure San Marcos salamanders were able to survive with and retain p-Chip tags. Although p-Chips were associated with high survival and retention in other salamander species at the SMARC, it was prudent to be certain there would not be any negative effects for San Marcos salamanders before tagging wild individuals. Therefore, SMARC staff tagged 23 F1 San Marcos salamanders and compared survival to 16 control salamanders. Salamanders ranged 26-34 mm SVL. Salamanders were tagged using methods established at the SMARC (Moore and Bockrath, unpublished data). The salamanders were monitored daily for mortality and scanned weekly for tag retention. As a result of a supersaturation event in early 2023, two tagged and two control salamanders perished. These mortalities were not considered to be related to tagging due to the circumstances. Additionally, one tagged salamander mortality was recorded on day 53 of the pilot study. No tag loss was recorded during the pilot study. SMARC staff determined tagging wild salamanders was acceptable due to the low mortality and high retention rates. A size limit of 20 mm SVL was selected based on the results of this pilot study and the tagger's ability to tag salamanders of that size without slowing the process.

Field Study

San Marcos salamanders were collected from three sites across Spring Lake and the headwaters of the San Marcos River. The three sites were near the Meadows Center (Hotel), surrounding the diversion pipe (Diversion), and in the San Marcos River just below the eastern spillway (Eastern Spillway). Divers collected salamanders for tagging from the floor of Spring Lake at the Diversion site once monthly in May and June 2023. SMARC

staff snorkeled to collect salamanders for tagging from the Hotel and Eastern Spillway sites twice each in May and once each in June. Additionally, divers joined snorkelers to collect salamanders for tagging at the deeper areas of the Hotel site at the first May collection and the June collection (Table 1). A tagging station was set up on the bank near each site.

Table 1. The number of tagged and recaptured San Marcos salamanders from each site each field day of the San Marcos salamander mark-recapture study. The number of untagged salamanders that were collected and released without tagging due to size restrictions or because tagging was completed is also reported. The presence of divers is reported, where no indicates only snorkelers conducted collections and yes indicated divers conducted collections in place of or in addition to snorkelers.

Date	Site	# tagged	# recaptured	# untagged	total catch	divers
9-May-23	eastern spillway	82	0	5	87	no
10-May-23	diversion area	33	0	0	33	yes
11-May-23	hotel area	53	0	8	61	yes
30-May-23	eastern spillway	53	0	16	69	no
31-May-23	hotel area	22	0	0	22	no
12-Jun-23	eastern spillway	75	6	20	101	no
14-Jun-23	hotel area	74	6	25	105	yes
20-Jun-23	diversion area	62	2	8	72	yes
26-Jun-23	hotel area	0	9	21	30	no
27-Jun-23	eastern spillway	0	4	90	94	no
10-Jul-23	hotel area	0	3	19	22	yes
12-Jul-23	diversion area	0	2	78	80	yes
13-Jul-23	eastern spillway	0	4	53	57	no
8-Aug-23	eastern spillway	0	2	95	97	no
10-Aug-23	hotel area	0	3	54	57	yes
22-Aug-23	hotel area	0	1	101	102	no
24-Aug-23	eastern spillway	0	0	108	108	no
6-Sep-23	diversion area	0	5	79	84	yes
13-Sep-23	hotel area	0	3	23	26	no
14-Sep-23	eastern spillway	0	1	59	60	no
25-Sep-23	hotel area	0	0	51	51	no
27-Sep-23	eastern spillway	0	1	94	95	no
10-Oct-23	eastern spillway	0	3	145	148	no
11-Oct-23	diversion area	0	5	87	92	yes
12-Oct-23	hotel area	0	1	43	44	no
23-Oct-23	hotel area	0	0	60	60	no
24-Oct-23	eastern spillway	0	1	104	105	no
8-Nov-23	diversion area	0	4	95	99	yes
14-Nov-23	eastern spillway	0	2	90	92	no

16-Nov-23	hotel area	0	0	14	14	no
11-Dec-23	hotel area	0	0	8	8	no
12-Dec-23	eastern spillway	0	0	66	66	no
13-Dec-23	diversion area	0	5	84	89	yes
3-Jan-24	hotel area	0	0	7	7	no
23-Jan-24	diversion area	0	2	55	57	yes
7-Feb-24	hotel area	0	0	75	75	yes
14-Feb-24	diversion area	0	1	98	99	yes
27-Feb-24	eastern spillway	0	0	82	82	no
29-Feb-24	hotel area	0	0	13	13	no
12-Mar-24	eastern spillway	0	0	39	39	no
13-Mar-24	diversion area	0	2	51	53	yes
14-Mar-24	hotel area	0	1	77	78	yes
14-Mar-24	crater bottom	0	0	3	3	yes
14-Mar-24	salt and pepper 1	0	0	1	1	yes
14-Mar-24	salt and pepper 2	0	0	0	0	yes
14-Mar-24	cabomba	0	0	0	0	yes
25-Mar-24	hotel area	0	0	29	29	no
26-Mar-24	eastern spillway	0	0	19	19	no
9-Apr-24	hotel area	0	0	86	86	yes
10-Apr-24	diversion area	0	1	100	101	yes
11-Apr-24	eastern spillway	0	1	80	81	no
23-Apr-24	eastern spillway	0	0	42	42	no
24-Apr-24	hotel area	0	0	36	36	no
6-May-24	hotel area	0	0	42	42	no
8-May-24	riverbed	0	0	33	33	yes
8-May-24	cream of Wheat	0	0	15	15	yes
8-May-24	ossified Forest	0	0	6	6	yes
8-May-24	diversion area	0	0	49	49	yes
20-May-24	hotel area	0	0	189	189	yes
20-May-24	crater bottom	0	0	1	1	yes
20-May-24	salt and pepper 1	0	0	5	5	yes
20-May-24	salt and pepper 2	0	0	0	0	yes
20-May-24	cabomba	0	0	0	0	yes
21-May-24	eastern spillway	0	0	41	41	no
21-May-24	eastern spillway +	0	0	36	36	no

SMARC staff tagged wild San Marcos salamanders with p-Chips to individually identify the salamanders upon recapture. First, staff anesthetized salamanders by immersion in tricaine methanesulphonate (MS-222, 0.5 g/L) buffered with sodium bicarbonate. Staff then examined salamanders and rejected any with visible injuries to prevent harming them further through tagging. Each salamander was measured to obtain the snout-to-vent length (SVL), sexed if possible, and injected with a p-Chip subcutaneously at the base of the tail near the left hindlimb. Tail clips were also collected

from each salamander and will be used for eventual population genetic analyses. Salamanders were placed in a container of fresh river water to recover from anesthesia and tagging. Staff released the salamanders after they began swimming normally again. The divers and snorkelers returned the salamanders to the interstitial spaces among rocks in the general area where they were collected to provide cover for optimal healing and protection from predators.

San Marcos salamanders were sampled to determine recapture rate and movement patterns. Divers and snorkelers collected salamanders for recapture at Spring Lake and the Eastern Spillway similarly to the collections for tagging. Recapture collections occurred at the Diversion site once monthly after tagging was completed except in August, when divers were unavailable (Table 1). Recapture collections at the Hotel and Eastern Spillway site occurred twice monthly when staff were available and water levels allowed. A wider area was sampled at each site compared to during tagging, when possible, to create a buffer area around the initial tagging area to account for possible movement away from the tagging area. Due to staff availability, a wider collection area was not always possible. To sample the wider area, divers joined snorkelers to recapture at the deeper areas of the Hotel site on four occasions (Table 1). Additional sites surrounding each site were sampled at the final collections to attempt to examine salamander movement.

At each recapture event, collected salamanders were scanned for p-Chips by an experienced tag scanner, the number of tagged and untagged salamanders was recorded, and the amount of time spent searching for salamanders was recorded. All salamanders were released back to their capture location. Staff used these data to calculate the capture rates of tagged and untagged salamanders collected at each collection event and the movement distance between the capture and recapture locations of tagged individuals.

To analyze these data, SMARC staff developed summary statistics to analyze salamander recapture rates and sizes across sites. Only the three main sites (eastern spillway, diversion, and hotel) were used in these analyses due to a low number of visits to other sites and few salamanders collected. Because salamander collectors targeted salamanders ≥20mm SVL, size analyses were conducted twice, once with all salamanders collected and once with only salamanders ≥ 20mm SVL to ensure our results are not affected by this bias. A one-way ANOVA and post-hoc pairwise t-tests were used to determine differences in salamander size among sites. Net movement directionality was not examined due to no movements being recorded. The distribution of SVL values was

normal, but the data did not have equal variances. To account for the data not meeting the assumption of homogeneous variances, we used Welch's ANOVA.

SMARC staff used a chi-square test to determine if sex ratios varied across sites or differed from an equal sex ratio. Staff did not include salamanders whose sex was not able to be identified with certainty.

Modeling

A linear model was developed to estimate the total number of salamanders that would be collected given sampling conditions. Variables initially considered in modeling were the sampling method (snorkeling, SCUBA diving, or a combination of the two), manpower (calculated by multiplying the number of samplers by the number of minutes spent searching for salamanders), site (Hotel, Diversion, or Eastern Spillway), and season where December, January, and February were considered winter, March, April, and May were considered spring, June, July, and August were considered summer, and September, October, and November were considered autumn. However, the site was correlated with sampling method and manpower and could not be used in modeling. Models with all possible combinations of the remaining variables were fit and ranked using Aikake's Information Criterion adjusted for small sample sizes (AICc; Sugiura 1978). The continuous variable (manpower) was standardized to a mean of 0 and standard deviation of 1, and all assumptions were met for the modeling process.

Similarly, a linear model was developed to estimate the number of tagged salamanders that would be recaptured given sampling conditions. The variables initially considered in modeling were the sampling method, manpower, site, season, weeks since tagging, number of tagged salamanders at that site, and total catch of salamanders for that day. For the same reason as above, site could not be used in modeling. Models with all possible combinations of the remaining variables were fit and ranked using AICc. The continuous variables (manpower, weeks since tagging, number of tagged salamanders, and total captured) were standardized to a mean of 0 and standard deviation of 1, and all assumptions were met for the modeling process. All modeling was conducted using the program R version 4.3.3 with the packages ggplot2 (Wickham 2016), car (Fox and Weisberg 2019), and MuMIn (Bartoń 2024).

RMARK was used to estimate population size at Hotel Springs, Diversion Springs and Eastern Spillway. The other sites were eliminated from consideration due to the low number of recapture efforts. A range of models were defined and tested to identify the best fit values for the parameters of Φ, p, p_{ent} and N. Φ is the probably of an individual surviving from one sampling event to the next, p is the probably of detection, p_{ent} is the probably of an individual entering the system, and N is the number of uncounted individuals in the inferred superpopulation. These three parameters were allowed to vary or remain constant across sampling events and locations. All possible models were ranked using Akaike Information Criterion (AICc), and the top ranked model parameters were used to run a final MARK model to estimate population size and probably of survival at each sampling location.

Results

A total of 453 San Marcos salamanders were tagged across sites, with 46% tagged at Eastern Spillway, 33% tagged at Hotel, and 21% tagged at Diversion (Table 2). The recapture rate across sites was 14%, with the highest rate occurring at the Diversion site (21%). The recapture rate at Hotel was 15%, and the lowest recapture rate was 10% at the Eastern Spillway (Table 2). Fifteen of the tagged salamanders were recaptured twice, eight at Diversion, four at Eastern Spillway, and four at Hotel. Additionally, one salamander was recaptured four times at Diversion. All 16 of these salamanders were recaptured at the site where originally marked.

Table 2. The snout-vent lengths (SVL) of tagged and recaptured individuals, where the mean and standard deviation (Mean ± SD), minimum (Min), and maximum (Max) of the lengths are reported. The number of recaptures does not include multiple recaptures of the same individual.

	Tagged				Recaptures			
Site	#	Mean SVL (mm) ± SD	Min SVL (mm)	Max SVL (mm)	#	Mean SVL (mm) ± SD	Min SVL (mm)	Max SVL (mm)
Eastern spillway	209	28.7 ± 4.3	20	40	21	26.9 ± 4.3	21	33
Diversion area	96	27.2 ± 3.2	20	32	20	27.7 ± 2.6	22	31
Hotel area	148	27.0 ± 3.1	20	35	23	26.9 ± 3.9	20	35
Total	453	27.8 ± 3.8	20	40	64	27.1 ± 3.6	20	35

There were 3,469 San Marcos salamanders collected for this study. Collections varied by site and month (Figure 1). The average number of salamanders collected per collection event was 78 at Eastern Spillway, 76 at Diversion, and 53 at Hotel.

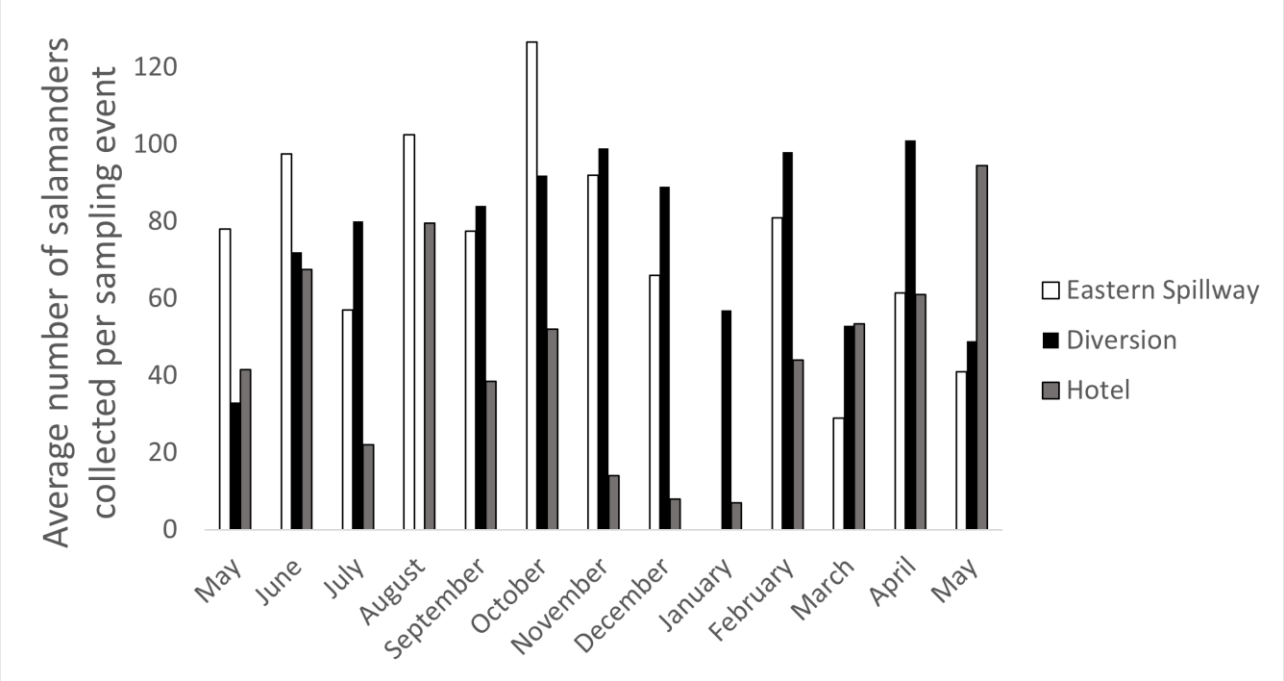

Figure 1. The average number of San Marcos salamanders collected each month at each site from May 2023 through May 2024. The total number of salamanders collected at each site each month was divided by the number of sampling events at that site over that month. No sampling events were conducted for the Diversion site August 2023 or the Eastern spillway site January 2024.

The lengths of all salamanders collected (Table 3) were different among sites ($F_{2, 1983}$ = 127.77, P < 0.001), and post-hoc pairwise t-tests were used to determine that salamanders at the Eastern Spillway site were larger than salamanders at the Diversion (P = < 0.001) and Hotel (P = < 0.001) sites. The salamanders collected at the Diversion site were larger than those collected at the Hotel site (P < 0.001). These results were the same when we used only salamanders ≥20 mm SVL.

Table 3. The snout-vent lengths of all salamanders ≥20 mm SVL collected for this project, where the mean and standard deviation (Mean ± SD), minimum (Min), and maximum (Max) of the lengths are reported. Additionally, the length data for all salamanders collected are reported. Salamanders <20 mm SVL are most likely underrepresented due to collection goals.

	Salamanders ≥20 mm SVL				All salamanders collected			
Site	#	Mean SVL (mm) ± SD	Min	Max	#	Mean SVL (mm) ± SD	Min	Max
Eastern spillway	1,324	28.2 ± 4.0	20	40	1,415	27.4 ± 4.9	8	40
Extended spillway	28	27.9 ± 4.3	20	35	36	25.3 ± 6.3	10	35
Diversion area	721	27.0 ± 3.6	20	36	855	25.2 ± 5.4	4	36
Cream of wheat	14	29.7 ± 3.1	27	39	15	28.4 ± 5.7	11	39
Ossified forest	6	27.7 ± 3.4	22	31	6	27.7 ± 3.4	22	31
Riverbed	35	29.5 ± 3.4	20	35	37	28.9 ± 4.2	18	35
Hotel area	853	26.6. ± 3.3	20	35	1,095	24.1 ± 5.4	8	35
Crater bottom	2	22.0 ± 2.8	20	24	4	16.0 ± 7.2	9	24
Salt and pepper 1	6	28.5 ± 3.2	24	32	6	28.5 ± 3.2	24	32
Total	2,989	27.4 ± 3.8	20	40	3,469	25.8 ± 5.5	4	40

Approximately 52% of collected salamanders could be sexed by staff (Table 4). Sex ratios were not significantly different among sites ($X^2 = 1.050$, $P = 0.592$). Additionally, sex ratios did not significantly differ from an equal sex ratio ($X^2 = 1.4376$, $P = 0.6967$).

Table 4. The number of salamanders identified as male, female, or unknown sex for each site. The percentage of salamanders of unknown sex is also reported. Sex ratios are reported as the number of identified males divided by the number of identified females.

	Male	Female	Unknown	% unknown	Sex ratio
Eastern spillway	398	383	634	45%	1.04
Diversion	230	227	398	47%	1.01
Hotel	269	235	591	54%	1.14
Total	897	845	1,623	48%	1.06

To estimate the total number of salamanders that would be collected given sampling conditions, seven models were ranked (Table 5). In the top model, San Marcos salamander catch increased with manpower (10.68; P = 0.025), and the combination of snorkeling and diving increased catch (61.35; $P = 0.013$). Conversely, snorkeling only decreased catch relative to diving only (-21.80; $P = 0.034$).

Table 5. Ranks of candidate linear models evaluating the number of San Marcos salamanders collected related to sampling conditions. For each model, Y is the number of salamanders collected, method is the collection method used (snorkeling, SCUBA diving, or a combination of the two), manpower is calculated by multiplying the number of samplers by the number of minutes spent searching for salamanders, and season is the season when the collection took place. Akaike's information criterion adjusted for small sample size (AICc) is reported. ΔAICc was calculated as the difference in AICc score between each model and the top model (bold).

Rank	Model	AICc	ΔAICc
1	**Y = manpower + method**	**521.70**	**0.00**
2	Y = method	524.88	3.18
3	Y = manpower + method + season	527.61	5.91
4	Y = method + season	530.65	8.95
5	Y = manpower	532.62	10.92
6	Y = manpower + season	539.10	17.40
7	Y = season	539.88	18.18

To estimate the number of tagged salamanders that would be recaptured given sampling conditions, 29 models were ranked (Table 6). In the top model, San Marcos salamander recaptures decreased with weeks since tagging (-1.22; $P = < 0.001$) and snorkeling decreased recaptures relative to diving (-1.07; $P = < 0.043$). However, combining snorkeling and diving had no effect on the number of recaptures (0.84; $P = 0.051$).

Table 6. Ranks of candidate linear models evaluating the number of tagged San Marcos salamanders recaptured related to sampling conditions. For each model, Y is the number of salamanders collected, method is the collection method used (snorkeling, SCUBA diving, or a combination of the two), manpower is calculated by multiplying the number of samplers by the number of minutes spent searching for salamanders, season is the season when the collection took place, weeks is the number of weeks since tagging, and total is the total number of salamanders captured. Akaike's information criterion adjusted for small sample size (AICc) is reported. ΔAICc was calculated as the difference in AICc score between each model and the top model (bold).

Rank	Model	AICc	ΔAICc
1	**Y = method + weeks**	**199.73**	**0.00**
2	Y = weeks	201.07	1.34
3	Y = method + weeks + total	201.11	1.39
4	Y = weeks + total	201.44	1.72
5	Y = manpower + method + weeks	202.32	2.59
6	Y = manpower + weeks + total	203.02	3.29
7	Y = manpower + weeks	203.22	3.49

8	Y = season + weeks	207.58	7.85
9	Y = method + season + weeks	207.82	8.10
10	Y = season + weeks + total	209.30	9.58
11	Y = manpower + season + weeks	210.43	10.70
12	Y = manpower + method + season + weeks	210.80	11.07
13	Y = method + season + weeks + total	210.95	11.23
14	Y = manpower + method + season + weeks + total	214.09	14.36
15	Y = season	214.76	15.03
16	Y = method + season	215.50	15.77
17	Y = season + total	216.76	17.04
18	Y = manpower + season	217.34	17.61
19	Y = manpower + method + season	217.65	17.92
20	Y = method + season + total	218.41	18.69
21	Y = manpower + season + total	219.54	19.81
22	Y = manpower + method + season + total	220.60	20.87
23	Y = total	230.10	30.38
24	Y = method	231.38	31.65
25	Y = manpower + total	232.41	32.68
26	Y = manpower	232.58	32.86
27	Y = method + total	232.84	33.12
28	Y = manpower + method	233.37	33.65
29	Y = manpower + method + total	235.25	35.52

To estimate the population size of San Marcos salamanders at Hotel Springs, Diversion Springs and Eastern Spillway, 54 models were estimated and ranked (Table 7). The top model was Φ(~site)p(~time)p_{ent}(~1)N(~site), where the probability of an individual surviving from one sampling event to the next varied by sampling location, the probably of detection varied by sampling event, the probably of an individual entering the population was constant and the estimated number of unmarked individuals in the population varied by site. A POPAN model was used to estimate population size because it incorporated estimates of unmarked individuals. Eastern Spillway is estimated to have the largest population size with 1,366 individuals while Diversion Spring has the smallest population at 354 individuals (Table 8).

Table 7. Ranks of candidate models incorporating Φ, p, p_{ent} and N where each were allowed to be constant or vary over sampling events and locations. Constant variables are noted by a value of ~1, variation across sampling events is noted by ~time and variation across sampling sites is noted by ~site. Npar is the number of estimated parameters. AICc is the Akaike Information Criterion corrected for small sample size. Delta AICc is the difference between the AIC score of the best model and the model being considered. Weight is the model's likelihood of being the best fit model.

Rank	model	npar	AICc	Delta AICc	weight

1	Φ(~site)p(~time)pent(~1)N(~site)	80	2274.45	0	9.43E-01
2	Φ(~site)p(~time)pent(~site)N(~site)	82	2280.062	5.611134	5.70E-02
3	Φ(~1)p(~time)pent(~1)N(~site)	78	2307.529	33.0787	6.19E-08
4	Φ(~1)p(~time)pent(~site)N(~site)	80	2313.09	38.6394	3.84E-09
5	Φ(~1)p(~time)pent(~1)N(~1)	76	2338.499	64.04897	1.17E-14
6	Φ(~site)p(~time)pent(~1)N(~1)	78	2342.281	67.8301	1.76E-15
7	Φ(~1)p(~time)pent(~site)N(~1)	78	2344.01	69.5599	0.00E+00
8	Φ(~site)p(~time)pent(~site)N(~1)	80	2347.841	73.3908	0.00E+00
9	Φ(~time)p(~1)pent(~1)N(~site)	77	2465.584	191.1335	0.00E+00
10	Φ(~time)p(~site)pent(~1)N(~site)	79	2470.449	195.9981	0.00E+00
11	Φ(~time)p(~1)pent(~site)N(~site)	79	2470.865	196.4142	0.00E+00
12	Φ(~time)p(~site)pent(~1)N(~1)	77	2473.683	199.2324	0.00E+00
13	Φ(~time)p(~site)pent(~site)N(~site)	81	2476.034	201.5839	0.00E+00
14	Φ(~time)p(~site)pent(~site)N(~1)	79	2479.219	204.7681	0.00E+00
15	Φ(~time)p(~time)pent(~1)N(~site)	149	2484.754	210.3035	0.00E+00
16	Φ(~time)p(~time)pent(~site)N(~site)	151	2492.632	218.1814	0.00E+00
17	Φ(~time)p(~1)pent(~1)N(~1)	75	2498.792	224.3417	0.00E+00
18	Φ(~time)p(~1)pent(~site)N(~1)	77	2504.278	229.828	0.00E+00
19	Φ(~site)p(~time)pent(~time)N(~site)	151	2510.593	236.1428	0.00E+00
20	Φ(~site)p(~1)pent(~1)N(~site)	8	2512.041	237.5904	0.00E+00
21	Φ(~site)p(~site)pent(~1)N(~site)	10	2516.171	241.7205	0.00E+00
22	Φ(~site)p(~1)pent(~site)N(~site)	10	2516.19	241.7397	0.00E+00
23	Φ(~site)p(~site)pent(~site)N(~site)	12	2520.353	245.9022	0.00E+00
24	Φ(~time)p(~time)pent(~1)N(~1)	147	2521.575	247.1249	0.00E+00
25	Φ(~time)p(~time)pent(~site)N(~1)	149	2529.371	254.9208	0.00E+00
26	Φ(~site)p(~site)pent(~1)N(~1)	8	2535.886	261.435	0.00E+00
27	Φ(~site)p(~site)pent(~site)N(~1)	10	2540.035	265.5843	0.00E+00
28	Φ(~1)p(~time)pent(~time)N(~site)	149	2541.354	266.9031	0.00E+00
29	Φ(~1)p(~1)pent(~1)N(~site)	6	2555.833	281.3826	0.00E+00
30	Φ(~1)p(~site)pent(~1)N(~site)	8	2559.933	285.482	0.00E+00
31	Φ(~1)p(~1)pent(~site)N(~site)	8	2559.95	285.4997	0.00E+00
32	Φ(~1)p(~site)pent(~1)N(~1)	6	2563.264	288.814	0.00E+00
33	Φ(~1)p(~site)pent(~site)N(~site)	10	2564.082	289.6313	0.00E+00
34	Φ(~1)p(~site)pent(~site)N(~1)	8	2567.382	292.9311	0.00E+00
35	Φ(~1)p(~time)pent(~time)N(~1)	147	2570.04	295.589	0.00E+00
36	Φ(~site)p(~time)pent(~time)N(~1)	149	2576.105	301.6544	0.00E+00
37	Φ(~1)p(~1)pent(~1)N(~1)	4	2589.221	314.7708	0.00E+00
38	Φ(~site)p(~1)pent(~1)N(~1)	6	2591.474	317.0237	0.00E+00
39	Φ(~1)p(~1)pent(~site)N(~1)	6	2593.307	318.8565	0.00E+00

40	Φ(~site)p(~1)pent(~site)N(~1)	8	2595.591	321.1408	0.00E+00
41	Φ(~time)p(~1)pent(~time)N(~site)	148	2651.479	377.0288	0.00E+00
42	Φ(~site)p(~1)pent(~time)N(~site)	79	2664.536	390.0857	0.00E+00
43	Φ(~time)p(~site)pent(~time)N(~1)	148	2667.916	393.4657	0.00E+00
44	Φ(~site)p(~site)pent(~time)N(~site)	81	2669.947	395.4962	0.00E+00
45	Φ(~time)p(~site)pent(~time)N(~site)	150	2673.786	399.3356	0.00E+00
46	Φ(~time)p(~1)pent(~time)N(~1)	146	2683.892	409.4419	0.00E+00
47	Φ(~site)p(~site)pent(~time)N(~1)	79	2686.78	412.3299	0.00E+00
48	Φ(~1)p(~1)pent(~time)N(~site)	77	2716.137	441.6867	0.00E+00
49	Φ(~1)p(~site)pent(~time)N(~site)	79	2721.657	447.2061	0.00E+00
50	Φ(~1)p(~site)pent(~time)N(~1)	77	2722.615	448.1647	0.00E+00
51	Φ(~1)p(~1)pent(~time)N(~1)	75	2747.981	473.5301	0.00E+00
52	Φ(~site)p(~1)pent(~time)N(~1)	77	2750.477	476.0263	0.00E+00
53	Φ(~time)p(~time)pent(~time)N(~site)	220	2831.789	557.3381	0.00E+00
54	Φ(~time)p(~time)pent(~time)N(~1)	218	2860.915	586.4642	0.00E+00

Table 8. Population size estimates using a POPAN model in RMARK. SE is standard error, LCL is lower confidence limit and UCL is upper confidence limit.

Location	Population Estimate	SE	LCL	UCL
Diversion Spring	353.93	55.20	266.08	486.12
Hotel Spring	753.02	112.97	568.80	1,017.50
Eastern Spillway	1,365.76	205.06	1,028.33	1,841.81

Discussion

The findings of this study are very informative for the San Marcos salamander refugia population at the SMARC, monitoring efforts, and a comprehensive reintroduction plan for the species. The results show a potential need for each of the three sites being represented separately in the refugia population, with the Eastern Spillway site having the highest number represented due to the largest population estimated there. A model was developed to inform monitoring efforts, and a model was developed to inform reintroduction planning of San Marcos salamander in the future.

Although salamander sex ratio was similar across sites, differences among sites were evident in salamander capture rates, recapture rates, and size differences. The Eastern Spillway site had the highest salamander capture rate, the highest number of

tagged salamanders, but the lowest recapture rate. These together could indicate a higher population of San Marcos salamanders at that site compared to the two others or that salamanders at this site are easier to capture, perhaps due to the shallow, wide nature of the site. Additionally, the salamanders were larger at the Eastern Spillway site. This could be due to the Eastern Spillway site resembling the historical salamander habitat (e.g., shallow, rocky, fast-flowing) more closely, temperature, nutrient availability, or some other site factor. Alternatively, larger salamanders at the deeper sites (Hotel and Diversion) might spend more time in the aquifer or evading capture some other way. However, salamanders at the Hotel site were also smaller than those at the Diversion site, indicating salamander size might increase in the downstream direction. More sites would need to be investigated to determine if this is a consistent relationship or something specific for these three sites.

No movement among sites was detected from recaptured salamanders, suggesting strong local site fidelity or very slow movement rates. After a year of recapture data, no salamanders were found to have moved from one site to another, but several salamanders were recaptured more than once at their tagging site. There were also salamanders that were recaptured at their tagging sites up to 11 months after tagging. These salamanders either stayed at their tagging site for those months or left and returned. Although it is common for movement studies to underestimate movement (i.e., it is easier to recapture something that did not move), it would be expected to see some evidence of movement during a study of this magnitude if movement was a common occurrence. An apparent lack of movement among subpopulations indicates the potential for significant spatial genetic structure, which will be examined in the paired genetic study using the tail clips preserved during tagging.

The model for total salamander catch is useful for informing monitoring and husbandry collections in the future. This model showed that including a component of diving, whether that is combined with snorkeling (optimal) or used exclusively, increased overall salamander catch. This could be a useful tool to include in monitoring or use to increase catch for meeting refugia stock numbers. The model itself could be used to determine if the salamanders collected during a monitoring effort is lower than expected. Additionally, the model could be used to estimate the amount of manpower needed to achieve a needed catch for the refugia.

Future reintroduction efforts can be assessed using the model for salamander

recapture catch. This model estimated the number of recaptured salamanders that one would expect given the collection method (snorkeling, diving, or both) and the time since tagging in weeks. This is very useful during a reintroduction effort where the reintroduced salamanders would be tagged prior to reintroduction and follow-up collections are conducted. During the subsequent collections, the method and time since tagging are entered into the model and the resulting recaptures are compared to the estimate given by the model. If a large portion of the reintroduced individuals do not survive to be recaptured, the actual recapture number would be much lower than the estimation given by the model. It would be beneficial to conduct a study of reintroduction success to inform the number of salamanders needed for a reintroduction in the case of catastrophe in the wild.

The population estimate is supported by observations in the field. The POPAN model estimated Eastern Spillway site as the most populous, which is supported by the salamander capture and recapture rates discussed above. These estimates at least show estimations of the population of salamanders that can be captured at each site. However, it is possible these are underestimating total population due to the relatively small number of individuals tagged with p-chips (N= 453) in comparison to the total number of salamanders collected for this study (N= 3,469). Additionally, the potential of salamanders to be unreachable in the aquifer spaces may have reduced our ability to recapture tagged salamanders. Despite the unknowns, these estimates can be used to determine the ratio of salamanders that should be held in the refugia from each of these sites to maintain a more representative captive population. More work, such as a genetic assessment of these populations, is needed to further refine refugia numbers needed and if other sites should be represented in the refugia population.

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Appendix C | **Comal Springs dryopid beetle research 2023-2024:** laboratory studies of habitat preferences and development of field methods for detection, collection, and monitoring (Final Report)

Comal Springs dryopid beetle (*Stygoparnus comalensis*) research 2023–2024: laboratory studies of habitat preferences and development of field methods for detection, collection, and monitoring

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Table of contents
To be added for final version

Executive summary
Understanding the life history of the Comal Springs dryopid beetle (*Stygoparnus comalensis*) is a key objective for the Edwards Aquifer Habitat Conservation Plan (EAHCP) refugia program. This study built upon our collective knowledge obtained for this endemic invertebrate species reported on by BIO-WEST in 2019 and 2022. The longevity and low rates of reproduction of this species in captivity, combined with the difficult in finding beetles for use in studies, suggested that we may be missing some fundamental understanding of the ecology of this species. There were two main objectives of this study: 1) experimentally assess habitat preferences in captive conditions and 2) develop methods that can be used for detecting, collecting, and monitoring *S. comalensis* in the wild.

Habitat choice experiments demonstrated that *S. comalensis* have a strong affinity for wood over other habitat types and have greater rates of consumption of wild-conditioned wood over captive-conditioned wood. There was no strong evidence that *S. comalensis* respond to water flow, but they are attracted to conspecifics and are more often found in groups than alone. Despite having reduced visual organs, *S. comalensis* avoided UV light, were seemingly attracted to red and white light, but did not respond to blue or green light.

Discs constructed out of conditioned wood found in Spring Lake were highly productive for finding and collecting *S. comalensis*, with 87 adults found in a six-month period, eclipsing the total ever found in 21 years of cotton lure biomonitoring. Variations on the structure of wood discs did not show that any variant was more effective than the other, so ultimately the most basic method – a wood disc 6–8 cm diameter, 2 cm thick – was effective and simple. Other than two *S. comalensis* found on wood stakes, none were found using small drift nets or cotton lures. For other species, paired study of wood discs with cotton lures further demonstrated wood discs were more effective at detecting the presence of the Comal Springs riffle beetle (*Heterelmis comalensis*) and equally effective at assessing their abundance as cotton lures.

These findings provide further evidence that *S. comalensis* is primarily a near-surface species that is potentially reliant on riparian vegetation to provide habitat and food. The wood disc method developed here is effective for finding and collecting beetles to support refugia populations. However, we still lake strong data-based knowledge of what contributes to the presence and abundance of *S. comalensis* in natural habitats, and we have largely been unable to find this species outside of the Spring Island area of Comal Springs. The use of wood discs should be explored for further longer-term studies of *S. comalensis* and investigations into the habitat characteristics that this species requires to maintain healthy populations. Currently, we do not know the population size of *S. comalensis*, and it would benefit from a population assessment to study its relative abundance in the Comal Springs system and potentially other sites where it occurs, particularly Fern Bank Springs. Findings of these potential studies could then inform longer-term studies or biological monitoring of *S. comalensis*, habitat restoration efforts, successful maintenance of captive populations, and reintroduction efforts if a catastrophic event were to occur.

Introduction

The Comal Springs dryopid beetle, *Stygoparnus comalensis* Barr and Spangler, 1992 (Coleoptera: Dryopidae) is a beetle known primarily from Comal Springs, Comal County, Texas, USA; it has also been collected from two springs in Hays County, Texas (Gibson et al. 2008). It is protected by the US Fish and Wildlife Service (USFWS) and has 22 hectares (ha) of designated critical habitat (United States Fish and Wildlife Service 1997, 2013). Like many other species in the Edwards Aquifer, *S*. *comalensis* faces numerous threats to its habitat including over-pumping of water, pollution, and potential effects of introduced exotic species (Bowles and Arsuffi 1993). A self-propagating captive refugia population of *S*. *comalensis* is a goal of the Edwards Aquifer Habitat Conservation Plan (EAHCP), and a better understanding of the habitat, ecology, and life history of this species is essential for meeting that goal.

Adults have been collected primarily from near-surface habitats, although their occurrence in drift nets and their vestigial eyes has led to the suggestion that they are a subsurface species (Barr and Spangler 1992; Gibson et al. 2008). Wild-caught adults have survived in captivity for as long as 21 months (Barr and Spangler 1992), and limited captive breeding efforts have indicated they have long larval stages that take over one year to reach the adult stage (BIO-WEST 2022). Studies of the lengths of the three immature life stages (egg, larva, pupa) have also shown low rates of egg laying by females, low rates of egg hatching, and even lower rates of pupation, with prior work producing only four adults from captive-laid eggs (BIO-WEST 2022).

Previous efforts to develop a system to maintain *S*. *comalensis* in captivity did not produce a setup that was notably better for this species than any other (BIO-WEST 2022). Field-based observations of this species have largely been anecdotal or coincidental (e.g., occurrence of *S*. *comalensis* near *Platanus* roots in Comal Springs). Clearly, more information on the habitats and biology of this species is needed, while assessment with rigorous experimental and statistical tests will help to better determine habitat preferences of *S*. *comalensis* in a variety of different conditions.

Project background

Work on this project in the San Marcos Aquatic Resources Center (SMARC) refugia and efforts to collect more individuals began in spring 2023 at which time the captive BIO-WEST population of *S*. *comalensis* consisted of fewer than 20 larvae and no adults. Because of the unknown origin and age of these mixed larvae, they had no utility for the original proposed objective of estimating larval growth. These larvae were allowed to remain in the original housing chamber, and their numbers dwindled over the following months as efforts focused on collecting new adults.

Prior observations that *S*. *comalensis* occurred on or around roots and on submerged wood served as the basis for collecting efforts. In addition to manually searching around existing wood, roots, and rocks in springs, conditioned wood (found submerged in locations away from spring opening in Landa Lake) was placed in or on springs at several dozen sites in Spring Runs 1, 2, and 3 during spring 2023; a more concentrated collecting effort was made around Spring Island. Each site was checked approximately weekly until the end of July 2023 when declining springflow left most of the sites in the spring runs dry at the surface. Given the scarcity of beetles in 2023 and the lack of records on locations of specific sites that were checked in previous studies (including the presence, absence, and/or abundance of beetles at those sites), field efforts in 2023 focused on gaining an understanding of the system while building the framework for potential future *in-situ* studies.

By early August 2023 fewer than five adults had been obtained and were alive at SMARC. The lack of adults, combined with the known low oviposition rate and long larval stage, meant that over the remaining timeframe of this project (just over 1 year), obtaining an adequate sample size of larval growth over the entire larval stage was unlikely. Therefore, research efforts under this project shifted away from the initial objective of understanding development across the lifecycle of *S*. *comalensis* towards determining responses to, and preferences for, various environmental conditions.

Collecting efforts continued throughout 2023, and from August 2023 through March 2024, zero to two adults were typically found each week. During this time, individuals collected were typically found at two sites north of Spring Island. A few dryopid larvae were collected during summer 2023 but none were observed after that time. Burrowing habits of larvae mean they are difficult to collect and their visual similarity to the many other larvae typically found on wood makes them difficult to coarsely id when quickly searching.

Adults were housed communally at SMARC during the time between the environmental choice experiments to allow opportunity for reproduction. What appear to be breeding attempts were observed when searching the housing chamber and sorting adults, but only a single egg was found and no larvae were produced. The egg was transferred to a separate housing container but after the egg disappeared, no larvae were found during monitoring over the following four months.

Following completion of environmental choice experiments in spring 2024, work shifted to the development of methods for detecting, collecting, and monitoring *S*. *comalensis* within the Comal Springs system. Testing of techniques and monitoring of sites was carried out from May through November 2024.

Part 1: Environmental choice experiments

To develop an understanding of the microhabitat preferences of *S. comalensis* and its responses to environmental conditions, a series of controlled laboratory experiments were conducted. In most of these experiments, adult *S. comalensis* were tested alongside adults and larvae of *Stenelmis sexlineata* Sanderson, 1938 (Coleoptera: Elmidae). *Stenelmis sexlineata* is an aquatic riffle beetle that reaches approximately the same size as adult dryopids and commonly co-occurs as both adults and larvae with *S. comalensis*, especially on wood on spring surfaces at sites near Spring Island where dryopids are common. Adults of both species are relatively similar in body size and morphology. However, while *S. sexlineata* has eyes and is a widespread species across the central United States (Schmude 1992), the populations in Comal and San Marcos springs are among the southernmost populations of this species and exhibit variation from their typical coloration. The inclusion of *S. sexlineata* primarily serves to provide a contrast to habitat preferences of dryopids since they are in different families and one has fully developed eyes while the other does not. If studies suggest similar habitat preferences among the two species, then *S. sexlineata* may serve as an imperfect surrogate to study habitat preferences with greater statistical replication in a non-threatened species.

The majority of the experiments were paired habitat choice experiments wherein beetles were held in an experimental chamber with one environmental condition at one side of the chamber and a different environmental condition at the opposite end (Fig. 1). After testing some initial designs, an experimental chamber was formed out of a plastic pasta container (30 cm long × 7.5 cm wide × 7.5 cm tall). This same general design was used for all but one of the experiments (leaf consumption). The design of these chambers had some slight modification depending on the purpose of the individual experiment and is noted for each study below. Generally, all sides of this chamber (including the lid) were blacked out to prevent any light from entering the chamber. Outflow holes were cut into each end of the chamber 1.5 cm below the chamber top and covered with fine mesh such that a filled chamber held ~1350 mL of water. The chambers were set up with a flow-through system using water from the Edwards Aquifer: water entered via a tube at the top center of each chamber at a rate of approximately 1.5 mL/s, flowed towards each end, and then out each side and was not recirculated (Fig. 1). The entirety of each experimental setup was covered in 6 mil (0.15 mm) thick black plastic to eliminate the influence of other light sources. The experiments were maintained indoors with climate-controlled conditions at SMARC.

In each experiment, as many aspects of the setup were randomized as possible, including treatment positions, beetle assignment to experimental chamber, chamber order, etc. The experimental chambers and most materials placed within the chambers were cleaned and dried between experiments to remove any debris, environmental cues, or any other buildup within the chambers; materials that were not cleaned (leaves, wood) were discarded between experiments. We aimed for a minimum replication of eight individuals of each species for each experiment, but this number varied by study based on the number of beetles we had at the time of the experiment. Multiple temporal rounds of the experiments were performed with new beetles if necessary to achieve greater replication. Data analysis was performed in R v. 4.4.1 (R Core Team 2024) using the lme4 v. 1.1-35.5 and lmerTest v. 3.1-3 packages (Bates et al. 2015; Kuznetsova et al. 2017).

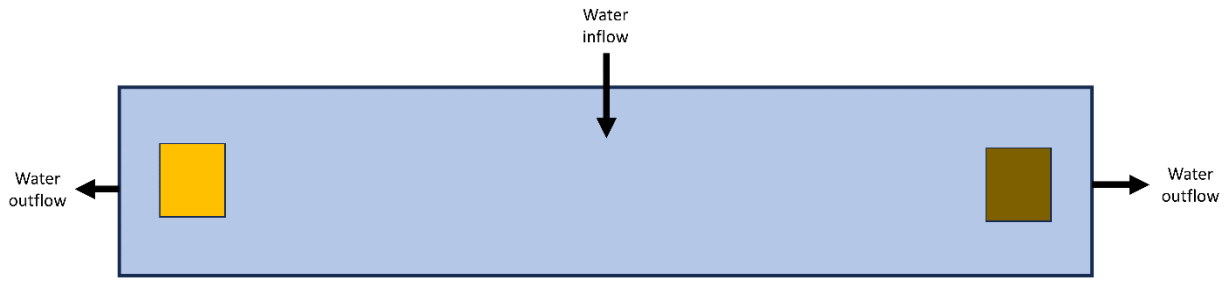

Fig. 1. Illustration of the layout of the primary design for paired habitat choice experiments. Figure is from the top-down perspective on a 30 cm × 7.5 cm × 7.5 cm chamber with water inflow at center and outflow at both ends. Colored squares represent two different objects on opposite side of the chamber (e.g., wood and rocks, etc.). Figure is not to scale.

Responses to light

Although *S. comalensis* have greatly reduced visual organs (Barr and Spangler 1992), no direct responses of this species to different light wavelengths have been tested. A basic understanding of responses to light can inform us as to why this species occurs in certain habitats and what conditions refugia populations should be kept in, as well as to inform how external light is handed in other experiments. To accomplish this, six separate and consecutively conducted experiments were performed to test the response of beetles to different wavelengths of light: a control (no light), white, red, green, blue, and UV light. The UV light was produced by 9W LED UVA 395–400 nm bulbs. The other bulbs were Great Value 9W LED bulbs (red, green, blue, and daylight).

For each of experiment, one end (7.5 cm × 7.5 cm) of the experimental chamber and a 7.5 cm × 5 cm section of the lid at the same end were not blacked out so that light could enter the chamber. Lightbulbs were placed outside of the chamber but underneath the black plastic sheeting and set with a timer to a 12:12 hour light:dark cycle from 06:00 to 18:00. Two bulbs were hung approximately 50 cm apart and about 20 cm above one end of the experimental chambers; all experimental chambers had the treatment (open light end) on the same side for this experiment. One piece of wood (~3 cm × 1.5 cm × 0.5 cm) was placed at each end of the chamber to serve as a food source and substrate; each piece of wood was placed below a small limestone rock (~1.5 cm diameter) to prevent movement. Beetles were placed in the middle of the chamber and checked between 09:00 and 11:00 the following two or three days. We recorded the location of the beetle in the chamber, as well as the position of the beetle relative to the habitat (on top of the wood/rock or below the wood/rock) each day. There were 10–14 replicate units for each species in each light combination.

Data from each of the six separate light experiments were combined for analysis. We conducted two mixed effects logistic regressions with a binomial distribution on each species/life stage. Although some beetles were reused in subsequent experiments, due to communal housing between experiments, we were unable to track individuals between the studies. However, we were able to track individuals between consecutive days of the same experiment, therefore we included experimental container and day as random effects. In the first analysis, the side of the experimental chamber (dark or light) was the response variable and light type was the predictor variable. In the second analysis, the position of the

beetle relative to the wood structure – hidden under the wood or in the open exposed to light (beetles 'on wood' and beetles 'walking in the open on the bottom of the container' were combined for analysis purposes) – was the response variable with light type as the predictor variable. The initial experiment used white light and served as the baseline from which all other light types were compared in both analyses.

Results indicated there was significant variation in the position of *S*. *comalensis* adults, *S*. *sexlineata* adults, and *S*. *sexlineata* larvae in response to different light types (Tables 1, 2). *Stygoparnus comalensis* adults were attracted to white and red light, with only 10% and 17% of recorded locations, respectively, being on the dark side of the experimental container. Significantly fewer *S*. *comalensis* were recorded on the light side for all other light types: approximately 50% (indicating no response) were observed on each side with blue, green, and no light (control). Ninety percent of *S*. *comalensis* were on the dark side in UV light, indicating avoidance of UV light. *Stenelmis sexlineata* adults selected the dark side of the experimental chamber when white, blue, and green light was present but showed no response to red, UV, or no light. *Stenelmis sexlineata* larvae were found on the dark side of the experimental chamber in the presence of all light types (white, blue, green, red, and UV), but when no light was present, they were found on both sides approximately equally (46% dark).

Stygoparnus comalensis adults showed no variation in their microhabitat use (above or below wood) in response the various light types (Tables 3, 4); overall, 71% of *S*. *comalensis* were on top of the wood or otherwise exposed to light. *Stenelmis sexlineata* adults showed a similar pattern of microhabitat use as they did when selecting side of the experimental chamber: when in the presence of white, blue, or green light >90% of *S*. *sexlineata* adults were found under wood with no variation among light types. *Stenelmis sexlineata* larvae were universally found under wood when any light type was present, with only 2 out of 24 occurrences in the open when no light was present.

Table 1. Number of total observations (counting the same beetle across multiple days in each experiment) beetles were observed on the dark and light side the experimental chamber and the percentage of those observations that were on the dark side (%D) when the light side was illuminated by white, blue, green, red, or UV light and the control (no light).

	Stygoparnus adults			*Stenelmis* adults			*Stenelmis* larvae		
	Dark	Light	%D	Dark	Light	%D	Dark	Light	%D
white	2	18	10.0	17	2	89.5	15	4	78.9
blue	13	14	48.1	26	7	78.8	17	11	60.7
green	15	11	57.7	22	12	64.7	26	13	66.7
red	3	15	16.7	8	9	47.1	12	6	75.0
UV	18	2	90.0	12	12	50.0	13	8	61.9
no light	9	10	47.4	11	13	45.8	11	13	45.8

Table 2. Results of mixed effects logistic regression on the responses of beetles to light types. Results are in reference to white (full spectrum) light and coefficient indicates direction and magnitude of response relative to white light with negative values indicating higher proportions in darkness. Bold indicates significant differences (P<0.05) from white light.

	Coef.	*z*	*P*
Stygoparnus adults			

blue	-3.84	-2.07	**0.038**
green	-4.82	-2.29	**0.022**
red	-0.65	-0.37	0.71
UV	-7.90	-3.15	**0.0017**
no light	-3.85	-2.02	**0.043**
Stenelmis adults			
blue	0.90	0.94	0.35
green	1.66	1.79	0.074
red	2.48	2.43	**0.015**
UV	2.36	2.43	**0.015**
no light	2.55	2.60	**0.0093**
Stenelmis larvae			
blue	1.05	1.32	0.19
green	0.79	1.04	0.30
red	0.84	0.82	0.41
UV	0.93	1.15	0.25
no light	1.66	2.07	**0.039**

Table 3. Number of total observations (counting the same beetle across multiple days in each experiment) beetles were observed under wood, on top of the wood, or walking in the open near the wood within the experimental chamber and the percentage of those observations that were under the wood (%U) when the light side was illuminated by white, blue, green, red, or UV light and the control (no light).

	Stygoparnus adults				*Stenelmis* adults				*Stenelmis* larvae			
	Under	On	Open	%U	Under	On	Open	%U	Under	On	Open	%U
white	6	13	1	30	19	0	0	100	19	0	0	100
blue	16	10	1	59	30	3	0	91	28	0	0	100
green	5	20	1	19	31	3	0	91	39	0	0	100
red	2	15	1	11	13	4	0	76	18	0	0	100
UV	2	16	2	10	18	4	0	82	21	0	0	100
no light	7	10	2	37	16	8	0	67	22	0	2	92

Table 4. Results of mixed effects logistic regression on the responses (hidden under wood or in the open exposed to light) of beetles to light types. Results are in reference to white (full spectrum) light and coefficient indicates direction and magnitude of the response with a higher proportion under wood (negative) or in the open exposed to light (positive) relative to the response to white light. Bold indicates significant differences (P<0.05) from white light.

	Coef.	*z*	*P*
Stygoparnus adults			
blue	1.22	1.95	0.051
green	-0.59	-0.84	0.40
red	-1.23	-1.38	0.17
UV	-1.35	-1.52	0.13
no light	0.31	0.45	0.65
Stenelmis adults			
blue	0.35	0.01	0.92

green	0.31	0.01	0.93
red	-0.12	-0.0	0.97
UV	-0.49	-0.1	0.97
no light	-0.88	-0.3	0.78
Stenelmis larvae			
No variation across 5/6 treatments, so no test performed			

Physical habitat structure

Given the long history of reported association with American sycamore (*Platanus occidentalis*) roots or occurrence on wood in the wild, we wanted to experimentally test whether *S. comalensis* has a preference for, or attraction towards, wood. Two paired habitat experiments were conducted to test the relative preference of beetles for conditioned dead sycamore wood relative to two other objects: leaves and rocks. The wood was conditioned for approximately 1 year and leaves for one month at SMARC using a flow-through system with water from the Edwards Aquifer. The initial experiment paired a limestone rock and a similarly sized piece of sycamore wood (~3 cm × 1.5 cm × 0.5 cm), while the second experiment paired sycamore wood with a similarly sized clump of sycamore leaves. In the second experiment, a limestone rock was placed on top of the leaves to hold them in place and also on top of the wood to maintain a symmetrical design. Beetles were set in the middle of the experimental chamber and checked after 24 hours when the side of the experimental chamber and the beetle position (on top of or below wood/rock/leaf) were recorded. After the initial check, beetles were reset by placing them in the middle of the chamber and then checking after another 24 hours before ending the experiment. A total of 12–24 replicates were tested for each species/life stage and habitat pair.

Data from the two paired habitat experiments were analyzed individually and together to compare responses among and within species, respectively. We used mixed effects logistic regressions with a binomial distribution for all analyses. Although some beetles were reused in subsequent experiments, due to communal housing between experiments we were unable to track individuals between the studies. However, we were able to track individuals between consecutive days of the same experiment, therefore we included experimental container and day as random effects. In the first two analyses, we tested whether the proportion of beetles on the wood side of the experimental chamber varied in *S. sexlineata* adults and larvae relative to *S. comalensis* adults for both the wood vs rock and wood vs leaf experiments. We then repeated this comparison but asked whether the proportion of individuals above the wood/rock/leaf varied among species in the two experiments. Then, we combined the data from both experiments and tested for differences in proportions within species between the two experiments. This analysis was to determine if the relative use of wood as a habitat changed when the alternative was a rock versus a leaf.

Stygoparnus comalensis adults were found on wood at high rates in both experiments: 96% of observations were on wood when rock was the alternative and 79% when leaf was the alternative (Table 5). These were significantly higher rates than either *S. sexlineata* larvae or adults in both experiments, and the occurrence of *S. comalensis* on wood when rock was the alternative was significantly higher than when leaf was the alternative (Table 7). Both *S. sexlineata* larvae and adults were found on/under wood >50% of the time when rock was the alternative. However, while use of wood did not significantly vary between experiments for *S. sexlineata* adults, there was a large shift toward favoring leaves among *S. sexlineata* larvae when offered the option. The position that *S. comalensis* adults were found in was “on top of the object” in >50% of the observations in both experiments, significantly greater than either *S. sexlineata* adults or larvae (Table 8). *Stenelmis sexlineata* were typically found underneath the wood, rocks, or leaves, or in the case of larvae, typically within the folds of the leaves. While the position of each taxon did not significantly vary between the two experiments, there was a marginally higher occurrence of *S. sexlineata* adults above the object when leaf was the alternative than when rock was the alternative.

Table 5. Number of total observations (counting the same beetle across multiple days in each experiment) beetles were observed on the wood or on the other material (rock or leaf) and the number of observations the beetle was above or below the object in the two paired habitat experiments. %W indicates the percentage of occurrences on the wood side of the experimental container and %A indicates the percentage of occurrences when the beetle was on or above the object.

	Stygoparnus adults			*Stenelmis* adults			*Stenelmis* larvae		
Side	Wood	Other	%W	Wood	Other	%W	Wood	Other	%W
Wood vs Rock	46	2	96	25	15	63	22	6	79
Wood vs Leaf	30	8	79	15	17	47	3	21	13
Position	Above	Below		Above	Below		Above	Below	
Wood vs Rock	30	18	63	10	30	25	1	27	4
Wood vs Leaf	22	16	58	16	16	50	2	22	8

Table 7. Results of species comparison mixed effects logistic regression on the responses of beetles in two paired habitat experiments, wood vs rock and rock vs leaf. Results are in reference to adult *S. comalensis*. Bold indicates significant differences ($P<0.05$).

	Coef.	*z*	*P*
Side			
Wood vs Rock			
Stenelmis adults	-2.62	-3.31	**0.0009**
Stenelmis larvae	-1.84	-2.14	**0.032**
Wood vs. Leaf			
Stenelmis adults	-1.73	-2.69	**0.0072**
Stenelmis larvae	-3.69	-4.13	**<0.0001**
Position			
Wood vs Rock			
Stenelmis adults	3.69	3.02	**0.0025**
Stenelmis larvae	7.97	3.08	**0.0021**
Wood vs. Leaf			
Stenelmis adults	0.33	0.66	0.51
Stenelmis larvae	2.74	3.33	**0.0009**

Table 8. Results of comparisons between experiments within each species of the side and position of beetles. For side, each row indicates whether the proportion of beetles on wood was lower (significant positive coefficient) when wood was paired with a leaf in reference to when wood was paired with a rock. For position, each row indicates whether the proportion of beetles above the object was higher (positive coefficient) or lower (negative coefficient) when wood was paired with a leaf in reference to when wood was paired with a rock. Results are those from mixed effects logistic regression. Bold indicates significant differences ($P<0.05$).

	Coef.	*z*	*P*
Side			
Stygoparnus adults	1.87	2.09	**0.036**
Stenelmis adults	0.63	1.17	0.24

Stenelmis larvae	3.16	3.98	**<0.0001**
Position			
Stygoparnus adults	0.28	0.43	0.67
Stenelmis adults	-1.25	-1.92	0.055
Stenelmis larvae	-0.90	-0.71	0.48

Beetle presence

This study assessed whether adult *S*. *comalensis* and adult *S*. *sexlineata* respond to the presence of caged conspecifics and heterospecifics. The experimental design utilized one caged adult beetle and one free-roaming adult beetle within the same experimental chamber. Cages were constructed out of 50 mL polypropylene tubes (25 mm diameter) with screw caps (Fig. 2). The tubes were cut to a length of 40 mm, fine mesh was hot-glued to cover the open end of the tube, and four 3-mm diameter holes were drilled into the cap and covered with fine mesh. This allowed movement of water and dispersion of cues between the cage and the rest of the experimental container. Cages contained one piece of wood (~3 cm × 1.5 cm × 0.5 cm) to serve as a food source and one small limestone rock (~1.5 cm diameter) to prevent the cage from floating. One cage was placed at each end of the experimental chamber for a symmetrical design; the side that received the beetle was randomly selected. One similar-sized piece of wood and rock were placed next to each cage (outside of the cage) in the experimental chamber to serve as food and additional substrate for the free-roaming beetle. At the start of the experiment, the free-roaming beetle was placed in the center of the experimental chamber. The location of the free roaming beetle was recorded once per day for the following three days before each replicate was terminated. After checking and recording the position of each beetle, its position was reset by placing the beetle at the center of the experimental chamber.

Six separate temporal rounds of the experiment were conducted to achieve at least eight replicates of each of the four species pairs: responding *S*. *comalensis* and caged *S*. *comalensis*, responding *S*. *comalensis* and caged *S*. *sexlineata*, responding *S*. *sexlineata* and caged *S*. *comalensis*, and responding *S*. *sexlineata* and caged *S*. *sexlineata*. Individual beetles only acted as the responding beetle or caged beetle at most once for each of the species pairs. We used mixed effects logistic regressions with a binomial distribution for two analyses. For each species, we separately tested whether the proportion of beetles adjacent to the cage containing a beetle was different between heterospecifics and conspecifics. Day and housing chamber were again included as random effects in these analyses.

Both species had a majority of occurrences of the free-roaming beetle adjacent to the conspecific cage but approximately half of occurrences with heterospecifics were adjacent to the beetle cage (Table 9). This was a significantly higher proportion of beetles next to conspecifics versus heterospecifics for *S*. *comalensis* ($z = 2.20$, $P = 0.027$), but this was a marginally non-significant difference for *S*. *sexlineata* ($z = -1.67$, $P = 0.095$). While we did not record all beetle positions (object they were crawling on) in this experiment, *S. comalensis* were observed crawling on the mesh side of the cage when another *S. comalensis* was in the cage, in contrast to typical positions for other species pairings (*S. comalensis* on wood and *S. sexlineata* under wood).

Fig. 2. Photograph of a beetle cage constructed out of polypropylene tubes used for the beetle presence study.

Table 9. Number of total observations (counting the same beetle across multiple days in each experiment) when beetles were observed on the side of the experimental chamber adjacent to the caged beetle or on the opposite side of the chamber, along with the percent of occurrences on the caged side. Table shows the observations when the responding beetle (free to roam the chamber) was *S. comalensis* and *S. sexlineata*, with data separated by species that was in the cage.

	Side responding beetle was on		
	Caged beetle	Opposite	% on caged
Responding beetle: *Stygoparnus*			
conspecific	30	9	77
heterospecific	13	13	50
Responding beetle: *Stenelmis*			
conspecific	25	12	68
heterospecific	11	13	46

Wood conditioning

We conducted two experiments to test beetle responses to captive- versus wild-conditioned sycamore wood. The captive wood was conditioned at SMARC using a flow through system with their aquifer water supply for over one year. The wild wood was collected from the Spring Island area the morning that the

experiment started and had been in the water for an unknown amount of time. The wild-collected wood was checked in the field for any invertebrates and then in the lab under a microscope to ensure they were clear of wild macroinvertebrates.

The first experiment was a selection experiment in which one piece of wood (~3 cm × 1 cm × 0.5 cm) was placed at each end of the experimental chamber. The position of the wild wood and the captive wood in each chamber was randomized. One *S*. *comalensis* adult and one *S*. *sexlineata* adult were randomly assigned to each experimental chamber and placed in the center and checked once per day on the following three days. After each daily check, the beetles were reset by placing them back at the center of the chamber. We analyzed differences between the two species using a mixed effect logistic regression with a binomial distribution that included day and housing chamber as random effects. The number of beetles on captive versus wild wood was approximately even and similar between species. There were 20 occurrences of *S*. *comalensis* on captive wood and 19 on wild wood, whereas there were 21 occurrences of *S*. *sexlineata* on captive wood and 18 on wild wood. There were no significant differences between species (z = -0.23, $P = 0.82$).

The second experiment investigated whether feeding rates by *S*. *comalensis* differed between the captive and wild-conditioned wood. Because of their translucent cuticles, we are able to see material within the abdomen of *S*. *comalensis* when backlit (similar to Kosnicki 2019; Fig. 3). After beetles were photographed, we could then quantify the proportion of the abdomen (excluding the pronotum) below the elytra that was full of gut contents using ImageJ (Schneider et al. 2012). This experiment consisted of two rounds with eight individual beetles (all females, no males were available at the time) that were tracked and repeated between rounds. Each experimental chamber received one piece of wood (~3 cm × 1 cm × 0.5 cm) and beetles were randomly assigned to experimental chambers during both rounds. During the first round, half of the chambers were randomly assigned as captive wood and the other half received wild wood, while during the second round the wood treatment was alternated such that individuals that received wild wood during the first round received captive wood during the second and vice versa.

On day 0 of the experiment (setup day), beetles were placed in the center of the experimental chamber with one limestone rock (~1.5 cm diameter) placed in the center of the chamber (rocks were cleaned and kept dry during the preceding months). No wood was initially placed in the chamber so that we could determine how much of their guts is cleared in one day. In all cases, beetles showed no signs of food in their guts after only 24 without a food source (Fig. 3). After 24 hours (on day 1), the wood was added to the center of the chamber and the rock was removed. After another 24 hours (on day 2) the wood was removed, beetles were photographed, and the rock was replaced. We repeated this process once more to obtain two measurements of gut contents, on days 2 and 4 of each round of the experiment. The proportion of the abdomen area occupied by gut contents (our response variable) was analyzed using mixed effects models in the lme4 and lmerTest packages in R. Wood conditioning was our fixed predictor variable, and beetle individual, round of the experiment, and day of each trial (2 or 4) were random effects.

Stygoparnus comalensis with wild-conditioned wood had significantly more of their abdomen full of gut contents (Fig. 4; 27.6 ± 1.2%; mean ± SE) than those with captive-conditioned wood (17.1 ± 2.6%) ($F_{1,30}$ =

2.79, $P < 0.0001$). The proportion of guts full did not vary between days 2 or 4 or between rounds of the experiment.

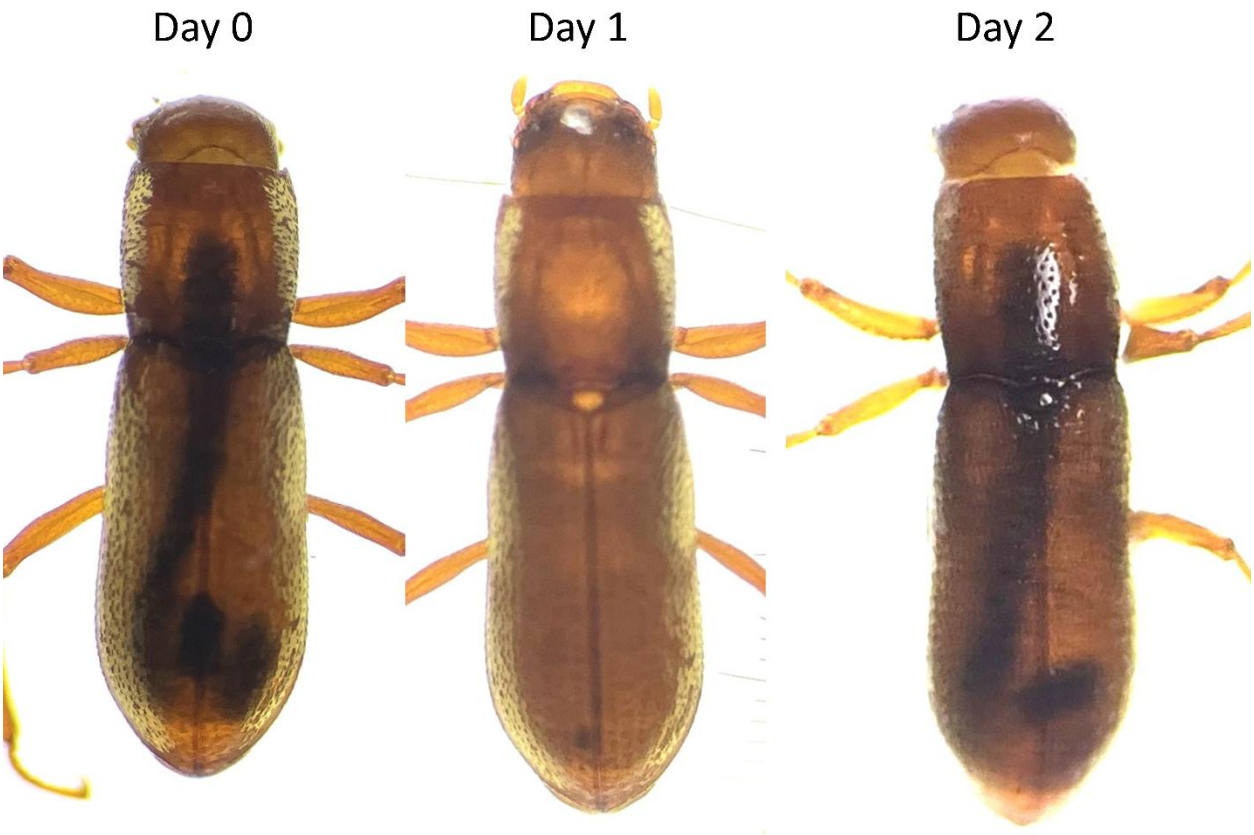

Fig. 3. Backlit dorsal photographs of the same beetle on day 0 (start of the experiment), day 1 (after 1 day without food), and day 2 (after 1 day with wild-conditioned wood). The dark areas in the abdomen on days 0 and 2 are gut contents, which are notably absent on day 1.

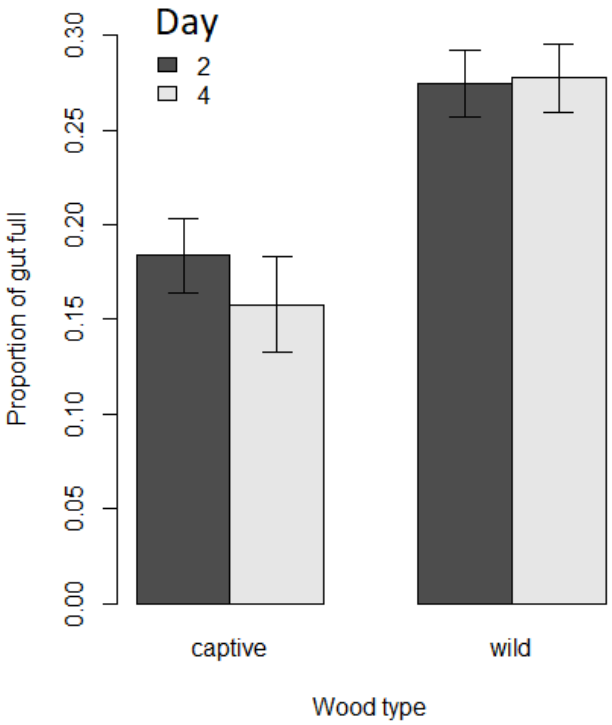

Fig. 4. The proportion of the abdomen (mean ± SE) of *S. comalensis* adults occupied by gut contents as inferred from dorsal backlit photos when beetles were provided with captive or wild conditioned sycamore wood.

Response to flow

As a spring-endemic species, we might expect *S. comalensis* to have an affinity for flowing water. We investigated whether *S*. *comalensis* occupied habitat within the experimental chambers based on where there was water flow. In all of the above studies, the water inflow tube was hanging above the center of the experimental chamber, inaccessible to any beetles. Additionally, previous observations and studies indicated that *S*. *comalensis* have a strong attraction to wood and preference for clinging to structures within the housing chambers. In this study, we blocked off the outflow at one end of the experimental chamber such that water would only flow out the opposite side (Fig. 5). At each end of the chamber, we placed a piece of wood vertically that stretched from the bottom of the experimental chamber to the outflow (1 cm below the top).

We then varied where the inflow tubing was placed. First (Fig. 5A), we placed it in the center just dangling into the top of the water as in the previous studies, with one end of the chamber having outflow and the other effectively no flow. Second (Fig. 5B), we placed the inflow adjacent to the piece of wood at the end of the chamber opposite of the outflow, creating unidirectional flow across the chamber. Lastly (Fig. 5C), we placed the flow directly adjacent to the piece of wood at the outflow, largely limiting any flow towards across the chamber. Beetles were placed in the center of the chamber and checked after 24 and 48 hours. After the first check, beetles were reset and placed in the center of the chamber. Only one beetle was placed in each experimental chamber and some beetles were repeated between the three iterations of the experiment; these individuals were tracked between iterations.

We analyzed the data twice with mixed effects logistic regressions with binomial distributions. First, we tested whether the proportion of beetles on the outflow varied between the three setups. Second, we tested whether the proportion of beetles at the location of strongest accessible flow varied between the three setups. We considered the strongest flow to be at the outflow in Fig. 5A because the inflow point was inaccessible. In Fig. 5B, the strongest flow was at the inflow because it is concentrated at the point of the small inflow tubing. In Fig. 5C, inflow and outflow were at the same side, so this was also the point of strongest flow.

Although there were no significant differences in where beetles were observed between the three setups (Tables 10, 11), there was a marginal difference in the proportion on the outflow between when the inflow was at the opposite end and when it was adjacent to the outflow. This indicates there may be some potential affinity for flow, but it is not as strong as with the affinities of *S*. *comalensis* for some other habitat conditions such as wood and light. In this experiment, beetles were always observed on the wood, but there was no consistent position in the water column where they occurred. Although the wood extended slightly above the water surface, no beetles were ever observed above the water surface.

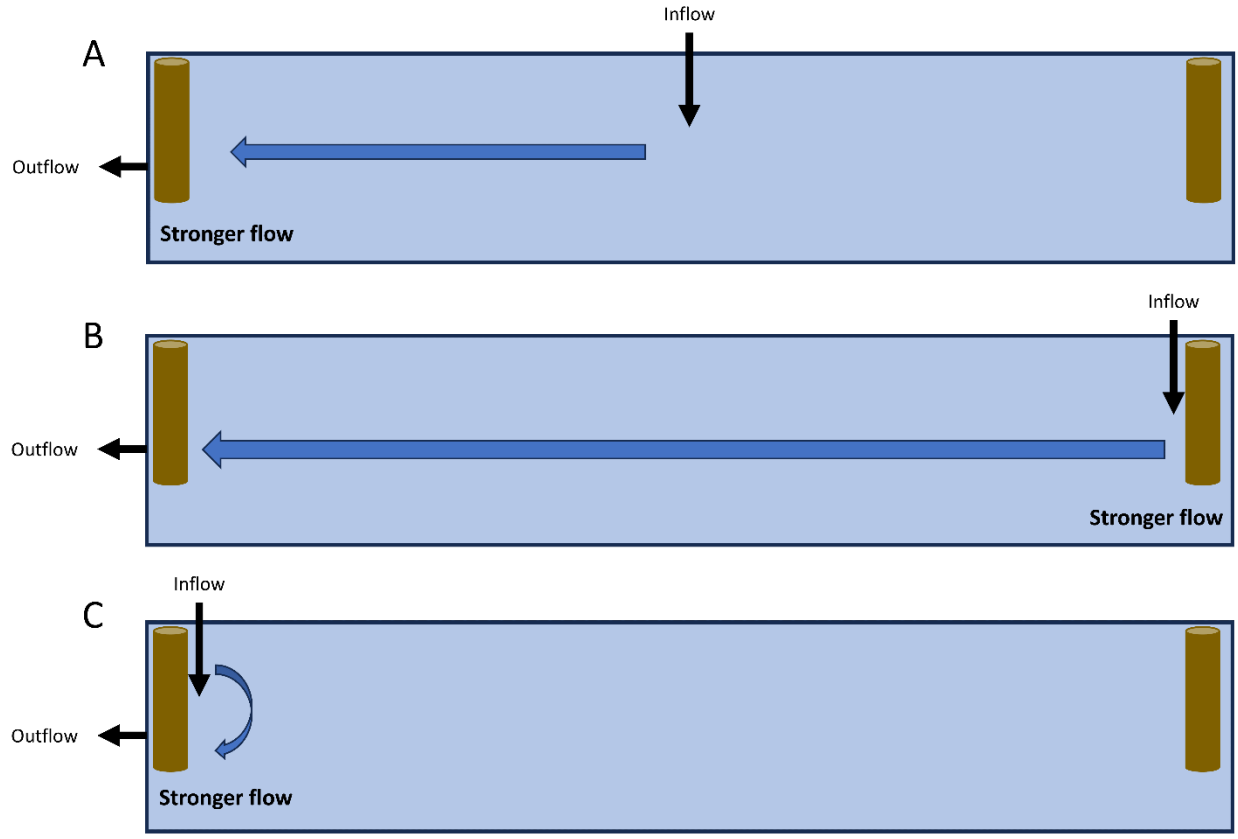

Fig. 5. Illustration of the three variations in the experimental setup in the flow experiment. A wood stick was placed vertically at each of the experimental chamber and water was allowed to flow out of the top of one end only. The inflow tube was considered the point of strongest flow (except in A where it dangled at the water surface and was not accessible to beetles). In B and C, the inflow was placed adjacent to the wood. Blue arrows indicate the dominant flow in each setup.

Table 10. Number of total observations (counting the same beetle across multiple days in each experiment) of where beetles were observed based on where the inflow was positioned (opposite, middle, or outflow; Fig. 5). The left three columns present the occurrences based on how many beetles were observed on the wood at the outflow versus the opposite end and the percentage of observations on the outflow. The right three columns consider this from the perspective of where the stronger accessible flow was located: this was the inflow when adjacent to a piece of wood (opposite and outflow) or the outflow when the inflow was in the middle.

Inflow location	Opposite	Outflow	% outflow	Stronger	Lower	% stronger
Opposite	16	8	33	16	8	67
Middle	11	13	54	11	13	46
Outflow	10	14	58	14	10	58

Table 11. Results of binomial logistic regressions assessing *S. comalensis* responses to flow. The top results are testing whether the proportion of beetles on the outflow side varies based on where the inflow was located (middle or experimental chamber or next to the outflow) and is relative to the inflow being placed at the opposite end of the inflow. The second set of results is assessing whether the

proportion of beetles at the location of stronger flow varies relative to when the stronger flow (inflow in this case) is at the opposite end to the outflow.

	Coef.	*z*	*P*
Proportion on outflow			
Middle	0.86	1.44	0.15
Outflow	1.03	1.72	0.086
Proportion on stronger flow			
Middle	-0.86	-1.44	0.15
Outflow	-0.36	-0.60	0.55

Larval feeding on leaves

Providing an appropriate food source to *S. comalensis* in captivity is certainly very important for survival and successful reproduction. Prior to this experiment, a preliminary study was conducted using four captive-raised *S. comalensis* larvae to assess whether they preferred different leaf species as habitat or food sources. The limited replication in this initial study did not produce any meaningful results but informed the design of a subsequent study. The study presented below was designed to test whether there was preferential feeding on one leaf species over another in a paired system. The initial round of this study was conducted using late instar *S. sexlineata* larvae since they were readily obtained from Landa Lake. However, we were never able to obtain sufficiently more *S. comalensis* larvae and results from the *S. sexlineata* study suggest that *S. sexlineata* larvae have no detectible effect on leaf mass, so further study was not pursued.

In this experiment, dead leaves of three plant species commonly found around Landa Lake were collected from terrestrial habitats: *Platanus occidentalis* (American sycamore), *Ungnadia speciosa* (Mexican buckeye), and *Quercus fusiformis* (Texas live oak). Leaves were cut into 2 cm × 2 cm squares (avoiding any major veins and the midrib) and autoclaved at 121°C. After autoclaving, leaves were weighed (analytical balance with 0.0001 g accuracy) and randomly assigned to experimental chambers (10 cm × 10 cm × 15 deep). Leaves were arranged in a square, with 0.5 cm between leaves. One small (~0.5 cm diameter) limestone rock was placed on top of each leaf to hold in place so that individual leaves could be tracked across the experiment. The experimental chambers were filled with continuously flowing water from the Edwards Aquifer. Leaves were allowed to condition and develop biofilm for two weeks before larvae were added. Larvae were randomly assigned to chambers with one larva per chamber; controls had no larvae. There were six replicate experimental chambers of each species pair with larvae and two replicates of each species pairs that were controls (24 total chambers measured on two dates).

After an additional two weeks, one leaf of each species was removed from each container (two adjacent leaves were randomly selected). Leaves were air dried for at least 48 hours and then weighed. After a further two weeks, the remaining leaves were removed, dried, and weighed. All larvae were removed and returned to a separate housing chamber. We analyzed the proportional change in the mass of each leaf using a mixed effects model with the leaf species, leaf pair, larva presence, and the interaction between species and presence as fixed effects with time and experimental chamber as random effects. Exploratory analyses showed no differences across time for any species, so it was only included as a random effect.

Our results showed only a significant effect of leaf species on the proportional change in mass (Table 12), with sycamore losing 56% of their mass on average, buckeye 25%, and oak 9% (Fig. 6). There were no differences in mass changes between species pairs or based on the presence/absence of larvae.

Table 12. Results of the mixed effect model on proportional change in leaf mass over the duration of the leaf consumption experiment. Bold indicates statistical significance ($P < 0.05$).

	df	*F*	*P*
Species	2, 88	35.2	**<0.0001**
Larva presence	1, 88	0.48	0.49

Leaf pair	2, 88	1.32	0.27
Species:Presence	2, 88	1.03	0.36

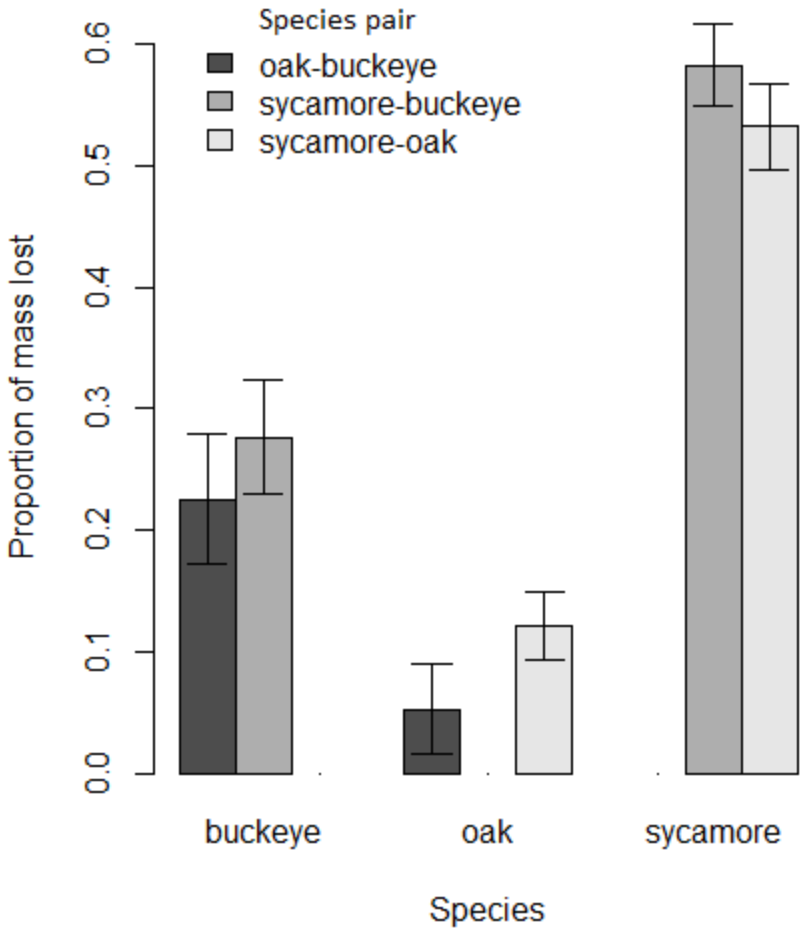

Fig. 6. Proportion of the leaf starting mass that was lost in the leaf consumption experiment (mean ± SE). Because neither time nor larval presence had effects in analyses, totals for each species-by-species pair are presented.

Discussion of environmental choice experiments

The most consistent observation across these experiments, along with the initial trials used to develop methods for these experiments, is that *S. comalensis* has a strong affinity for wood. The physical habitat structure experiment directly tested this and showed a strong preference for wood over leaves or rocks. However, observations from experimental and holding chambers indicate that in the absence of wood *S. comalensis* are seemingly both more active (observed crawling across the chamber several times) and have an affinity for clinging to some sort of object (i.e., not the smooth side of the chamber); these other occurrences were not tracked consistently and so are not presented here.

Biofilms that grow on wood serve as a food resource for *S. comalensis* and are seemingly the preferred food source over biofilms that may be growing on leaves. This is contrast to *S. sexlineata* larvae, which

were predominantly found within or under leaf packs when paired with wood; whether this is a true preference for leaves as a food source or a preference for the greater interstitial space they provide (between the many crevices in a folded leave versus only underneath the piece of wood) could not be discerned here. In the wood conditioning study, adult *S. comalensis* consumed more when paired with wild-conditioned wood versus wood conditioned in captivity. Whether this is a difference in the quality or quantity of wood/biofilms is unknown, but beetles were provided with equal sized pieces of wood from each source, so the amount of surface area biofilm to grow on each piece should have been relatively similar. Regardless, results of both of these studies indicate that *S. comalensis* maintained in captivity should be provided with wood, and the relative amount of captive-conditioned wood needed to sustain a population could be higher than would be provided from wild wood. Furthermore, lower consumption rates of captive-conditioned wood perhaps suggest there are issues with the quality of biofilm on captive-conditioned wood, which could have consequences for successfully maintaining captive populations and be one of the reasons behind why there has been so little success breeding this species and raising larvae to the adult stage in captivity.

There has been no clear response of *S. comalensis* to flow in our flow experiment above or in preliminary tests that used four or fewer beetles. Despite being a species that we only find in springs, this lack of response could be due more to the limitations of the experimental design rather than any true lack of affinity for flowing water. The small experimental chamber may not have provided sufficient variation in flow or associated differences in water quality that would elicit a response by *S. comalensis*. The affinity for wood may override any other factors with minor variation, such as flow, in these experiments.

The tendency of free-roaming *S. comalensis* and possibly *S. sexlineata* to occupy habitats closer to conspecifics but not heterospecifics suggests an attraction to beetles of the same species. In aquatic systems and among insects more broadly, semiochemicals (information-carrying chemicals) are widely used by animals to inform them of environmental conditions and select habitats (Eveland et al. 2016; Pintar and Resetarits 2020). Therefore, it should be expected that a species occupying dark habitats and lacking developed eyes would rely on chemicals produces by conspecifics for finding mates or to find favorable habitats that are already occupied by others of your own species. This is further supported by the field studies reported below in which 57% of samples where *S. comalensis* were found (representing 82% of beetles), more than one individual was on a wood disc.

Both beetle species displayed phototaxis (movement in response to light), and perhaps the most peculiar of the experimental results are the responses of *S. comalensis* to light: they avoided UV light, had no response to blue or green light, but were attracted to white and red light. The positive response to white light could just be a response to the red component of light emitted by those lights. The opposite responses to UV and red light also coincide to the wavelengths on opposite sides of the spectrum. Different insect species respond to different wavelengths of light in various ways, but attraction to red or infrared light seems to be somewhat common (Park and Lee 2017). Why *S. comalensis* seems to respond to this wavelength is unknown but is perhaps an adaptation that has not been lost with the reduction of its visual organs. However, whether there might ever be sufficient light of these wavelengths in the absence of UV light to affect behavior of this species is unknown. Similarly, responses to UV light are common in insects (usually attraction), but the avoidance of UV light by *S. comalensis* at least aligns with its occupancy of subsurface habitats. Detection of UV light during daylight

hours would be an indication that a beetle has strayed too close to the spring surface and away from favorable habitats. This could be useful for a species that seems to have little response to flow and in Comal Springs where there is relatively little variation in water quality that beetles could respond to across the small distances near spring openings they could traverse. In captive populations, it seems likely best that *S. comalensis* are kept in the absence of any light source. Exposure to any light source could potentially elicit responses or induce stress in the beetles that could affect behavior and reproduction.

The responses by *S. sexlineata* to light were not surprising: larvae nearly completely avoided all light and adults avoided white, blue, and green light. These experimental observations are consistent with field observations. When checking springs during the daylight hours, both adults and larvae of *S. sexlineata* have always been seen in dark conditions, such as on the underside of wood in springs. Larvae also have a strong affinity for occupying interstitial space within leaves or under wood, so these two aspects could not necessarily be separated with our results here, but we would expect larvae to avoid light to at least a similar degree as the adults do. Much more study would be required to fully understand the behavioral and physiological basis behind the responses to light in both of these beetle species.

These controlled habitat choice experiments have tested and confirmed some of the previously stated but untested ideas about the ecology of *S. comalensis*. These initial findings were used when developing methods for sampling wild populations (Part 2 below) and can be used for refining protocols for housing *S. comalensis* in captivity. The suggestions that we can clearly confirm here – that *S. comalensis* should be housed communally, with conditioned wood as food source, and probably in dark conditions – are not surprising or substantive departures from current protocols. However, some aspects of our findings, particularly the optimal food source (e.g., wild or conditioned wood, different species of wood, etc.), would require further investigation for developing optimized protocols when housing this species in captivity and creating a self-sustaining captive population. Our findings here regarding the responses to wood further support the need for not only maintaining healthy tree populations with extensive root networks through the Comal Springs system, but also potentially for maintaining submerged wood withing the springs as a food source, as it is not currently known whether live trees are directly important for *S. comalensis* survival and reproduction over short time scales.

Part 2: Field tests of detection, collection, and monitoring

Stygoparnus comalensis have historically proven difficult to find and collect, and this limited our ability to simultaneously conduct all replicates of experiments (some studies this was achieved via temporal replication over different months) and establish sufficient captive breeding pairs during the early stages of this project. Over the past several years of work conducted on *S. comalensis* at SMARC, collections were often made by placing and checking wood on the surface of springs, but systematic methods were never developed and detailed records of methods, locations, and abundances of beetles found and collected were never maintained.

A systematic method of using cotton cloth lures was established in 2014 for monitoring and collecting *H. comalensis*. Over 21 years of semiannual biomonitoring for *H. comalensis*, only 31 *S. comalensis* were found in contrast to over 9,800 adult *H. comalensis* using the cotton lures and preceding methods. Over the same time period, 24-hour drift net surveys during semiannual biomonitoring found 84 *S. comalensis*, 148 *H. comalensis*, 162 *Comaldessus stygius* (endemic subterranean diving beetle), 31 *Haideoporus texanus* (endemic subterranean diving beetle), and over 15,000 *Stygobromus* spp. (amphipods). Clearly, more effective methods are needed to collect *S. comalensis* as well as to monitor its population and understand environmental factors that may affect it.

During spring 2024, work under this project shifted towards developing and testing methods that could be used for collection and monitoring. Modified versions of previously used methods (cotton lures, drift nets) were tested alongside various iterations of wood devices.

Preliminary spring surveys

The occurrence *S. comalensis* on wood logs placed on top of springs in the Spring Island area was recorded from January through May 2024. The exact number of sites was not recorded, but tended to vary as conditions allowed, with at least ten sites typically checked every 1–2 weeks. Although other objects in springs such as leaves, rocks, or cotton lures were often checked, *S. comalensis* were only observed on wood. These surveys were performed through the end of March to collect *S. comalensis* for the environmental choice experiments and continued until the end of May as part of preliminary surveys of sites before more extensive surveys began. A total of 26 *S. comalensis* were found during these surveys, with 13 of those beetles found at two nearby sites in the Spring Island Backwaters (Fig. 7). The remainder of the beetles were found at five upwelling sites on the upstream side of Spring Island. All occurrences were between 29 February and 20 May.

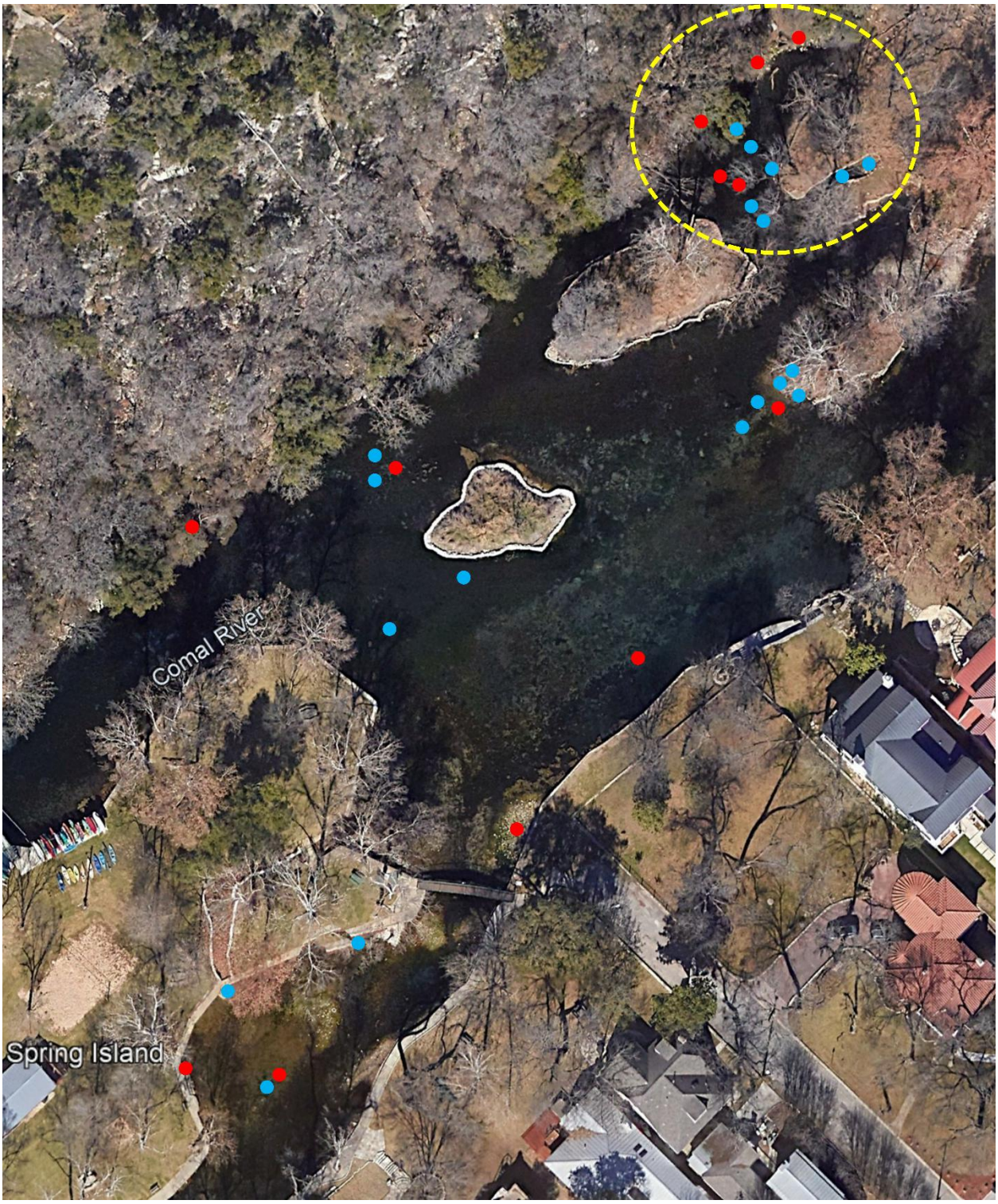

Fig. 7. Map of sites around Spring Island that were monitored with wood discs, cotton lures, and/or wood stakes for at least one month from June–November 2024. Blue dots represent sites where at least one *S. comalensis* was found and red dots are sites where no *S. comalensis* were found. The yellow ellipse encloses the Spring Island Backwaters.

Drift nets

Small drift nets were constructed out of PVC pipe (forming the frame) and 1.2 mm × 1.2 mm (opening) window screening (as the net). Initially, ten 20 cm × 20 cm (frame) nets were constructed and set starting 15 May 2024; 10 additional nets with a 12 cm × 12 cm frame were constructed and set starting in late May/early June. The net narrowed from the frame to a bottle opening; conditioned wood from Landa Lake was placed in the bottle to act as substrate and food for any beetles that may enter the net. The collection bottle was 'downstream' of the spring opening and covered with rocks to hold in place and maintain dark conditions. Nets were checked every 3–7 days and moved between sites. In total, the nets were set (for various amounts of time at each site) at 6 sites in Spring Run 3, 8 sites along the Western Shoreline, and 14 sites around Spring Island for a total of over 550 trap-hours. No *S. comalensis* or *H. comalensis* were ever found in these nets and all nets were removed from the system by early July.

The three large orifices in Spring Run 1, Spring Run 3, and along the Western Shoreline are the three sites that individually have the greatest flow under historically average flow conditions. These three sites are those where the semiannual biomonitoring is conducted; they were not tested using our modified drift net methods. We expect that the lack of any beetles in our drift nets is because the flow of water coming from the small spring openings that we monitored is far too low to wash out small beetles clinging to substrate within them. Additionally, the lack of beetles in the nets (which were typically checked starting around 08:30 each day, may suggest that at night these beetles do not crawl out of the springs into surface habitats exposed to daylight, but a definitive determination would require further study.

Wood stakes

To survey the numerous small upwellings in the Spring Island Backwaters that are covered in silt and sand, wood stakes were purchased from Lowe's (likely pine stakes). These stakes were inserted into ten springs in this area between 20 May and 11 September. Not all ten springs had stakes during the entire study, and some stakes did not remain in springs and floated away. During the first month of the study, cellulose cotton sponges were attached with cable ties to one side of the stake, either alone or in addition to sycamore sticks. Cellulose sponge was not used after the first month due to degradation and high numbers of *H. comalensis* larvae (up to 80 on one stake) that burrowed into the sponge.

Wood stakes proved productive for detecting and collecting *H. comalensis* and also produced two *S. comalensis* (Table 13) and also suggest that various species of trees may all be effective. Wood stakes had drawbacks that the wood disc method did not (see below): stakes were often difficult to insert into springs (they could not really be inserted easily into sites that did not have a lot of silt or sand) and on several occasions they floated out of the spring. Additionally, while wood discs are buried and not visible, wood stakes could be visible to people recreating around Spring Island or at least potentially subject to being trampled, though algal and biofilm growth over the stake did help provide camouflage. After removing the stakes, wood discs or pieces of wood stake of similar size to the wood discs were placed into most of the sites that had stakes.

Table 13. Total numbers of beetles and *Stygobromus* spp. amphipods found on wood stakes by month during sampling from June through September.

Month	Material	# of stakes	*Stygoparnus* adults	*Heterelmis* larvae	*Heterelmis* adults	*Stygobromus* spp.	*Microcylloepus* larvae	*Microcylloepus* adults
June	sponge	3	0	20	36	7	0	2
June	sponge + sticks	3	1	90	33	2	0	2
July	none	5	0	8	10	1	46	8
Aug.	none	7	1	54	11	0	7	2
Sept.	none	8	0	45	4	1	1	3
Total			**2**	**217**	**94**	**11**	**54**	**17**

Wood discs

Methods

Our intent with this study was to create a durable object that could be buried into springs and be attractive to *S. comalensis*; other taxa found were recorded but generally a secondary consideration. Wood logs found in Spring Lake away from active spring openings were used to create wood discs (Fig. 8). The wood had been in the lake for an unknown amount of time and was selected for its size (6–8 cm diameter) and apparent level of conditioning (not freshly in the lake, sunk to the bottom, solid and not disintegrating upon touch). While we do not know which species of tree the wood belonged to, three different logs were chosen and are potentially different species given they all had different coloration when they were cut (light brown, dark brown, brownish-red), but this coloration was no longer apparent after the first couple of months they were set in the springs. The logs were sawed to create circular discs; the initial set of discs were cut to widths of ~1.5 cm, but later discs were cut to ~2 cm. The surface area of the flat side of each disc was measured for use as a covariate in analyses (42.1 ± 0.5 cm^2; mean ± SE). A 2 mm wide hole was drilled through the disc approximately 1 cm from the edge through which a wire with a name tag was attached to the disc.

Fig. 8. Photograph of a wood disc with eight *S. comalensis* adults on the underside (flipped over after retrieving from the spring).

The structure of the wood discs was modified throughout most of the study. Initially other objects were tied using plastic cable ties to the flat side of the wood disc and placed such that the added objects faced down (into the spring flow in upwellings; downward facing but angled slightly upstream in terrestrial margin springs). These variations included: wood disc only, cellulose sponge, sycamore sticks (3 sticks ~0.5 cm diameter), and cellulose sponge + sycamore sticks. The variations were alternated within some sites between monthly checks.

Initially, the cellulose sponge on discs that had been set for only two weeks seemed to be a viable method, but after four weeks the sponge degraded much more (often breaking apart and/or slimy) and was perhaps unattractive to any beetles that might have colonized the wood. At most sites, sponge was not used after checking sites during July, and after checking in August neither sticks nor sponge were used. Beginning in August, three or four 1.5 mm wide × 2 mm deep grooves were cut with a saw into the flat face of some wood discs to act as permanent structural complexity (grooves placed face down in the spring) to compare to flat-faced discs (no grooves). Following the removal of wood stakes in September,

we cut stakes into pieces of wood with a similar surface area to the wood discs and cut grooves into the bottom of the stakes (Fig. 9). These stake pieces were then placed into springs in a similar manner as the wood discs.

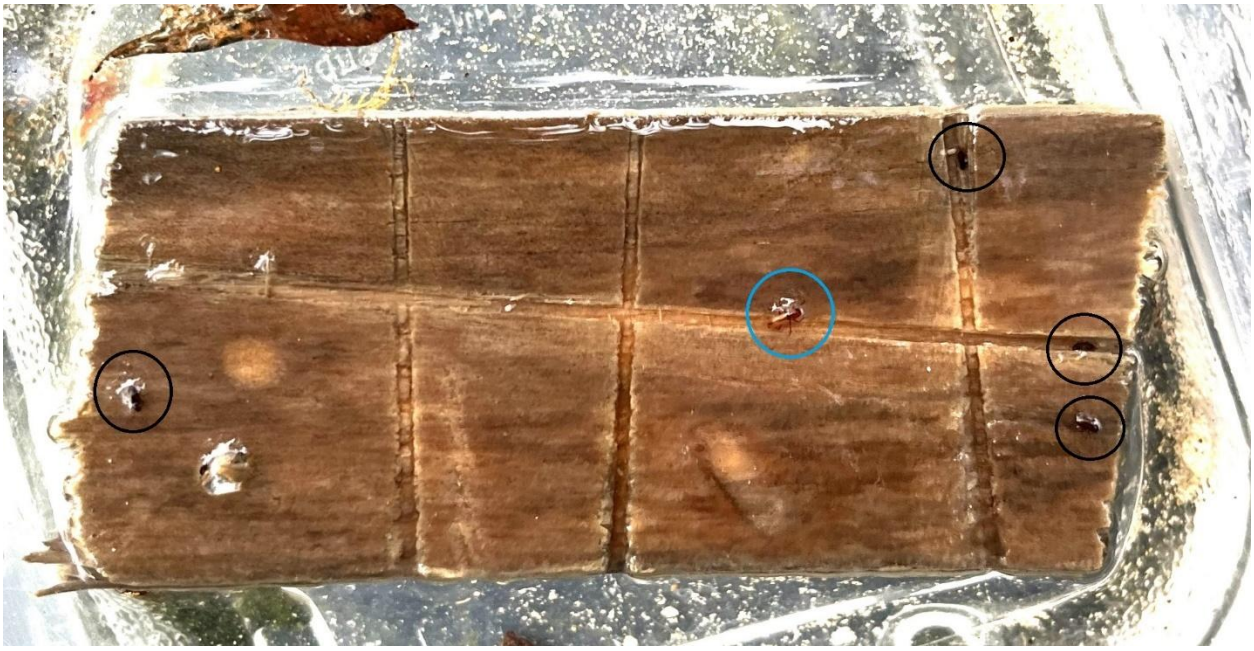

Fig. 9. Photograph of a piece of wood stake (with grooves) with one *S. comalensis* (blue circle) and four *H. comalensis* (black circles). The wood was placed such that grooves faced downward into the spring.

Following some limited tests during April and May, the discs were first set into sites around Spring Island and at the Western Shoreline on 20 May and checked two weeks later; later checks were performed approximately every four weeks. Additional discs were set at Spring Island, the Western Shoreline, and Spring Runs 1, 2, and 3 during June. Four discs were set in the Upper Spring Run area during August, though one disc was lost, flows were continually low, and no beetles were ever observed there. Most sites were monitored continuously from July through November, though some discs were lost (particularly in the heavily recreated area around Spring Island), and some discs were set and removed to survey different sites or to test modified methods.

At the start of *H. comalensis* semiannual biomonitoring in October, all discs set in those sites were removed so as to not compete with cotton lures for beetles. During each sampling period, nearly all *S. comalensis* adults and *H. comalensis* adults, along with some *H. comalensis* larvae and larger *Stygobromus* spp., were removed from the system and incorporated into the USFWS refugia populations in San Marcos and Uvalde.

The abundance of the following taxa/life stages were individually analyzed: *S. comalensis* adults, *H. comalensis* adults, *H. comalensis* larvae, *M. pusillus* adults, *M. pusillus* larvae, *Stygobromus* spp., and *Lirceolus* spp. Analyses were restricted to Spring Island, the Western Shoreline, Spring Run 2, and Spring Run 3 for all taxa except *S. comalensis* because no invertebrates were found in Spring Run 1 or the Upper Spring Run. Analyses with *S. comalensis* were restricted to only Spring Island sites because only three individuals were found at just a single site at the Western Shoreline. Device type served as our primary

factor of interest, with six categories: wood disc only, wood disc with grooves, wood disc with sponge, wood disc with sticks, wood disc with sticks and sponge, and piece of wood stake with grooves. The area of the flat face of each wood disc was included as a fixed effect covariate. The abundance of *H. comalensis* larvae was included as a covariate for the adult *H. comalensis* analysis and vice versa; the same was tested for *M. pusillus* but neither factor improved the model fit so they were not included. Time (days since discs were cut) was included as a covariate in exploratory analyses but was not a meaningful factor in any analyses except *M. pusillus* larvae, which may have been more indicative of phenological variation in that species. Characteristics of each spring site were not measured during these initial surveys, so we include spring type (upwelling, terrestrial margin) as a fixed effect and location (site nested within region) as a random effect; sector [spatial grouping of sites around Spring Island] replaced region for the *S. comalensis* analysis. Date of sampling was also included as a random effect. Analyses were mixed effects generalized linear models fit with negative binomial distributions using the glmmTMB package v. 1.1.10 (Brooks et al. 2017) in R.

Results

Over the course of six months of sampling, 247 wood disc samples were collected (include wood stake pieces placed like wood discs but excluding discs above the water). Other than three individuals found at one site along the Western Shoreline, all other 87 *S. comalensis* adults found were in the vicinity of Spring Island; no larvae of this species were observed (Table 14). *Stygoparnus comalensis* were found at 14 separate spring sites around Spring Island (Fig. 7; 19 total sites where *S. comalensis* were found when including other methods); four of those sites produced 10–13 beetles each, while all other sites had 7 or fewer. The wood discs were also productive for detecting and collecting *H. comalensis*, with 1,004 adults and 817 larvae found across four of the six regions. At the Spring Island area, *S. comalensis* were roughly $1/10^{th}$ as common as *H. comalensis*. Although over 400 *M. pusillus* were found on discs, relatively few (69) larvae were found. The wood discs also produced some *Stygobromus* and *Lirceolus* (Table 14), among other less common, non-focal invertebrates.

Table 14. Total number of samples and focal invertebrates found on wood discs at each of the six regions from June to November 2024.

Region	Samples	*Stygoparnus*	*Heterelmis*		*Microcylloepus*		*Stygobromus*	*Lirceolus*
		adults	adults	larvae	adults	larvae		
SI	120	87	815	732	429	69	65	13
SR1	8	0	0	0	0	0	0	0
SR2	14	0	54	23	22	15	1	4
SR3	31	0	13	14	24	8	2	9
USR	4	0	0	0	0	0	0	0
WS	70	3	122	48	46	37	14	30
Total	**247**	**90**	**1,004**	**817**	**521**	**129**	**82**	**56**

Most (127) samples collected were from devices consisting of only wood discs; the remaining were wood discs with sticks (38), discs with grooves (37), discs with sticks and sponge (24), discs with sponge (15), and stake pieces (6). Among samples from the Spring Island area, we found no significant variation in the numbers of *S. comalensis* between any of the device types (Fig. 10); there was also no variation in their abundance based on wood disc area or spring type (Table 15). Although raw data showed some variation among the other taxa (Fig. 11), *H. comalensis* larvae, *M. pusillus* larvae, and *Lirceolus* spp. did not significantly vary among device types. *Heterelmis comalensis* adults, *M. pusillus* adults, and *Stygobromus* spp. all had significant variation among device types with the most individuals on discs that contained both sponge and sticks (greatest structural complexity). Both *Stygobromus* spp. and *M. pusillus* adults had higher abundances on discs with sticks than most remaining device types. Discs with grooves tended to have lower average abundances, but this is likely in part due to this form of disc only being deployed in the spring runs and Western Shoreline, where overall numbers were lower. Only *M. pusillus* larvae showed any significant variation in abundance with disc size; they were found in higher abundances on larger discs.

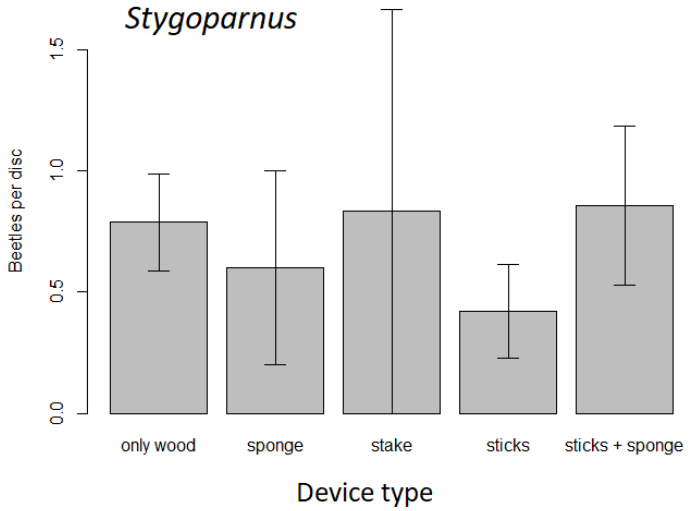

Fig. 10. Mean (±SE) number of *S. comalensis* per wood disc sample in the Spring Island area over the course of the study.

Table 15. Overall analysis results for the comparison of different wood disc types for each invertebrate species. Coefficients are included only for significant continuous variables to show directionality of effects. Bold indicates statistical significance ($P < 0.05$).

	Coefficient	χ^2	*P*
Stygoparnus comalensis			
Device type		2.0	0.74
Area		1.9	0.16
Spring type		0.19	0.67
Heterelmis comalensis adults			
Device type		17.1	**0.0044**
Area		0.4	0.53
Spring type		0.7	0.41
H. comalensis larvae	0.271	4.7	**0.031**
Heterelmis comalensis larvae			
Device type		3.4	0.64
Area		0.3	0.62
Spring type		0.0	0.87
H. comalensis adults	0.392	7.4	**0.0065**
Microcylloepus pusillus adults			
Device type		15.0	**0.010**
Area		0.32	0.57
Spring type		5.4	**0.020**
Microcylloepus pusillus larvae			
Device type		5.6	0.35
Area	0.582	9.5	**0.0021**
Spring type		0.16	0.69
Stygobromus spp.			
Device type		42.7	**<0.0001**
Area		0.5	0.46
Spring type		1.9	0.17
Lirceolus spp.			
Device type		6.8	0.23
Area		1.6	0.20
Spring type		1.9	0.17

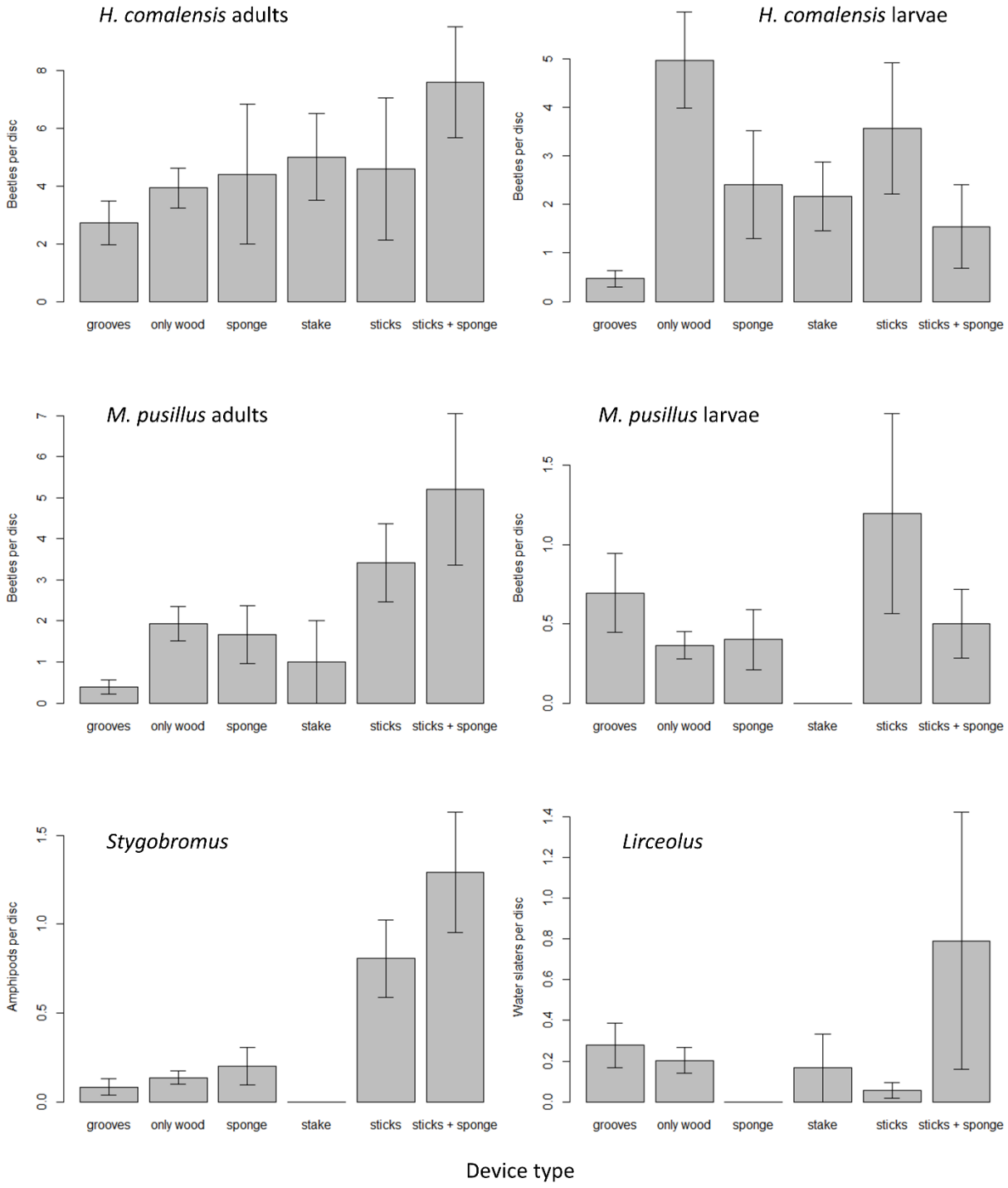

Fig. 11. Mean (±SE) number of invertebrates per wood disc sample in the Spring Island area over the course of the study.

Paired cotton lure and wood disc study

During two of the monthly sampling periods (July–August, September–October), we conducted a paired test of cotton lures and wood discs. Within a spring, one cotton lure and one wood disc were placed adjacent to each other and separated by 2–4 cm at their edges. Otherwise, lures and discs were set and checked in the same manner as described previously. Eight sites were repeated during both sampling periods (6 at Spring Island, 1 at the Western Shoreline, 1 in Spring Run 3). Two sites at Spring Island were set only in July and three only in September; one site in Spring Run 3 was set only in July and two only in September; three sites at the Western Shoreline were set only in July and two only in September; and three sites in Spring Run 2 were set only in September. In total 64 total samples were taken (each device counted separately during each sampling period); 17 of the sites were terrestrial margin springs and 15 were upwellings.

Each of the five beetle species/life stages were analyzed separately using mixed effects models with month and site nested within region as random effects using the lme4 package in R. Device type (cotton lure, wood disc) and spring type (upwelling, terrestrial margin) were fixed effects and the natural log-transformed abundances were response variables. Spring type was dropped from the model when ΔAIC > 2.

There were no significant differences in the numbers of *H*. *comalensis* adults, *M*. *pusillus* adults, or *M*. *pusillus* larvae between device types (Table 16, Fig. 12). There were significantly more *H*. *comalensis* larvae and *S*. *comalensis* adults on wood discs than on cotton lures. In the case of *S*. *comalensis*, all 10 individuals in this paired comparison study were found on wood discs.

Although there were no statistical differences in the natural log-transformed mean number of *H. comalensis* adults, the number observed on wood discs was more consistent (lower variance; 2.63 ± 0.72 beetles per disc; mean ± SE) compared to cotton lures (3.06 ± 1.76 beetles per lure). *Heterelmis comalensis* adults and larvae were encountered on wood discs more often (adults = 16/32 sites; larvae = 18/32 sites) than cotton lures (adults = 11/32 sites; larvae = 5/32 sites). One site had much higher abundances of *H. comalensis* adults on cotton lures during both August (N = 47) and October (N = 33) than any other site (N ≤ 6), but excluding this site did not change the statistical conclusions so it was maintained. This same site also had two of the highest abundances on wood discs in this comparative study (N = 13, 10 *H. comalensis* adults).

Table 16. Results of the mixed effects models for abundances of each species and life stage in the paired collection device comparison study (cotton lure versus wood disc). Bold indicates statistical significance ($P < 0.05$).

Species/life stage	Factor	df	*F*	*P*
Heterelmis adults	Device	1, 41	2.8	0.10
Heterelmis larvae	Device	1, 43	16.1	**0.0002**
Microcylloepus adults	Device	1, 37	1.1	0.30
	Spring	1, 22	5.7	**0.026**
Microcylloepus larvae	Device	1, 45	0.1	0.82
Stygoparnus adults	Device	1, 47	7.3	**0.0098**
	Spring	1, 22	5.1	**0.034**

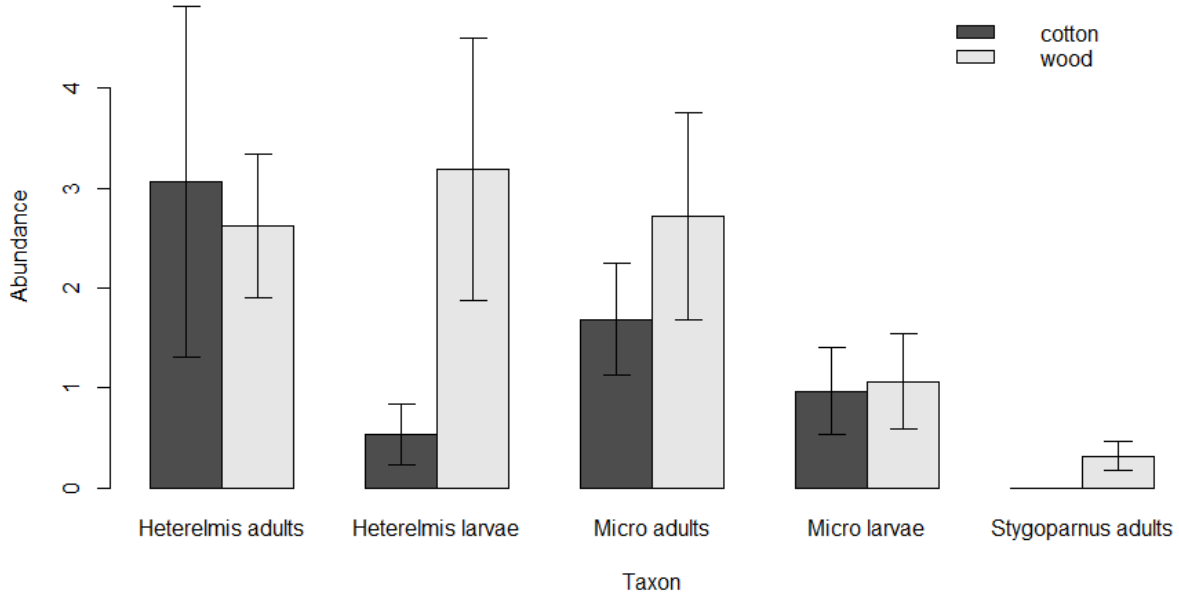

Fig. 12. Mean (±SE) abundances of each beetle species/life stage per collection device (cotton lure, wood disc) in the paired comparison study. Micro = *Microcylloepus pusillus*.

Comparison of non-paired cotton lures and wood discs

To address the possibility that wood discs increased the attractiveness of the area around them in the paired cotton lure study, we combined data from Spring Island from the paired comparison study with data on single device sites when both lures and discs were set in the system for fall biomonitoring (October–November). This was restricted to the Spring Island area because during November because no *S. comalensis* or *H. comalensis* were observed in Spring Run 3 during this time and the number beetles (*S. comalensis* = 0; adult *H. comalensis* = 6) and wood discs (N = 5) at the Western Shoreline was low.

Each of the five beetle species/life stages were analyzed separately using mixed effects models with sector as a random effect using the lme4 package in R. Device type (cotton lure, wood disc), spring type (upwelling, terrestrial margin), and pairing (yes, no), plus the device:pairing interaction were fixed effects and the natural log-transformed abundances were response variables. Spring type was dropped from the model when ΔAIC > 2.

Here, the device:pairing interaction was our primary result of interest. This interaction was not significant for any comparisons (Table 17; Fig. 13), although there was a marginal non-significant interaction with *M. pusillus* adults reflecting a shift towards more beetles on cotton lures when not paired with wood discs. This generally indicates that placement of wood discs next to cotton lures did

not alter colonization rates of the device for either *S. comalensis* or *H. comalensis*. However, results of this comparison should be interpreted with caution as it was conducted at a different time than the paired study and the cotton lure and wood sites were by necessity different locations without alternating replication within those sites (e.g., replacing cotton lures with wood discs in the same site for the following month).

Table 17. Results of the mixed effects models for abundances of each species and life stage for non-paired wood discs and cotton lures collected in November 2024. Results indicate whether there were statistically significant ($P < 0.05$) results between wood discs and cotton lures.

Species/life stage	Factor	df	*F*	*P*
Heterelmis adults	Device	1, 53	3.2	0.082
	Spring	1, 53	1.8	0.19
	Pairing	1, 53	0.3	0.57
	P:D	1, 53	2.5	0.12
Heterelmis larvae	Device	1, 54	18.0	**<0.0001**
	Pairing	1, 54	0.3	0.56
	P:D	1, 54	1.0	0.33
Microcylloepus adults	Device	1, 53	2.1	0.15
	Spring	1, 53	4.7	**0.035**
	Pairing	1, 53	0.3	0.57
	P:D	1, 54	3.3	0.073
Microcylloepus larvae	Device	1, 53	3.3	0.076
	Spring	1, 53	1.9	0.17
	Pairing	1, 53	1.9	0.17
	P:D	1, 53	0.1	0.82
Stygoparnus adults	Device	1, 53	10.4	**0.0021**
	Pairing	1,1	0.4	0.69
	P:D	1, 53	0.7	0.42

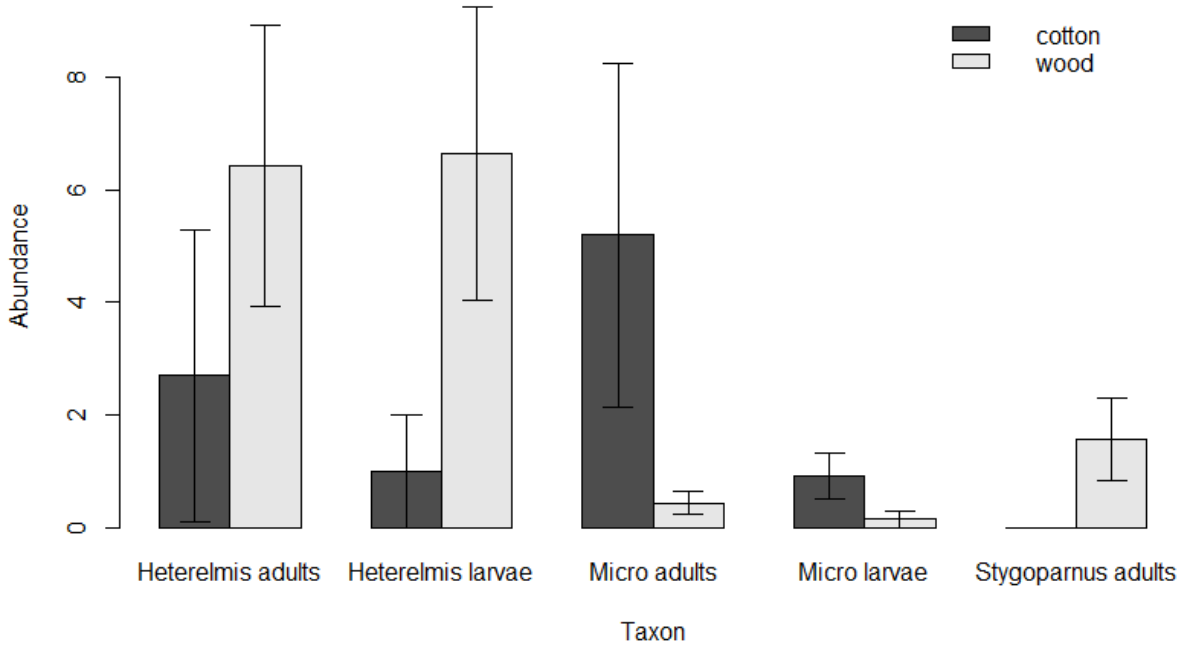

Fig. 13. Mean (±SE) abundances of each beetle species/life stage per collection device (cotton lure, wood disc) in November 2024 on non-paired wood discs and cotton lures around Spring Island. Micro = *Microcylloepus pusillus*.

Discussion of detection and collection methods

During six months of study in Comal Springs, more *S. comalensis* were found using wood discs than have ever been collected using cotton lures. Although there seemed to be no variation in the ability to detect *S. comalensis* using any of the variations in wood discs, additional material that provided the most structural complexity seemed to be more effective at also detecting other species *(H. comalensis*, *Stygobromus*). However, this additional material is much more difficult to manage as the sponge does not retain its structural integrity much beyond two weeks and sticks do not always remain in place on the discs. The size of the discs used here (~42 cm^2) were versatile – they could easily be placed in small spring openings near roots, under rocks, or in silty upwellings. In all of these cases, the entire disc was able to remain in the area of optimal spring flow; larger pieces of wood often extend beyond these spring openings with their distant sides harboring non-spring invertebrate taxa or covered in dense silt or sand.

On several instances throughout the year, wood discs at some of the sites were visually checked as little as one week after last set and several *S. comalensis* were already observed on the wood (specific data was not recorded). This, along with the data collected throughout the year, indicate that these wood discs likely have a stable level of attractiveness to these spring-associated beetles, which contrasts with cotton lures that must first develop biofilm and then be checked before they degrade too much and become unattractive. The same wood discs can remain in springs from month-to-month, regardless of

frequency with which they are checked, and perhaps for a year or more at a time before they degrade to the extent that they are no longer useful. Care must be taken when selecting wood to use for discs that it is solid, lacks any soft sections, and does not have cracks that could result in discs that easily break.

While some structural complexity seems beneficial, there does not seem to be a need to intentionally carve grooves into the wood, as wood the discs naturally developed some microstructure over time. Similarly, while wood stakes and pieces of wood stakes placed into springs in the same manner as wood discs seemed to produce beetles at similar rates to wood discs, both versions of wood stakes were more difficult to manage than wood discs. In both cases the wood often floated out of position: the full stakes sometimes floated away after leaving the site and the stake pieces would float away while placing or collecting them and often be difficult to find in turbid water. Ultimately, the simplest and easiest to manage method – a wood disc (6–8 cm diameter, 2 cm thick) made of out well-conditioned wood – was just as effective as other wood disc methods for detecting *S. comalensis* and is a durable method for collecting and monitoring this and other co-occurring species. While we did not produce discs of equal size, they were similar enough that we did not observe variation in either *S. comalensis* or *H. comalensis* with disc size. However, we would expect some variation in abundance if a wide range of wood discs were used.

While we tested a limited number of stakes and found them to be effective for detecting *H. comalensis*, they only produced two *S. comalensis* from a region (Spring Island Backwaters; Fig. 7) that, despite representing 16% of sites monitoring in the wood disc study, has been the most productive area for *S. comalensis* (36% of occurrences on wood discs) and highly productive for *H. comalensis* (27% of occurrences on wood discs) in this study. This is despite the prevailing sentiment that silt is detrimental for these species. This idea probably originates from observations that cotton lures that are covered with silt following heavy rainfall often had few or no beetles. Springs that exist alongside normally occurring silt and maintain springflow may reasonably support high numbers of beetles. The Spring Island Backwaters are surrounded by numerous trees, contain a moderate amount of dead wood in the water and within the silt, and are protected from recreation that affects the area around Spring Island. The depth of silt in this area (~15 cm at several sites) may reduce the amount of light reaching the rocky surface below, allowing beetles that have adverse responses to light to occur closer to the 'surface' in higher abundances. Furthermore, the amount of silt and sand in this area may insulate these springs from other animals, particularly fish and crayfish, that may otherwise disturb spring sites and potentially prey on beetles.

Conclusions and next steps

These studies in 2023–2024 experimentally confirmed the affinity of *S. comalensis* for wood and established the basics of a method that can be used for further in situ studies of this species. While we have confirmed the continued presence and distribution of *S. comalensis* around Spring Island, we have largely been unable to find this species elsewhere in the Comal Springs system. This may be in part hampered by the persistence of low-flow conditions since 2022, especially in Spring Runs 1 and 2 and the Upper Spring Run. Regardless, we still lack a data- and statistics-based knowledge of environmental factors that may contribute to the occurrence and abundance of *S. comalensis* in wild populations.

Developing a preliminary understanding of what environmental characteristics are associated with *S. comalensis* presence and abundance is important to form the baseline for longer-term studies of this species. If you do not know what habitat characteristics to monitor for, then it may be difficult to interpret the changing presence/abundance of a species over time if you measure the wrong variables. Most of the wood discs deployed in 2024 remain set in the Comal Springs system, and we have proposed that the initial data can be combined with continued monitoring and detailed habitat characterizations of the sites around Spring Island to determine what environmental factors may contribute to sustaining populations of *S. comalensis*. These findings could then inform longer-term studies of *S. comalensis* across the Comal Springs system, potential habitat restoration efforts, strategies for maintaining the species in captivity, and potential reintroduction efforts if a catastrophic event were to occur or if *S. comalensis* continue to not be found in other regions of Comal Springs.

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Appendix D | **Evaluating survival and tag retention of cave amphipods and riffle beetles** (Interim Report)

Evaluating survival and tag retention of cave amphipods and Comal Spring Riffle Beetles

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Importance of the research

Populations of Peck's Cave Amphipod (*Stygobromus pecki*) and the Comal Spring Riffle Beetle (CSRB, *Heterelmis Comalensis*) are maintained at the San Marcos Aquatic Resources Center so that wild populations can be enhanced if recovering from unfavorable conditions such as severe drought. As part of the propagation program, the USFWS and partners work to refine propagation methods and increase knowledge of the species. Tracking individuals over time would allow biologists to estimate survival (i.e., a conservation priority, EA Recovery Implementation Program, 2021) and examine their behaviors in the center (e.g., effects of flow changes on their movements). Moreover, tagged individuals could correspond to different collection sites (e.g., spring locations) or populations kept at the center. Tagged individuals would also allow biologists to conduct controlled laboratory studies to better understand the CSRB and amphipods' reaction to changes in flow, energy availability (i.e., loads of particulate and dissolved carbon, EA Recovery Implementation Program, 2021), water temperature, and other environmental parameters- these parameters can be controlled individually in the lab; thereby, increasing our understanding of likely population responses to perturbations under field conditions.

Justification

Peck's Cave Amphipod is a diminutive (< 11-mm, USFWS, 2013), federally endangered species that occurs in the Edwards Aquifer and is the focus of ongoing monitoring efforts. The amphipod

is endemic in groundwater springs and nearby habitats of the Edwards Aquifer. Peck's Cave Amphipod is uniquely adapted to groundwater ecosystems where it tends to occur in the highest densities. The amphipod is adapted to these habitats via a laterally flattened body, and they lack eyes and pigment; however, much of the rest of the species life history in wild populations is unknown.

The CSRB is a federally endangered species that occurs in the Edwards Aquifer and is the focus of ongoing monitoring efforts. The beetle is endemic to the Comal and San Marcos spring systems. The CSRB is uniquely adapted to spring ecosystems where it tends to occur in the highest densities. The beetle carries a thin layer of air on its underside that allows it to breathe while it swims. Concerns related to groundwater pumping and extended dry periods are significant given the associated loss of water quality and quantity.

Because of the listing status and with the limited available habitat, refuge populations have been established that would also benefit from tagging in some cases. These populations are maintained so the wild population can be enhanced if recovering from unfavorable conditions (e.g., severe drought). As part of the propagation program, the USFWS and partners work to refine propagation methods and increase knowledge of the species. An opportunity to mark collected animals would allow them to be tracked over time and survival and reintroduction success could be evaluated. The challenge for these organisms is their size- tagging very small animals is more difficult when compared to larger animals and Peck's Cave Amphipod is < 11-mm long (estimated maximum size) and the adult CSRB is only ~2-mm long.

As tags have become smaller over time, their use has increased where individual or batch identification is needed. Passive integrated transponders (PIT), for example, have several characteristics that increased the accuracy of mark-recapture studies (Gibbons and Andrews 2004; Hewitt et al. 2010). Recaptures of small animals have been used for a variety of purposes including estimating sampling efficiency (Price and Peterson 2010), estimating population size (Pine et al. 2013), estimating survival (Moore et al. 2021) and growth (Walters et al. 2012), evaluating movement (Steffensmeier et al. 2022) and habitat use (Teixeira and Cortes 2007), and even studying animal behavior (McCormick and Smith 2004). Technological advancements have been impressive in recent years (Musselman et al. 2017). P-Chips are a relatively new tagging technology that has been used on small, endangered fish with success (Moore et al. 2021). Additionally, p-Chips have been successfully used on insects like the Western Honeybee *Apis*

mellifera (Tenczar et al. 2014) and Rock Ant *Temnothorax albipennis* (Robinson et al. 2014) by external adhesion. P-Chips are micro-transponder tags (500 x 500 x 100 µm) that are powered by a handheld laser wand that is connected to a computer. They are lightweight and have high retention in small fishes. The p-Chip could be attached externally using a non-toxic adhesive or internally on larger amphipods to allow individual identification.

Using p-Chips to tag Peck's Cave Amphipod and the CSRB including an examination of attachments procedures could be very beneficial for managing a refugia population. Therefore, our study goal was to evaluate the methods of tagging with p-Chips on both species and assess the response of tagging the species via the specific objectives listed below.

Objectives

1) Our first objective was to evaluate attachment of p-Chips and short-term tag retention on CSRB and Peck's Cave Amphipod. There are multiple ways to attach tags- both internal and external. We began simply by determining the appropriate location and material used to attach the tag. We developed an approach for tagging CSRB and assessed the feasibility of tagging amphipods using an alternative tag that was chosen based on our literature review.

2) Our second objective was initially to tag Peck's Cave Amphipod to determine longer-term retention of the tag and survival of the tagged animal. We had planned to use amphipods held at the San Marcos Aquatic Resources Center for this evaluation. However, after our initial evaluation, we moved forward using a surrogate beetle to meet this objective.

Methods

Evaluation of tagging amphipods

Laboratory studies were conducted at the San Marcos Aquatic Resources Center in conjunction with USFWS biologists because the source water is ideal for the species. Because Peck's Cave Amphipod was anticipated to molt every ~50 days, we planned to evaluate relatively short-term tag retention of externally placed and internal tags through a single molt. Although little is

known about their biology, these amphipods are assumed to reach adulthood in a year going through several instars; thus, examining tags through a molting cycle is critical to determine if the tags could only be used through one molt or longer. For this objective, p-Chips were affixed on the dorsal side of the amphipod (we used *Hyallela aztecta*, collected from Spring Island) using two different types of glue (non-toxic, cyanoacrylate-free superglue such as Loctite or Hopson and dental cement). We did not internally tag the amphipod because we decided that it was too likely to result in high mortality. Instead, we evaluated whether pChips could be externally attached and still allow for proper swimming.

Based on our initial evaluation, we decided to try a different tag based on our review of other tag types and the difficulty of tagging with pChips on the amphipod due to the body morphology (see evaluation of different tags).

Evaluation of tagging CSRB using pChips

The beetles used in our experiments were raised in a hatchery or collected from the wild by USFWS biologists. Adult CSRB were collected by USWFS staff using poly cotton lures following the methods of Gibson et al. (2008) and Hutson et al. (2015) for refugia stocks. Since 2000, a captive assurance colony of Comal Springs Riffle beetles has been housed at SMARC (Mays et al 2021). We used adult beetles from SMARC housed within custom aquatic holding units and fed matured biofilms. Due to an ongoing drought, availability of *H. comalensis* was relatively low; thus, we primarily used a surrogate species. *H. glabra* is hypothesized to be phylogenetically and ecologically similar as both species inhabit spring areas containing woody debris where they feed on biofilm (Bowles et al. 2003). *H. glabra* were collected from Finnegan Springs along the Upper Devils River, Val Verde County, Texas the week prior to our experiments.

We set up our first set of experiments to evaluate p-Chips. P-Chips are small (500 x 500 µm), laser-activated transponder tags that carry a unique nine-digit code. The laser is attached to a computer prior to reading tags. When activated by laser light from the externally powered ID reader wand (PharmaSeq), a unique identification number is transmitted from the reader and displayed on the tracking software. P-Chips can be repeatedly and rapidly read by the reader and its tag information recorded. This software is compatible with other computer programs; thus we can export and log data directly into MS Excel (Microsoft). One limitation of p-Chip technology

is that the p-Chip read range, is less than or about 7mm (Pharmaseq Inc. 2012). The ID reader wand can be placed on a stand and set up to continuously read tags. P-Chips have successfully been used in diverse taxa (e.g. ants (Robinson et al. 2009), crayfish (Huber et al. 2023), honeybees (Tenczar et al. 2014), mice (Gruda et al. 2010) and fish (Moore and Brewer 2021)) demonstrating its applicable potential across taxa.

Construction of treatment and control chambers

The treatment chambers which were used to house the experimental beetles were constructed to allow p-Chips to be read when the beetles moved from one chamber (clear PVC, see below) to the next. The chambers were constructed to allow the direction of water flow to be changed on a regular basis. The underlying assumption was that rheotactic organisms would move toward the direction of water flow; thus, allowing themselves to be actively scanned when moving from one tube to the other.

First, we constructed the center piece of the treatment chamber (Table 1; Figure 1). We joined the two schedule 40 polyvinyl chloride (PVC) reducing bushings (5.08cm x 1.27cm) using the schedule 40 female adapter fitting (5.08cm) on one end (this was not glued yet, see next paragraph). We then inserted one side of a thin polycarbonate tube, cut into a 12.7 -cm piece, into the 1.27cm opening of the PVC bushing. We placed two O-rings (1.746cm OD x 1.429 cm ID x 0.159cm) that were tightly wedged around the polycarbonate tube to create a tight fit with the tube and bushing. Next, we glued (polyurethane adhesive, The Gorilla Glue Company) around the polycarbonate tube allowing it to sink between the tube and the bushing. We added another bead of glue after the first one sank around the tube. We allowed it to dry for an additional 12 h. Lastly, a thin piece (~3-4 mm) of white (easier to see the beetles and a better walking material), loop Velcro was placed on the inside of the polycarbonate tube (our first trial used cotton cloth but was adjusted to this final design, see below). We removed the sticky backing for the portion of the Velcro that would be affixed to the bottom of the polycarbonate tube and left the backing on both ends that extended from each side. We left a tag end on each that was approximately 14-mm long.

Next, we attached the two clear PVC schedule 80 threaded tubes (5-cm diameter 10.16-cm long, hereafter clear PVC) to the center piece of the treatment chamber. We added a 5-cm wide strip of white Velcro (Velcro Brand, loops) on the bottom of each clear PVC tube. We made a thin cut in each piece prior to attaching the end of the Velcro (closest to the polycarbonate tube) to the inside of the clear PVC. We pulled the thin Velcro (i.e., tag end) that extended from the inside of the polycarbonate tube through the cut in the larger Velcro in the clear tube. We affixed the end of the thin Velcro beneath the larger Velcro before we removed the backing and attached the remaining portion of the Velcro to the bottom of the clear PVC. The latter step ensured that there was a continuous piece of Velcro from the bottom of the clear PVC through the polycarbonate tube and to the other side. After we completed this step, we attached the PVC bushing and adaptor using PVC cement. All PVC was attached using PVC primer and cement. Cementing the busing and adaptor had to be completed after the Velcro tag ends were run beneath the larger Velcro strips prevent the Velcro from twisting when the clear PVC tube was screwed to the bushing.

Lastly, we created the end pieces of the treatment chamber that would be where water enters and leaves the chamber. We compressed a piece of steel wire mesh (0.35mm) between a schedule 40 PVC reducing bushing (5.08cm x 1.27cm) and a PVC adapter to measure the amount of mesh needed for each fitting. We cut around the rigid indented portion and inserted the trimmed piece inside the PVC. The PVC adapter and bushing were then cemented together. This process was repeated to create for the other side. Then both threaded tubes were screwed onto the center structure. Lastly, we screwed two Nylon barb fitting onto each end.

We constructed the control chambers to ensure our experimental results were based on tagging and not extraneous factors (i.e., survival in the lab otherwise). The control chamber consisted of a clear, plastic container (1.84 L Rubbermaid), and the two PVC bulkhead water tank adapters (Table 2; Figure 2). First, we drilled two 3.81 cm holes on the shorter ends of each chamber, one near the top and one closer to the bottom to improve flow within the chamber. This is where the two bulkheads would be attached. Next, we placed the same wire mesh (as with the treatment chambers) into the female end of the bulkhead to prevent beetles from leaving the chamber when connected to the water source. Next, a hole was drilled (2.54-cm drill bit) into the flat portion of the 12.7 mm to allow water flow. The male end was then screwed into the female

portion to hold the mesh into place. To place the bulkhead onto the chamber, one of the larger flat pieces was placed on the outside with a gasket and one of the larger pieces, gasket, and male end were placed on the inside of the chamber. This arrangement left minimal places for a beetle to hide within the bulkhead for easier weekly beetle checks. A barbed polypropylene male hose fitting (12.7mm x 6.35mm, Proline series, wrapped in Teflon as with the treatment chamber) was then attached to the opposite end of the bulkhead that would later connect to the water inflow or outflow. Lastly, we added two, 5 cm wide and approximately 15-cm long pieces of white, loop Velcro to the inside of the chambers to serve as a non-slick substrate for the beetles. Although the control chambers were not the "same" as the treatment chambers, they required a different set up so that the beetles could be easily checked without much disturbance (would not have been possible in the other chambers).

Experimental design

We designed our experiment to estimate the survival of *H. comalensis* in a controlled, laboratory setting. For our first trial, we used 4 experimental chambers, two containing the endangered beetle, *H. comalensis*, and the other two chambers held tagged *H. glabra*. Each chamber housed 15 tagged beetles. No more than 20 beetles should be housed in a chamber of similar size to those designed (Bio-West 2016); thus, we wanted to be conservative by housing only 15 beetles in each chamber. After randomly assigning beetles to chambers, a hose was connected to the inflow side of the chamber and water allowed them to fill the chambers and discharge via a hose into a tank below. A p-Chip reader was mounted on a stand in the middle of each chamber. The laser was centered on the middle of the polycarbonate tube directly onto the piece of Velcro or the path of the beetle (Figure 1). The laser was set to continuously read during the duration of each trial. The first trial was run for ~90 days.

We made some adjustments after trial one due to some observations that led to an improved design. For our first trial, we used three control chambers to ensure survival estimates were related to a tagging effect. One control chamber housed untagged *H. comalensis* and two chambers housed untagged *H. glabra*. Each chamber contained 15 beetles that were randomly assigned to their respective chamber (except *H. comalensis* as it had only one chamber). Because of the low number of detections by tagged beetles in trial 1 (n = 7), we made some adjustments to the chambers. First, we only tagged *H. glabra* after the initial trial since they are a little larger,

more readily available for experiments, and appear to have higher survival (see results). Second, we adjusted the location of the reader each week (i.e., simply moving it up or down the clear connecting tube, to prevent the laser reading at the exact same location repeatedly. We also adjusted and used Velcro on the bottom of the chambers to ensure a better walking path for the beetles and improve the likelihood that they would be scanned. We also changed the metal mesh size from 74 microns to 400 microns to improve water flow but still not allow the beetles to be lost from the chambers. Lastly, we reduced the number of control beetles since we decided to move forward with one species, and we needed more beetles for the treatment chambers than the controls.

pChip tag Attachment

Cold exposure beetles

We first evaluate if we could use cold to slow the beetles' movements during the tagging process. Cold anesthesia is commonly used to temporarily immobilize individuals (i.e. honeybees, flies, etc.) during experimental treatments (Gooley and Gooley 2021, Nilson et al. 2006). Beetles exposed to cold environments will move slower (Overgaard and MacMillian 2017) thus, allowing us to quickly tag the beetle and allow the glue to dry. To determine if the effect of putting *H. comalensis* into the freezer had any effect on mortality, we set up an additional control chamber to house these beetles. We tagged 12 beetles, where approximately half were put in the freezer for 2-min prior to tagging and the other half were not. We hypothesized that there would be no effect as not chilling the beetles resulted in increased handling time while tagging. The beetles were tagged using the methods below. Because our results indicated there was no difference in mortality of chilled or non-chilled beetles (only 2 chilled beetles died, and 1 non-chilled beetle died after 52 days), we chose to move forward with chilling the beetles prior to tagging.

pChip experimental beetles

We tagged the beetles with p-Chips to evaluate both tag retention and estimate survival of the tagged beetles. We first tagged the surrogate species *H. glabra,* then tagged *H. comalensis*. We removed several *H. glabra* from the flow-through colony tube at SMARC and placed them into a separate, sterilized container filled with approximately a third full of well water which was

maintained cooled by placing a tub containing ice water under it. A digital thermometer was placed in the container with the beetles to ensure consistent cool water temperature (~13.2 C). Next, using entomology soft-tip forceps (wide tip 107.95mm, DR Instruments) individual beetles were haphazardly selected and placed onto a small receptacle containing cotton cloth wetted with deionized water to prevent desiccation under the microscope light. Under the microscope, (Nikon SMZ18 Research Stereo Microscope, 0.75-13.5x) we inspected each beetle to make sure it appeared healthy and mobile. Next, we took the receptacle containing *H. glabra* and placed it into a freezer (-18 C) to cold anesthetize the individual for 2-2.5 min. After 2 min, the receptacle was placed back under the microscope to ensure the beetle was relatively immobile. If the beetle was still active, the receptacle was placed back into freezer for another 30 sec.

We tagged each treatment beetle, ensured the tag was readable, and placed them in a recovery chamber. First, we used Kim Wipes to remove any excess moisture from the elytra of *H. glabra.* Next, using a pre-cut piece of metal wire (18-gauge), we added a small amount of super glue (cyanoacrylate glue) off center of the elytra of the beetle. Next, we used a wooden stick with a p-Chip attached to the end using water soluble glue (See Table A1) to accurately place the tag on top of the drop of superglue with the readable side upward. The glue was designed by Pharmaseq to attach tags in their injectors. The glue consisted of sodium carboxymethyl cellulose, water, and glycerol (rolled in a tube mixer for 24 h). The glue is water soluble; thus, it can be easily dissolved from the pChip and dissecting pointer once the tag has adhered to the beetle. We glued several of these tags and wooden sticks in advance to save time during tagging. We held the tag firmly in place for about 3 sec. Next, we used deionized water and gently rinsed the CMC glue so that it would dissolve and leave the p-Chip attached only to the beetle. In many instances, the wooden stick would pop off the glue without the need to add water. We checked the beetle for mobility as well as proper tag adherence. Finally, we scanned the tag to ensure it was readable and recorded it on our data sheet. Tagged beetles were kept in a recovery container. The recovery container had water from SMARC and was held in a second container that contained ice water to maintain the water temperature ~10-13 ℃. After the final beetle was tagged and recovered (i.e., actively moving), we randomly assigned to their treatment chambers.

Evaluation of additional tags

We searched the Web of Science database for publications (search years 2010-2023) that used tags to study insect ecology using the following search terms: (Insect AND Tag OR Tagging OR Telemetry OR Biotelemetry) OR Beetles OR Bees OR Ants OR Flies OR Invertebrates. We checked the title and abstract of retrieved papers and eliminated those with unapplicable content. Additional publications were discovered by examining reference lists of appropriate articles and through additional investigative searches using Google Scholar.

BEEtag attachment to beetles

Due to the presumed discontinuation of p-Chips (at least temporarily), other tagging options were necessary to secure conservation efforts involving long-term monitoring of CSRB. Another tagging option that would also allow for unique identification was preferable. We reviewed several tags that are suitable for certain small organisms based on our literature review (see Results), but one option seemed like it would have some flexibility for modification, was lightweight, and would allow individual identification.

The BEhavioral Ecology tag (BEEtag) is an open source, image-based tracking system in Matlab (Matrix Laboratory, MathWorks) that allows unique identification of a specific binary image printed on paper (Crall et al. 2015). Tags consist of a 5x5 code matrix of black and white pixels unique to each tag. This simple binary image matrix can then be visualized by any camera (i.e. phone camera) and subsequently identified. The BEEtag system was initially used for tracking individual honeybees, thus we modified BEEtags for use with CSRB. Due to their visual design, tags can be scaled to different sizes as needed. The primary advantage of BEEtags is their lightweight design (i.e., printed on waterproof paper) allowing for minimal tag weight on the organism. We believe that the lightweight design, scalability and lack of a homogenous background for tracking makes BEEtags an attractive choice for tagging specialized organisms such as CSRB.

We printed BEEtags on a single 8.5 x 11 sheet of waterproof, tear resistant paper printed on a high resolution (1200 dpi) laserjet printer. We used the Duracopy Waterproof Printer Sheets (Item No. 6511, JL Darling) for this experiment. This type of paper is made from synthetic resins and was chosen for its durability in water and resistance to degradation. BEEtags can be saved until used (as a .png file) and a single sheet contains 100 tags. Currently, there are 1800 unique tags that can be created. For the first tag attempt, we simply printed the tags as provided in the

original description (but on our specified paper). We adjusted the tag size to 1.1. x 1.1 mm in our second attempt. The BEEtag package can be downloaded from github.

Finally, we made the tags even smaller and improved their resolution. We reconstructed the BEETags by pixel using software available at Piskel.com. A blank canvas was opened on their webpage. After creating a black border (square), we copied the same pixel pattern from the PDF of known BEETags using the software paint tools. This was repeated until we had 100 tags. The file was saved as a png and printed (size was set to 67). The final tags were cut out with a razorblade under the dissecting microscope.

BEEtags were read using MATLAB (see Appendix A). The raw input for tracking is an image in color or grayscale. Software converts the values of an image to a binary (i.e. black and white) image, where zeroes are represented by black and ones represented by white. The software then finds all unique regions of white in the image and checks to see which are rectangular. Next, the software reads the converted binary pixel values within each white square and references them against the list of all viable tag codes. The tag codes are recorded and then returned to the user.

Survival analyses of pChipped beetles

We qualitatively evaluated tag retention of the different tags. The retention of PChips was evaluated throughout the tagging process and at the end of each trial. The retention of BEEtags on amphipods was only evaluated short term.

We developed survival curves to determine how pChips affected the survival of the beetles. Analysis- Kaplan-Meier curves (Goel et al. 2010) were used to visualize survival over time. We used a log rank test to compare the survival curves of each trial including the control. We used the “survival” package in Program R (Therneau 2020).

Results

Evaluation of tagging amphipods

We initially tagged 8 amphipods using pChips. After 24 h, all of the tags remained on the amphipods except for one. We did not assess survival of the cave amphipods (see beetle survival) and feel that pChips might be an option for amphipods given they are available, and survival is first assessed.

We also tagged 28 amphipods using BEEtags (a series of 8 and 20) (Figure 5). This tag was chosen based on our review of available tags (see review results below). The tags appeared to adhere to the amphipods fine. We obtained video showing that the amphipods did not have trouble swimming with the tag attached, assuming the tag was placed approximately central on the body. If the tag was offset too much, the amphipod had trouble swimming due to the drag created. Again, we feel this could be alleviated with a smaller and different shaped tag (which is very possible). Also, the smallest amount of glue possible distinguishes success from failure as too much glue results in morality.

Evaluation of tagging beetles using pChips

We were able to successfully tag beetles and figure out the best strategy for moving forward with the design (Figure 3). The tagged beetle displayed no issues with walking with the tag attached. The tagging procedure was best completed by chilling the beetle for 2 minutes and then tagging the beetle under the microscope. The beetle quickly regained activity as it was warmed by the microscope light. Using the water-soluble glue to release the tag once it adhered to the beetle was a good investment.

We began tagging our first set of experiments in January 2024. We successfully tagged 51 beetles using p-Chips, *21 H. comalensis* beetles and 30 *H. glabra* beetles, a surrogate species. A surrogate species was chosen due to lack of necessary CSRB to complete the trial and was also immediately available on site. *H. glabra* shares a similar ecology and morphology to CSRB, further justifying its use during this tagging trial. We ended trial 1 in April 2024 with the experiment lasting a total of 86 days. There were 7 living beetles in our treatments chambers at the end of the first experiment (all *H. glabra*). Alternatively, we had 10 living CSRB in chamber 1 and 14 living *H. glabra* beetles in control chambers 2 and 3. All of the living beetles retained their p-Chips, indicating high tag retention. Data collected from our scanners indicated detection

(n = 7 beetles) primarily during the first 3 weeks of the trial, with no beetles scanned in any of the subsequent months. Because of the low "recapture" rate, we could not use these data to estimate survival. However, we did adjust our chambers based on some of our observations (as reported in the methods).

We began our second series of experiments in June 2024. We were able to successfully tag 60 *H. glabra* beetles using p-Chips. We shipped our modified treatment chambers (see methods) and control chambers to SMARC three weeks prior to tagging as to promote internal biofilm before housing beetles. The beetles were tagged similarly to the previous trial. After tagging, we again randomly assigned all beetles to each of the four treatment chambers, with 15 beetles in each chamber. We placed 10 untagged beetles in a control chamber. We connected all chambers to proper flow and scanners to correct configuration. We ended trial 2 in October 2024 and the experiment lasted 140 days. Modifications to the mesh size of each chamber proved to help as beetle detections by scanners became much more frequent. We found only 2 beetles alive in the treatment chambers and 4 beetles alive in the control chamber at the end of this experiment.

Evaluation of additional tags

We found 10 additional tags (not including p-Chips) that have been used on a variety of small organisms (Table 3). Because small is relative, many of the tags would not be appropriate for tagging the CSRB or the amphipod. However, the BEEtag or VIE tag may be useful for very small organisms with some modifications. For example, the BEEtag could be printed on a more durable surface that may allow the tag to last longer and be read with camera equipment. VIE tags are used on small fishes and amphibians and would likely just need to be applied thinned with a fine mist sprayer or thin dropper (i.e., jewelry dowel). It is unclear the longevity of either tag given their retention was only evaluated in a single study (BEEtags) and on a different organism.

BEEtag attachment to beetles

We began our evaluation of BEEtags on beetles during our third tagging trial at SMARC in October 2024. We successfully tagged 25 beetles using BEEtags: 15 *H. glabra* and 10 CSRB**.**

We used the same tag procedure (Experiment two) only using BEEtags. We printed and cut out BEEtags prior to tagging. Aquarium glue was also used because it does not run in water. Once successfully glued, we took a picture of the tagged beetle using the microscope's photo capture software (Figure 6). After tagging *H. glabra* beetles, we placed them inside a control chamber. We then placed tagged CSRB beetles inside a treatment chamber.

We then uploaded pictures of beetles with BEEtags onto Matlab using code for tag identification (see Appendix A for code). In Matlab, the pixels in the image are converted to a binary one (i.e. black and white) where zeroes are represented by black and ones represented by white. The software then finds all unique regions of white in the image and checks to see which are rectangular. Next, the software reads the converted binary pixel values within each white square and references them against the list of all viable tag codes. The tag code is identified and then returned to the user as the image with ID code in red (Figure 6). Using this system, we were able to successfully identify beetles that were tagged.

With the BEEtag attached, the beetle showed no initial issues in carrying the tag when walking. However, we noted that when beetles were upside down, beetles had issues trying to right themselves. The tag was not too heavy but the shape of the tag (i.e., square) left the edged off the beetle and created some drag (Figure 6). We felt that making these tags even a little smaller and perhaps rounding the edged would make them a better option for beetle mobility.

Survival analyses of pChipped beetles

From the scanner detections, we were able to successfully construct 3 Kaplan-Meier treatment curves in R (version 4.4.0, R core team), for T1 (Figure 7)**,** T2 (Figure 8) and T4 (Figure 9) and our control chamber (Figure 10) (R code provided in Appendix A). Due to chamber T3 only having 2 detections during the experiment, we decided this was not enough information to be able to construct a survival curve for analysis. The treatment Kaplan-Meier curves indicated survival.

When analyzing all treatment plot graphs, we found that survival probability was approximately 0.50 after 25 days for the treatment beetles (with quite a bit of uncertainty). There was variability in survival among treatment chambers (Figures 7-9). Beetle survival was quite

low after 50 days indicating there is no need to extend experiments beyond that time. Alternatively, the survival probability of 0.50 in the control chamber was not reached until approximately day 100. We performed a log-rank test and found that all three Kaplan-Meier treatment curves were statistically similar to one other (Chisq= 0.5, df= 2, P=0.80). Further, when comparing treatment survival curves to the control survival curve, we found treatment Kaplan-Meier to be statistically different (i.e., lower) than the control (Chisq=6.8, df= 3, P=0.08).

We began a third series of experiments in October 2024. We tagged 45 *H. glabra* beetles using p-Chips. Due to our observations during previous trials on the effects of handling, we opted for soft-tip paintbrushes instead of forceps when moving individual beetles. We believe that this approach would result in less handling stress. We maintained our tagging procedure similar to previous trials except we replaced superglue with aquarium glue (Cyanoacrylate glue, WoldoClean) because it does not have a tendency to run if it touches water. Due to the relatively high number of mortalities discovered in our second set of experiments, we increased the number of control beetles to 15. This set of experiments is currently ongoing.

Conclusion

After evaluation of two tags, we feel the BEEtag has the most potential for future efforts related to tagging. The future availability of pChips is uncertain and using them appeared to reduce survival of the beetle almost 50%. The BEEtag is lighter weight and can be made to be smaller. The BEEtag also allows for individual identification. Other options that we are exploring are 1) the idea of printing the BEEtags onto waterproof sticker material which would eliminate the need for glue (and likely reduce mortality). However, retention would need to be evaluated to determine feasibility. Another option is to use silicone material to print BEEtags that would eliminate the need for glue. Lastly, changing the shape of the BEEtag (circular) and reducing the size more would reduce issues with the paper creating drag and possibly reducing mobility or even survival (if they cannot right themselves).

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Table 1. The supplies needed to construct the treatment chambers for evaluating tag retention and survival of tagged beetles. We provided the use of each piece (Description), the dimensions, quantity needed for each chamber, and the location (Purchased) and cost at the time that we purchased (US$).

Supply	Description	Dimensions	Amount needed for 1 chamber	Purchased	Cost (USD)
Stainless Steel wire mesh screen	Allows water flow but prevents beetle escape	40 mesh size (0.45 mm opening)	(2) 6cm sheets	AggFencer (Amazon)	$9.99
Nylon barb fitting	Allows for hose to be inserted into chamber allowing for water flow	6.35 x 12.7mm	2	Proline Series (Lowes)	$2.78
Sch[1] 40 PVC female adapter fitting	Allows for connections between	5.08cm	4	Charlotte Pipe (Lowes)	$3.12

	bushing and Sch 80 tube				
Sch 40 PVC[2] reducing bushing	Connects to poly carbonate rigid tubing	5.08 x 1.27cm	2	Charlotte Pipe (Lowes)	$4.70
Sch 40 PVC reducing bushing	Is connected to adapter and nylon barb	5.08 x 2.54cm	2	Charlotte Pipe (Lowes)	$3.65
Poly carbonate rigid tubing	Clear plastic tube allowing for laser to pass through	1.91 OD x 1.59 ID x 0.159 cm L= 12.7 cm	1	Small Parts (Amazon)	$17.02
PVC Sch 80, clear threaded tube	PVC pipe that is between 2 PVC adapters	5 cm diameter 10.16 cm long	2	Alsco Industrial Products	$42.33
White Velcro	Is placed inside rigid tubing	5 cm wide	NA	Velcro Brand (Walmart)	$8.99
Original Gorilla Glue	To connect clear polytube to the two subchambers	NA	NA	Gorilla Brand (Lowes)	$6.98
# 35 O-ring	Provides a waterproof seal around tube and tighter fit	1.746 OD x 1.428 ID x 0.159 cm	4	Danco (Lowes)	$3.13
Wire brush	A wire brush used for cleaning the mesh vents	NA	NA	Lavaxon (Amazon)	$8.49

PVC Purple Primer	8 fluid oz can of PVC primer	NA	NA	Oatey (Lowes)	$9.38
Medium Clear PVC Cement	8 fluid oz can of PVC cement used for PVC pipe	NA	NA	Oatey (Lowes)	$8.18

1. Sch – Schedule
2. PVC – Polyvinyl chloride

Table 2. The supplies needed to construct the control chambers where survival of control beetles will be evaluated. We provided the use of each piece (Description), the dimensions, quantity for each chamber, and the location (Purchased) and cost (US$) at the time that we purchased.

Supply	Description	Dimensions	Amount needed for 1 chamber	Purchased	Cost (USD)
Rubbermaid Brilliance plastic container	Container where control beetles will be kept	1.84 L	1	Newell Brands (Amazon)	$18.99
PVC Bulkhead water tank connector	Keeps mesh screen in place & connects to hose fitting	12.7mm female, 12.7 mm male, 38mm diameter	2	QuQuyi (Amazon)	$11.99
NPT barbed hose fitting	Polypropylene fitting that connects to hose	12.7 x 6.35mm	2	Banjo Corporation (Amazon)	$6.40
Teflon tape	Prevent water leaking	NA	NA	VOTMELL (Amazon)	$5.99
White Velcro	Placed inside container	5 cm wide	2 strips	Velcro Brand	$8.99

Table 3. List of tags resulting from our review. We list the general advantages and disadvantages and characteristics of each tag. Retention is based on the referenced study and could vary across studies and with changes to the tag location, placement, etc.

Tag Name	Overview	Advantages	Disadvantages	Est. cost	Size	Weight	Signaling	Retention
Photoluminescence (PL Tags)[1]	Made using paper coated with lead sulfide dots (PBS QDs)	Can be used to track distances >1000 ft	Vegetation obscures detections	$0.10	5mm	12.5mg	Detector that is sensitive to the same wavelength of tag	~ 4 days
eDNA[2]	Uses genetic material to determine presence	Can cover relatively large sample areas	False positives; Influenced by water volume & chemistry	Varies ~$159	N/A	N/A	PCR technique	N/A (but DNA can degrade)
Behavioral Ecology Tag (BEEtag)[3]	Binary matrix printed on paper	32,000 code combinations. High correct identification	Intense computational activity. High data storage	$0.15	2.1mm x 2.1mm	1.83mg	Picture is uploaded to software for identification.	~ 5 days
Harmonic Radar Tags[4]	Uses a high-powered microwave source to energize tag	Durable, inexpensive, easily applied	Possible entanglement	$1.00	13mm	2.7mg	Harmonic radar technology	~ 5 days
Metal Detection[5]	Pieces of aluminum foil	No impact on survival, can be detected up to 8 cm	Corrosion, most useful on sedentary organisms	$0.05	15 x 15mm	0.3 g	Metal detection equipment	3-6 mo

Radio Frequency Identification (RFID Tags)[6]	Transmits data using specific frequencies to a reader	Small size, can track movement	Short detection distance	$0.45	8mm x 1.4mm	30mg	Transponder reading device	3 mo
Visible Implant Elastomer (VIE Tags)[7]	Brightly colored liquid polymer tag	Non-toxic, highly visible	Difficult to apply, size limitations can occur	$0.06	Any	<10 mg	Visual mark	Varies
Acrillex Ink[8]	Ink applied to insect surface	Cheap, quick drying, water-based, easy to apply	No unique ID's	$0.05	N/A	<5mg	Visual Mark	6 mo
Retroreflective Tags[9]	Any reflective tag is analyzed by computer to detect tag in real time	Low-cost, lightweight, precision tracking	False positives	$0.05	Any	12mg	Retroreflective based tracking system (RTS)	5 days
Milligram-scale Multi-Modal Sensor (mSAIL)[10]	Miniaturized lightweight tracker	Can record light and temperature data	Custom tag	~$100	8 x 8 x 2.6mm	62mg	Motus Wildlife Tracking System	3 mo
Milligram-scale Multi-Modal Sensor (mSAIL)[10]	Miniaturized lightweight tracker	Can record light and temperature data	Custom tag	~$100	8 x 8 x 2.6mm	62mg	Motus Wildlife Tracking System	3 mo

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Figure 1. Image of treatment chamber where tagged beetles were housed during experiments evaluating tag retention and survival of tagged CSRB. The image on the top is a close up of the pChip reader positioned directly on top of the polycarbonate tube. The image on the bottom shows the treatment chamber (without the pChip reader).

Figure 2. Image of the control chamber designed to hold CSRB.

Figure 3. Image of a beetle tagged with a pChip.

Figure 4. Example of different sized BEEtags (QR codes).

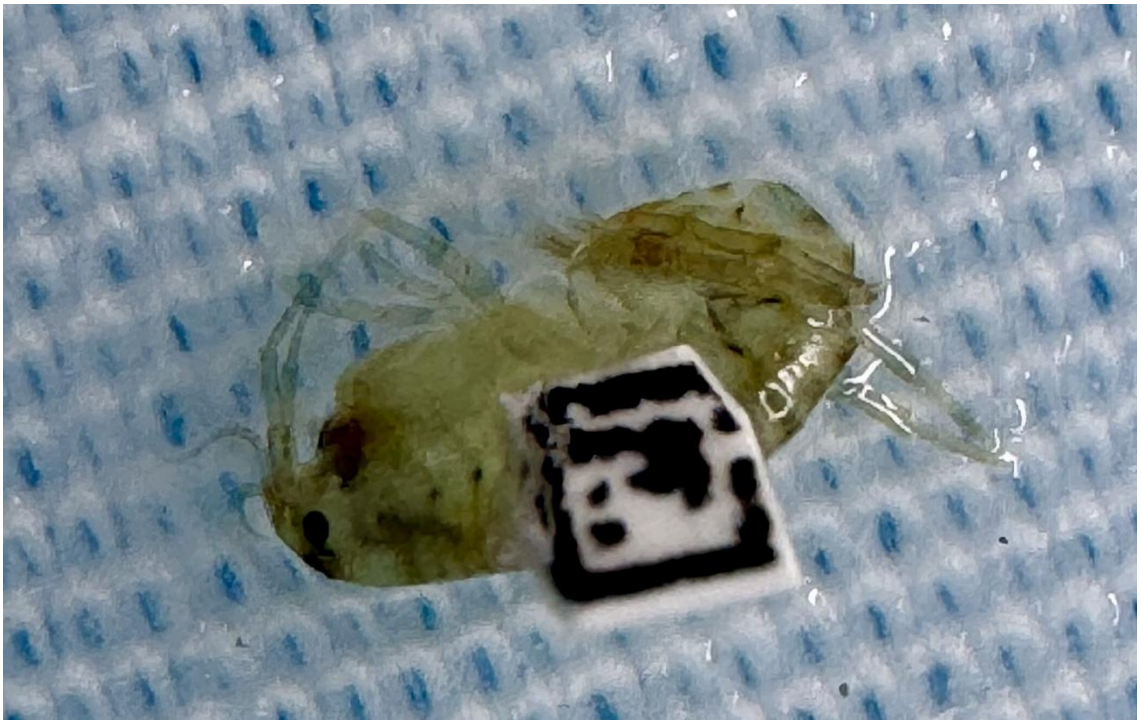

Figure 5. Example of an amphipod tagged with a 1.1-mm (1.3 mm with outside edge) BEEtag (QR codes).

Figure 6. Example of a riffle beetle with a BEEtag attached.

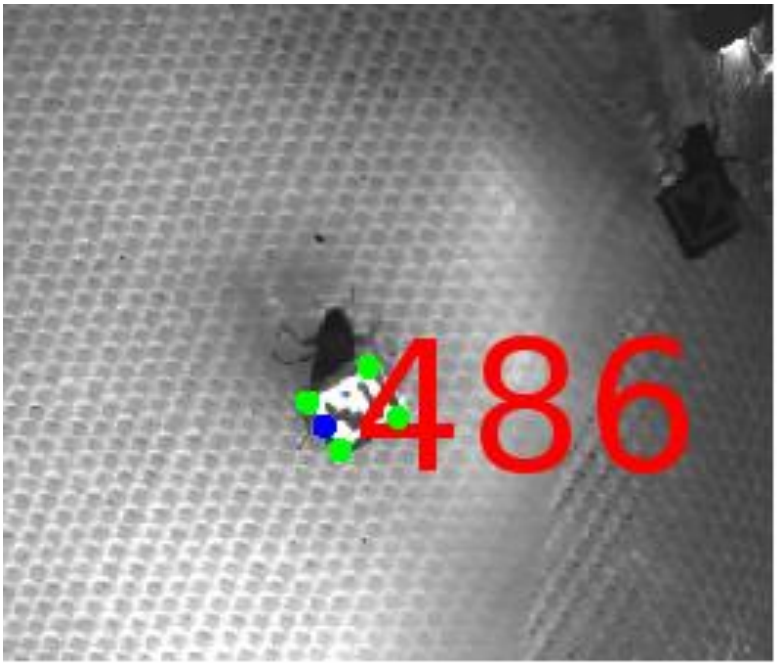

Figure 7. Image of BEEtag decoding performed using Matlab.

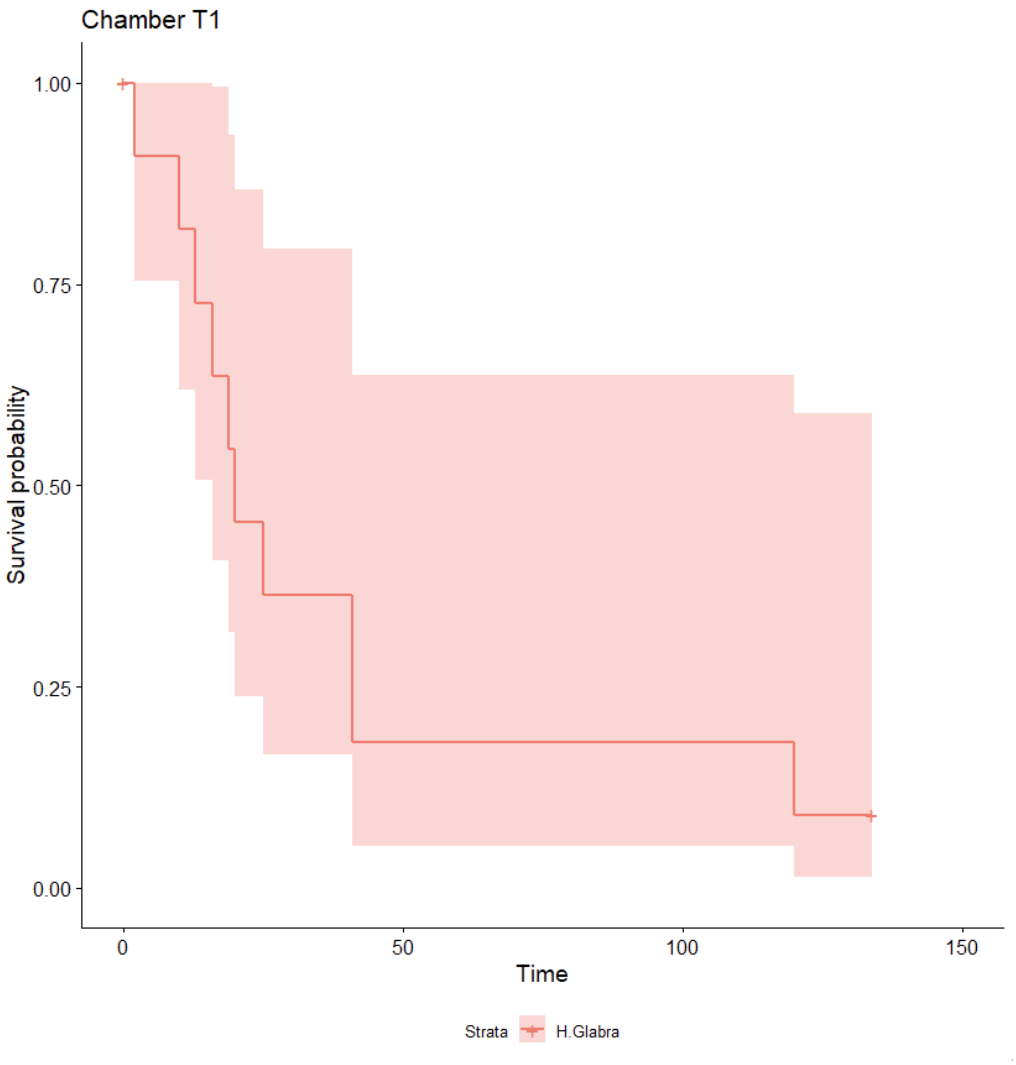

Figure 7. Kaplan-Meier plot for chamber T1. A plot of the Kaplan–Meier estimator is a series of declining horizontal steps which is assumed to approach the true survival function for that population, with a large enough sample size. The red line is the average survival, whereas the shaded area indicates the 95% confidence limits.

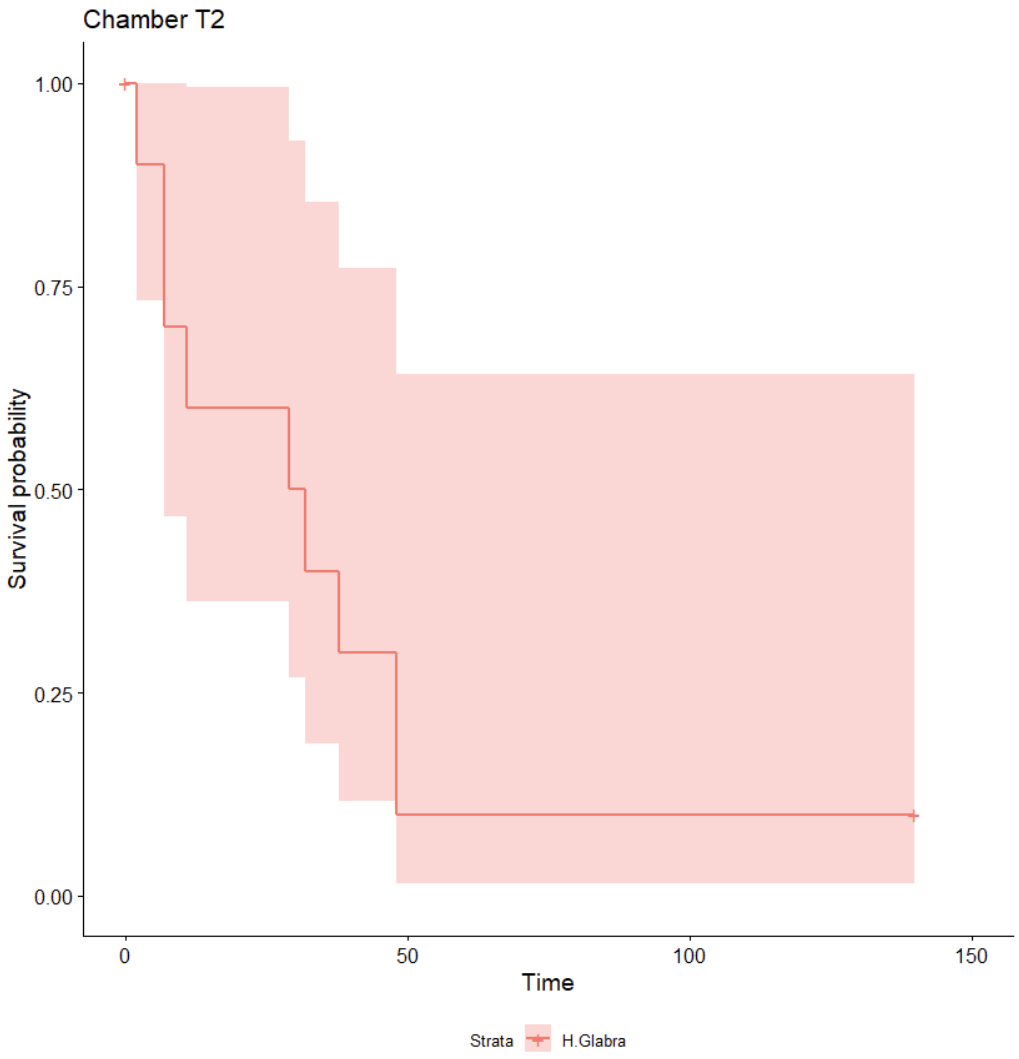

Figure 8. Kaplan-Meier plot for chamber T2. A plot of the Kaplan–Meier estimator is a series of declining horizontal steps which is assumed to approach the true survival function for that population, with a large enough sample size. The red line is the average survival, whereas the shaded area indicates the 95% confidence limits.

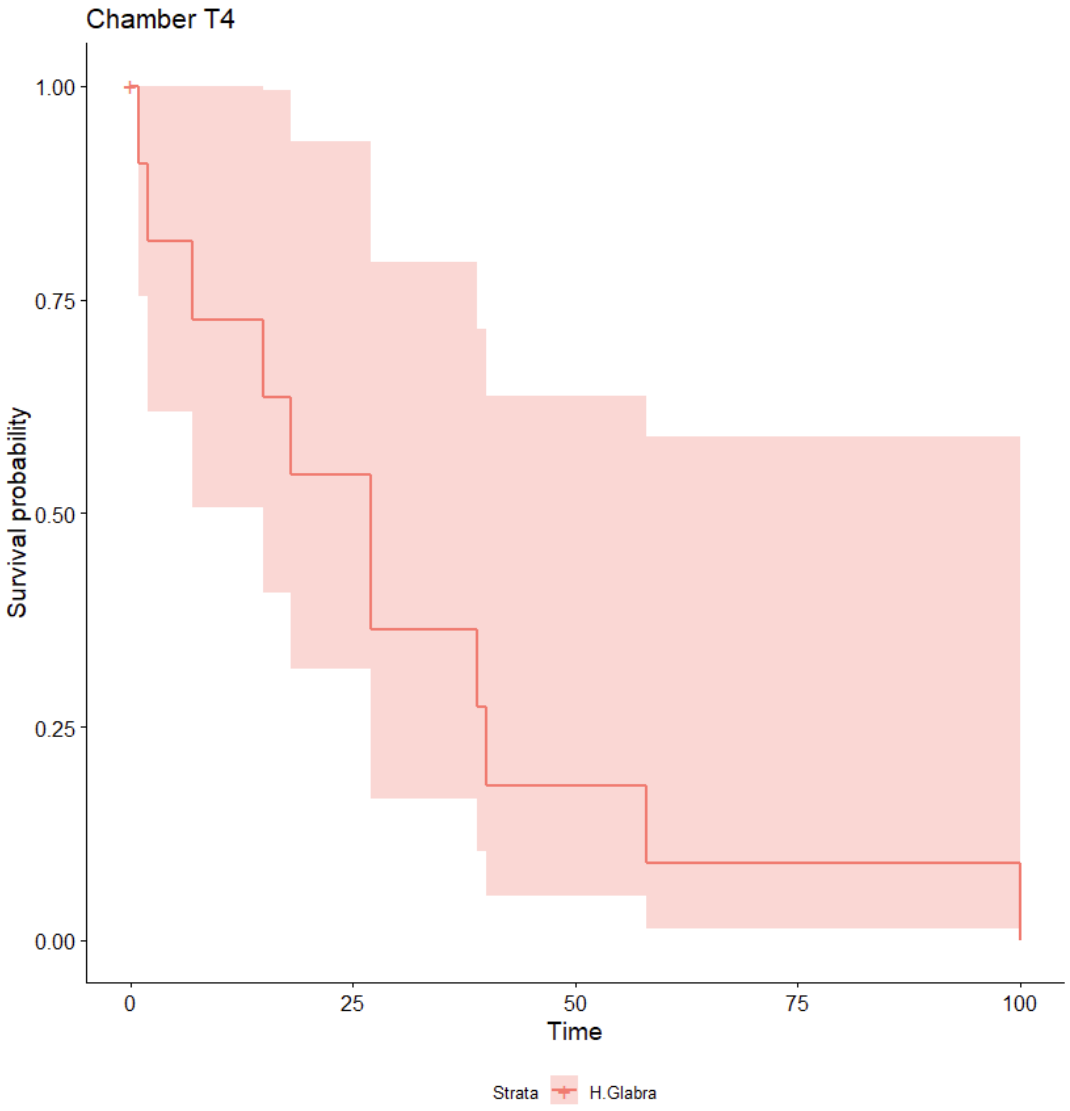

Figure 9. Kaplan-Meier plot for chamber T4. A plot of the Kaplan–Meier estimator is a series of declining horizontal steps which is assumed to approach the true survival function for that population, with a large enough sample size. The red line is the average survival, whereas the shaded area indicates the 95% confidence limits.

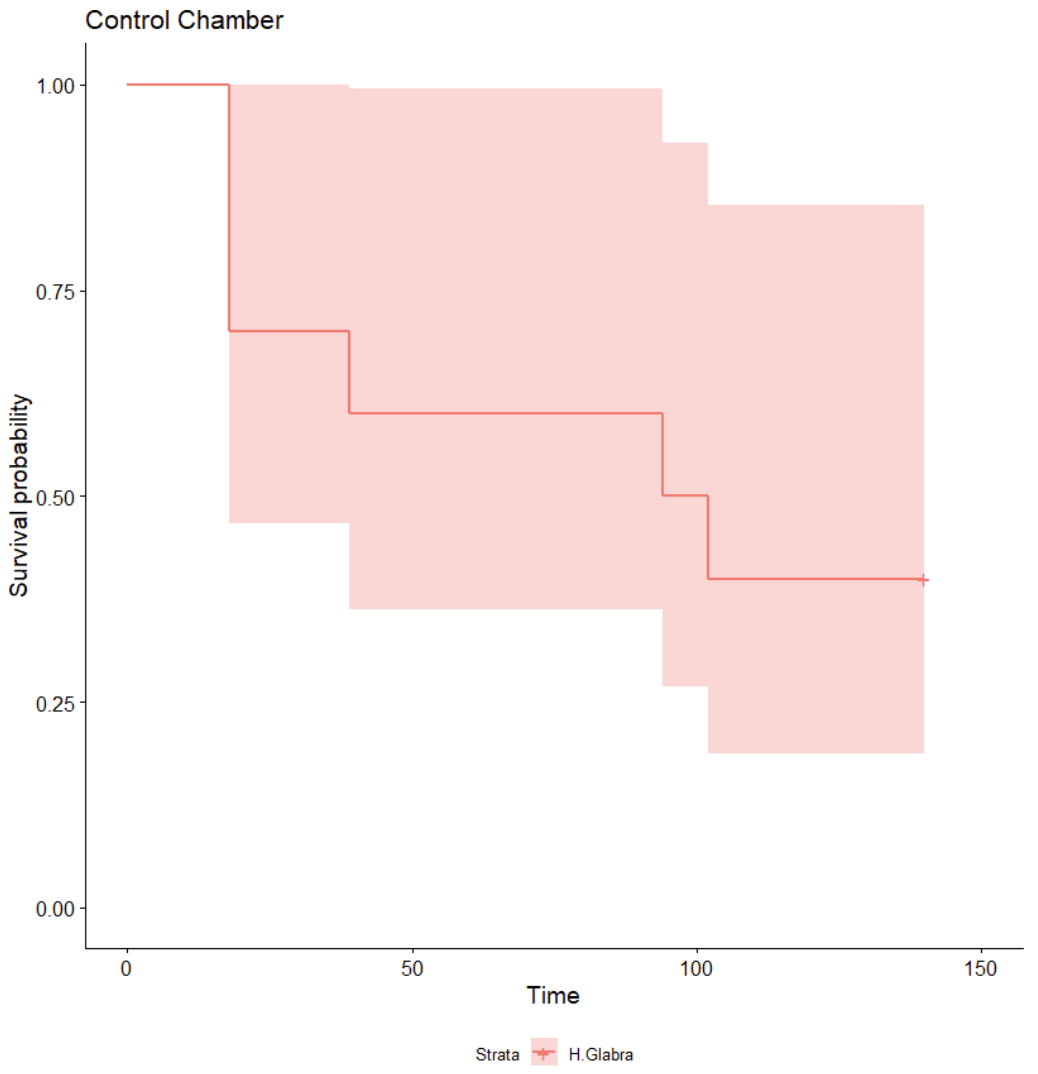

Figure 10. Kaplan-Meier plot for beetles held in the control chamber. A plot of the Kaplan–Meier estimator is a series of declining horizontal steps which is assumed to approach the true survival function for that population, with a large enough sample size. The red line is the average survival, whereas the shaded area indicates the 95% confidence limits.

Appendix A. Instructions for using BEEtag software.

The BEEtag package consists of a small library of functions that allow for the identification of the unique pattern found on each of the tags. To run BEEtags users must:

1. Download a repository of the code as a zip file. The entirety of the code is available at https://github.com/jamescrall/BEEtag.
2. After download, unzip file. Then create and name a personal file within the downloaded file. (This will serve as the location where one can upload tag pictures for later).
3. Users will need Matlab to run code. Matlab can be accessed either through the online web browser or by downloading the Matlab desktop application.
4. Once Matlab has been accessed, the user must add the downloaded file to the Matlab path, making sure to "Add with Subfolders"
5. Confirm that that the Image Processing and Statistics and Machine Learning Toolboxes are installed in Matlab, and if not install these (Select Home tab and then select Add-Ons -> Explore Add-Ons)
6. Run "trackingExample.m" to check functionality (example will be provided below)

Paper and printing

BEEtags can be printed on a single 8.5 x 11 sheet of waterproof, tear resistant paper printed on a high resolution (1200 dpi) laserjet printer. The type of paper we suggest is the Duracopy Waterproof Printer Sheets (Item No: 6511) available at: https://www.riteintherain.com/. For printing tags at a 1.1 x 1.1mm scale, the .png files provided must be printed by:

1. Find the .png file needed, right click print, and open it in your preferred printing browser
2. In the printing options, under quality select 1200 DPI
3. Then select scale, under custom enter scale = 65
4. Ensure that waterproof paper has been loaded into printer
5. Hit print

BEEtag Analysis

Matlab User Interface

After tags have been printed and cut out successfully. Tags can be identified with the software package primarily through the "locateCodes" function. This function takes a grayscale or color image and returns the locations and relevant information (e.g. ID, orientation) of any BEEtag located in the image. This process involves:

1. Take a picture of the desired tag (no more than two tags per photo) with a phone camera or digital camera. Ensure that the tag in the picture is properly focused.

2. Upload the picture to the computer where you are running Matlab. (Note: it is preferable to give each picture an individual and short name)
3. Next in Matlab under your Matlab path on the lefthand side, open the file that was created previously. Right click and select "upload files". Upload the desired picture.
4. Open a new document (script file) and run the code provided below:

```
im = imread('File name here.jpg');
figure(1);
subplot(2,2,1);
imshow(im);
title('Original image 1');
```

    - Note: The entire file name must be copied exactly (next to imread) to function including the file extension (.jpg or .png)
    - If successful, your image should show up on the right-hand side
    - If unsuccessful, refer to the software instructions above
5. Next, run the second half of the code

```
subplot(2,2,2);
codes =
locateCodes(im,'colMode',1,'threshMode',0,'thresh',0.4,'sizeThresh',[200
20000])
title('Tracked image 1');
```

    - If successfully done, image containing tag(s) should be identified through 4 green dots at the corners of each tag. The tag ID number should be displayed in red.
    - If no green dots or ID appear, refer to Troubleshooting below

Troubleshooting

Since BEEtag depends on visual information, performance can be substantially affected by:

- Uneven lighting (see below for more information)
- Tag or animal posture
- Tag cleanliness

Issues of uneven lighting can be computationally overcome by identifying codes at different threshold values, for example:

1. Recopy the second half of the Matlab code provided (drag and drop, there is no copy function in Matlab)
2. Next to the 'thresh' variable, delete the default amount (0.4), and type either a higher or lower value at 0.05 value increments
3. If the picture is suspected to have been taken in a low light environment, select a lower value, if in a well lit one, select a higher value

4. Repeat steps 1-3 until the correct ‘thresh’ value has been found. (Note: you are getting close if green dots appear)

Appendix A (continued). Code from our survival analyses completed using Program R.

R code for survival analyses

```
install.packages('survminer')
install.packages('survival')
library("survival")
library('survminer')
datum=read.csv(file.choose())
datumsurv=survfit( Surv(Time,Death)~1,
                type="kaplan-meier", data=datum)
datumsurv
ggsurvplot(datumsurv, legend.labs="H.Glabra", legend="bottom",title="Control Chamber")
summary(datumsurv)
T1_times=c(2,10,13,16,19,20,25,41,41,120,134,0,0,0,0)
T1_status=c(1,1,1,1,1,1,1,1,1,1,0,0,0,0,0)
T2_times=c(2,7,7,11,29,32,38,48,48,140,0,0,0,0,0)
T2_status=c(1,1,1,1,1,1,1,1,1,1,0,0,0,0,0)
T4_times=c(1,2,7,15,18,27,27,39,40,58,100,0,0,0,0)
T4_status=c(1,1,1,1,1,1,1,1,1,1,1,0,0,0,0)
control_times=c(18,18,18,39,94,102,140,140,140,140)
control_times_fix=c(control_times,rep(NA,5))
control_status=c(1,1,1,1,1,1,0,0,0,0)
control_status_fix=c(control_status,rep(NA,5))
survival_data=data.frame(
  time=c(T1_times,T2_times,T4_times,control_times_fix),
  status=c(T1_status,T2_status,T4_status,control_status_fix),
  group=factor(rep(1:4, each=15),labels=c("T1","T2","T3","Control"))
)
log_rank=survdiff(Surv(time,status)~group,data=survival_data)
log_rank
```

Matlab Code for Identifying Visual Photos of Beetags

```
im = imread('name here.jpg');
figure(1);
subplot(2,2,1);
imshow(im);
title('Original image 1');

subplot(2,2,2);
codes = locateCodes(im,'colMode',1,'threshMode',0,'thresh',0.4,'sizeThresh',[200 20000]);
title('Tracked image 1');
```

Appendix A (cont).

Table A1. Components needed to make water soluble glue (CMC glue) for attaching a tag to a stick for later placement on the organism. The glue is designed to come off the tag when it comes in contact with water

Chemical/ equipment	Description	Dimensions	Amount needed	Purchased	Cost (USD)
CMC[1]	Chemical component	N/A	2.5 g	Millipore sigma	73.20 (1x 100g)
Water[2]	Chemical component	N/A	48 ml	Thermo Fisher Scientific	118.00 (1x 1000ml)
Glycerol[3]	Chemical Component	N/A	2 ml	Millipore sigma	49.50 (1x 500ml)
Beaker	Container used for mixing components	250 mL	1	N/A	N/A
Centrifuge tube	Stores CMC glue after mixing	50 mL	1	N/A	N/A
Graduated cylinder	Used for measuring water	N/A	1	N/A	N/A
Digital hot plate/stirrer	Used for stirring mixture	N/A	1	N/A	N/A
Spatula	Measuring and breaking up clumps	N/A	1	N/A	N/A
Magnetic stir bar	Mixes components	NA	1	N/A	N/A

Weigh boat	For separating components into parts	N/A	8	N/A	N/A
Mixer/tube roller	Roller used for continual mixing of glue	NA	NA	BT Lab Systems	$666.00

1. CMC - Sodium carboxymethyl cellulose, Cat #419303-100G
2. Water – Invitrogen nuclease free water, Cat #AM9932
3. Glycerol – Cat #G7757-500ML
4. Mixer, tube roller, Cat #BT914

Procedure for making CMC adhesive

1. Ensure that the beaker, spatula, and stir bars to be used are sterilized before use. Make sure to wear gloves when preparing adhesive or handling reagents and glassware.
2. Using a 50ml graduated cylinder measure 48 ml of water and pour into the 250 mL beaker, containing a magnetic stir bar.
3. Set the stirrer to 450 rpm.
4. Divide the total CMC quantity (2.5g) to 8 parts, each of 0.3125g
5. Measure 8 near parts of 0.3125g in 8 separate weigh boats
6. Add each part slowly into the beaker at equal time intervals of 7.5 minutes.
7. Monitor the solution for clumps and use a spatula to dissolve the clumps during the above step.
8. Add 2ml of glycerol into the beaker and keep stirring the solution for an additional 20 minutes.
9. Pour the contents of the beaker into a 50ml centrifuge tube and rotate the tube on the roller mixer at 20rpm for 24hr, before moving at 4C for long term storage.
10. The solution at this stage is ready to be used.

Appendix E | **Conservation genetics of *Stygobromus* in Texas** (Final Report)

Conservation Genetics of *Stygobromus* in Texas

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Abstract

We generated genotying-by-sequencing data to explore genetic variation within and among nominal species of *Stygobromus* cave amphipods from the Edwards Plateau in central Texas. This region is home to at least 10 species of cave amphipods, including the endangered *Stygobromus pecki*. We found support for all of the nominal species in addition to some cryptic variation within at least one of the species. However, morphological characters were observed to be frequently unreliable or inaccurate, presumably because of convergence or variation within lineages. *S. pecki* from Comal Springs, New Braunfels, Texas, was found to be monophyletic with little detectable structure within the species and therefore no evidence of restricted gene flow within the Comal Springs complex. Temporal samples from two spring sites within Comal Springs that were sampled twice, more than 15 years apart, show little signs of differentiation suggesting the maintenance of relatively large effective population sizes over the interval. Another group of individuals from several localities south and west of Comal Springs were observed to be closely related, but differentiated from *S. pecki*. More extensive sampling of this group will be required before a clear understanding of their taxonomic status and relationship to *S. pecki* is possible.

Keywords: Stygobromus, phylogenetic inference, genetic diversity, conservation genetics

Introduction

Amphipod crustaceans of the genus *Stygobromus* (Amphipoda, Crangonyctidae) are commonly called cave amphipods and occupy subterranean aquatic habitats in North America (Culver *et al.* 2010) where at least 137 species have been described (Väinölä *et al.* 2008, Gibson *et al.* 2021), although new species continue to be described (e.g. Taylor & Holsinger 2011, Cannizzaro *et al.* 2019, Niemiller *et al.* 2024). Much of the diversity in the genus comprises isolated lineages (i.e. short-range endemics) confined in aquifers, caves and other hyporheic areas. The amphipods generally exhibit dispersal limitation which presumably contributes to the evolution of their biodiversity through allopatric speciation (Holsinger 1967). These stygobionts also share several morphological characteristics attributable to their subterranean habits, including eyelessness, a general lack of pigmentation, narrow bodies, and elongated appendages. Thus, isolation in similar, rather homogenous habitats, has contributed to substantial convergence within *Stygobromus* (Culver & Pipan 2015, Devitt 2019). Despite detailed morphological treatment of the genus by J. R. Holsinger and colleagues (Holsinger 1966, 1967, 1969, 1972, 1978, Holsinger *et al.* 2009, Culver *et al.* 2010), the combination of short-range endemism and convergent morphology suggests that the current estimate of diversity in *Stygobromus* is an underestimate (Gibson *et al.* 2021).

The Edwards Plateau region of south-central Texas is a region of uplifted limestone containing aquifers, numerous springs and other karst features. The area has a concentration of *Stygobromus* endemism in North America and is home to at least ten species (Gibson *et al.* 2021). Included in this diversity is the endangered *Stygobromus pecki*, the Peck's cave amphipod, which has been found only along an approximately 1,300m reach of Landa Lake in Comal Springs, New Braunfels, Texas and at the nearby Hueco Springs, Texas (Gibson *et al.* 2008). To further complicate the assessment of diversity in *Stygobromus* amphipods in the Edwards Plateau, some species are known to co-occur in specific localities. For example, the Artesian Well on the Texas State University campus (TSU Artesian Well) in San Marcos, Texas has been monitored for more than 125 years during

which four species of *Stygobromus* have been recorded (*S. bifurcatus*, *S. flagellatus*, *S. longipes*, and *S. russelli*) (Holsinger & Longley 1980, Hutchins *et al.* 2021). The newly described *S. bakeri* is sympatric with at least three congeners (*S. bifurcatus*, *S. russelli* and a member of the *S. flagellatus* species group) at Jacobs Well in central Texas (Gibson *et al.* 2021). Sympatry creates the potential for hybridization and presents challenges for identification and accurate diagnoses of species and species boundaries.

Molecular genetic investigations might facilitate more accurate biodiversity assessment for *Stygobromus*. Indeed, genotyping-by-sequencing (GBS) data have been employed to address questions about population structure in *Stygobromus* in karst habitats (Lucas *et al.* 2016, Ritter *et al.* 2023). Previous molecular investigations of central Texas *Stygobromus* using mitochondrial DNA (mtDNA), a single copy nuclear gene sequence marker (ITS) and AFLPs indicated that some nominal species might harbor cryptic diversity (Ethridge *et al.* 2013). MtDNA variation in particular indicated the potential of multiple lineages within *S. russelli*, *S. flagellatus*, *S. longipes* and *S. dejectus*. Further, two groups of mtDNA cytochrome oxidase I haplotypes were detected within *S. pecki*, which, while forming sister clades in phylogenetic analyses, were quite distinct with mean uncorrected sequence divergence of 2.3%. AFLP data suggested structure within *S. pecki* that very roughly paralleled the mtDNA division (Ethridge *et al.* 2013). Lucas *et al.* (2016) used genotyping-by-sequencing methods to generate a SNP data set for *S. pecki* and found very low levels of differentiation across the range of the endangered species and no evidence of the structure detected in mtDNA sequence data (Ethridge *et al.* 2013). However, despite these previous studies, we do not have a clear picture of *Stygobromus* diversity in the Edwards Plateau region or an understanding of the placement of *S. pecki* within that diversity. Here, we extend these previous investigations by generating genome-level GBS data from a broad, though still not exhaustive, sampling of *Stygobromus* diversity in the Edwards Plateau region of central Texas. We consider this a first genome-level exploration of the species richness of *Stygobromus* diversity in the region.

We also focus on *S. pecki*. Exploration of standing genetic variation within *S. pecki* is especially relevant due to the recent establishment of captive propagation of this endangered species by the U.S. Fish and Wildlife Service at the San Marcos Aquatic Resources Center, San Marcos, Texas. A clear understanding of the range of *S. pecki* and population structure within the species will facilitate effective propagation by providing baseline information. These data can be used to measure genetic variation by which the efficacy of captive propagation can be evaluated. They can also be used to design propagation methods that minimize inbreeding and avoid outbreeding depression.

Given the richness of cave amphipod species in the central Texas Edwards Plateau region, the likelihood of convergent evolution, the evidence of cryptic diversity and the critical need for molecular data to inform conservation management of the endangered *S. pecki*, we initiated an investigation using genotyping-by-sequencing (GBS) methods. We collected population genomics data from nine nominal species to explore evolutionary relationships and genetic structure. We attempted to include as much of the *Stygobromus* diversity of the Edwards Plateau region as possible, including many samples from Ethridge *et al.* (2013) plus additional sampling. We asked whether patterns of genomic differentiation correspond to the nominal taxonomy of *Stygobromus* in the region and whether there is evidence for cryptic variation or any admixture among sympatric lineages. We also focused specifically on *S. pecki* to describe population structure within this endangered species that would inform the captive management strategies and to clearly delineate its range.

Methods

DNA sequencing and data collection

We collected *Stygobromus* amphipods from 36 locality by taxon combinations (Table 1, Figure 1) from 2004 to 2024. Collections were made using drift nets, a cloth-capture technique (Gibson *et al.* 2008) at surface springs, bottle traps in wells, and hand collection with dip nets in caves. Specimens were typically stored in 95% ethanol until DNA extraction. Sampling of *S. pecki* was conducted under permits from the United States Fish and Wildlife Service (permit TE676811) and the Texas Parks and Wildlife Department (permits SPR-0390-045, SPR-0622-090). These samples included individuals from Ethridge *et al.* (2013) as well as new collections. Two *S. pecki* sites (Comal Springs Run 3 (17, 18) (we use locality number in parentheses to identify localities following Table 1 and Figure 1) and Comal Springs Spring Island (21, 22)) were collected twice: in 2008 and again in 2023-2024. These temporal samples of *S. pecki* were used to estimate any changes in diversity over time.

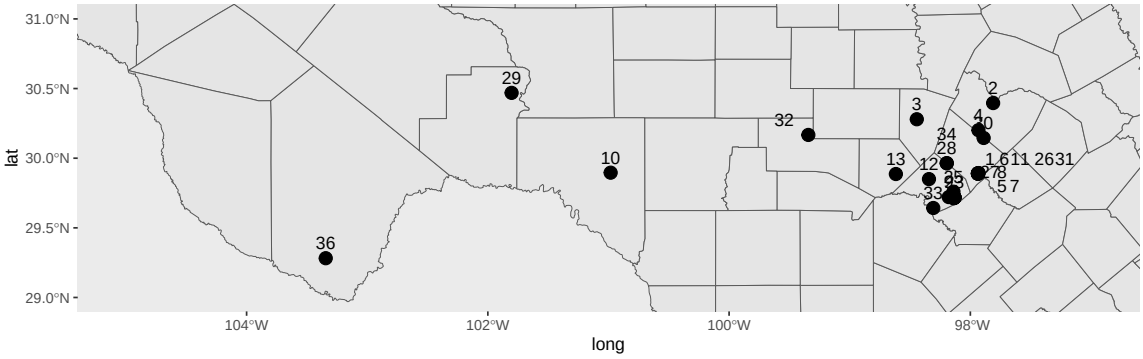

Figure 1: Map of *Stygobromus* sampling localities in the Edwards Plateau region of central Texas. Site numbers correspond to Table 1.

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Table 1: Sampling details. Locality numbers correspond to those in Fig. 1.

Locality x Taxon Number	**Nominal Taxonomy**	**Locality**	**County**	**n**	**Revised Taxonomic Hypothesis**
	S. bifurcatus				
1		TSU Artesian Well	Hays	1	*S. bifurcatus*
2		Bull Creek	Travis	2	*S. bifurcatus*
3		Cold Spring	Travis	2	*S. bifurcatus*
4		Faurie Well	Travis	15	*S. bifurcatus*
5		Sessom Spring	Hays	1	*S. bifurcatus*
	S. flagellatus				
6		TSU Artesian Well	Hays	50	*S. russelli* (1), *S. longipes* (29), *S. bifurcatus* (2), *S. flagellatus* (18)
7		Diversion Spring	Hays	16	*S. longipes* (2), *S. russelli* (1), *S. flagellatus* (13)
8		Sessom Spring	Hays	2	*S. flagellatus*
9		Mission Valley	Comal	2	*S. flagellatus* (1), *S. nr. dejectus*? (1)
10	***S. hadenoecus***	Devil's River	Val Verde	14	*S. hadonecus* (13), *S. tenuis* 1 (1)
	S. longipes				
11		TSU Artesian Well	Hays	13	*S. longipes*
12		CM Cave	Comal	2	*S. tenuis* 2 (1), *S. nr. dejectus*? (1)
13		Cave without a name	Kendall	4	*S. nr. dejectus*?
	S. pecki				
14		Panther Canyon Well 08	Comal	6	*S. pecki*
15		C Spr Run 1 23	Comal	3	*S. pecki*
16		C Spr Run 2 23	Comal	7	*S. pecki*
17		C Spr Run 3 08	Comal	16	*S. pecki*
18		C Spr Run 3 23	Comal	43	*S. pecki*
19		C Spr West Shore 23	Comal	35	*S. pecki*
20		C Spr upwelling 08	Comal	1	*S. pecki*
21		C Spr Spring Island 08	Comal	18	*S. pecki*
22		C Spr Spring Island 23	Comal	33	*S. pecki*
23		C Spr Yule	Comal	3	*S. pecki*
24		Need info	Comal	12	*S. pecki*
25		Hueco Springs	Comal	12	*S. russelli* (3), *S. nr. dejectus*? (9)
	S. russelli				
26		TSU Artesian Well	Hays	10	*S. russelli* (5), *S. longipes* (5)
27		Sessom Springs	Hays	20	*S. russelli*
28		John Knox Ranch	Comal	15	*S. russelli*
29	***S. tenuis***	Caroline Springs	Terrell	14	*S. tenuis* 1 (6), *S. tenuis* 2 (8)
30	***S. balconis***	Autumn Woods Well	Hays	9	*S. balconis* (4), *S. russelli* (5)
31	unknown (juvenile)	TSU Artesian Well	Hays	55	*S. flagellatus* (10), *S. longipes* (45)
32	unknown	Fessenden Spring	Kerr	11	*S. nr. russelli* (2), *S. nr. flagellatus* (1), *S. nr. dejectus*? (8)
33	unknown	Garden Ridge Well	Comal	16	*S. nr. dejectus*? (15), *S. flagellatus* (1)
34	unknown	John Knox Ranch	Comal	2	*S. russelli* (2)
35	unknown	Jacobs Well	Hays	1	*S. flagellatus* (1)
36	***S. hubbsi***	Oak Springs	Brewster		*S. hubbsi*?

Sampled individuals were identified to nominal species using the characters, descriptions, and keys for *Stygobromus* (Holsinger 1967), with an emphasis on taxa known from the central Texas area (Gibson *et al.* 2021). Because the key characters are found in mature individuals, some juveniles were classified as "unknown". Further, some apparently mature individuals were also classified as "unknown" when species-level identifications were not possible or clear, or when specimens were damaged and missing parts. DNA was then extracted. Samples from Ethridge *et al.* (2013) were extracted using the Gentra Systems Puregene DNA Purification kit. The remaining samples were extracted using the DNeasy Blood and Tissue Kit (Qiagen Inc., Alameda, CA, USA).

We created GBS libraries following the methods of Parchman *et al.* (2012) and Gompert *et al.* (2014). In brief, DNA was digested with EcoR1 and Mse1 restriction enzymes. Then, Illumina adapters with 8-10bp multiplex identifier sequences (MIDs) were ligated to fragments which were then amplified in two rounds of PCR with the high fidelity iProof polymerase (BioRad, Inc.). Individual libraries were pooled and the pooled library was shipped to the University of Texas at Austin Genome Sequencing and Analysis Facility (Austin, Texas) where size selection of fragments between 250-400bp was performed with a Blue Pippin (Sage Science Inc., Beverly, MA, USA) and the resulting reduced representation libraries were sequenced on two lanes of Illumina Novaseq 6000 (single read, 100bp).

Assembly, alignment and variant calling

PhiX sequences were removed from the resulting sequence reads by assembly to the PhiX genome using bowtie version 1.1.2 (Langmead et al., 2009). We used a custom script to remove MIDs from each read and to filter short reads and reads that contained Mse1 adapter sequence. Sequence reads were written to individual files in fastq format. The median number of sequence reads per individual was 1,488,039. At this stage, 26 individuals with less than 300,000 total reads were removed from further analysis.

Because previous data suggested that some divergences between lineages of *Stygobromus* cave amphipods in central Texas might be relatively deep (Ethridge *et al.* 2013), we adopted a hierarchical approach to our analyses. First, we assembled and called SNP loci across the entire set of samples and used these SNPs to infer phylogenetic relationships among individuals. Then, based on the topology of our phylogeny, we subdivided the samples into smaller groups for population-level analysis where the probability of restriction site evolution was minimized.

For the phylogenetic SNP set, sequences were assembled following the methods described in *dDocent* (Puritz *et al.* 2014). First, we identified unique sequences that had at least four reads within an individual and then identified those reads that were also present in at least four individuals. Next, we used CD-HIT (version 4.8.1) to cluster these filtered sequences and required a minimum sequence homology of 0.9 (Li & Godzik 2006, Fu *et al.* 2012). This resulted in 25,555 unique contigs that we used for a reference-based alignment using the ALN and SAMSE algorithms in BURROWS-WHEELER ALIGNER (BWA version 0.7.18-r1243-dirty) (Li & Durbin 2009). We aligned all reads and allowed a maximum number of differences of four, a seed length of 20, a maximum of two differences in the seed, and set the quality threshold to 20 for trimming sequences to 35bp.

After assembly and alignment, we identified single nucleotide polymorphisms (SNPs) using BCFTOOLS (version 1.19 (using HTSLIB 1.19)) and the commands MPILEUP and CALL (Li *et al.* 2009). For MPILEUP, maximum raw per-file depth was set to 8,000 and indels were skipped. For CALL we used the consensus-caller, again skipped indels, kept only variant sites, set the p-value threshold to 0.05, and set the prior mutation rate to 0.001. These were further filtered using custom

perl scripts requiring the minimum number of sequences per site to be 2 x number of individuals, the absolute value of the Wilcoxon-Mann-Whitney rank sum tests for base quality bias, mapping quality bias, and the read position bias to be less than 1.96, minor allele frequencies to be greater than 0.05, minimum mapping quality of 30, and the maximum number of individuals with missing data for a site to be 50% of individuals. We used a custom script to extract SNP data from the variant call format file and write them in a nexus format file that contained the concatenated SNP alignment for all individuals. At this point, an additional 37 individuals with greater than 20% missing data in the phylogenetic matrix were removed from further analysis. This left a total of 466 individuals (Table 1). SNPs were re-identified and filtered for this final set of individuals: similar to above, we required the minimum number of sequences per site to be 932 (2 x number of individuals), the absolute value of base quality, mapping quality, and read position rank sum tests to be less than 1.96, minor allele frequencies to be greater than 0.05, minimum mapping quality of 30, and the maximum number of individuals with missing data for a site to be 233 (50% of individuals). Finally, we removed any sites where the read depth exceeded 234,546 (equal to the mean sequence depth across sites plus two standard deviations; this filter is designed to remove potentially paralogous reads). This resulted in 23,103 variable sites.

Phylogenetic Analysis

We used these SNPs to construct a phylogenetic tree using approximate maximum likelihood with the program FASTTREE (version 2.1.11 Double precision (No SSE3)) (Price *et al.* 2010). We ran FASTTREE using the generalized time-reversible model for a nucleotide evolution and 10,000 re-samples to estimate bootstrap values for each split. The resulting tree file was imported into R (version 4.4.2) to visualize the tree using GGTREE (R Core Team 2022, Yu *et al.* 2017). Given the diverse evolutionary history of the individuals included in this project, we used the resulting phylogenetic tree to partition the individuals into smaller groups, where we expect greater overlap in genetic markers produced by our reduced representation approach (e.g. minimizing the extent of missing data due to restriction site evolution). This resulted in three sub-groups. The first partition included mainly individuals nominally identified as *S. bifurcatus*, *S. balconis*, *S. russelli* and *S. longipes* plus others including unknown individuals (Group 1) (Figure 3). The second partition included the rest of the samples, including individuals nominally identified as *S. flagellatus*, *S. tenuis*, *S. hadenoecus*, *S. pecki* and others including unknown individuals (Group 2) (Figure 3). Lastly, we specifically focused on the clade that included individuals identified as *S. pecki* and very closely related individuals (Group 2a) (Figure 3).

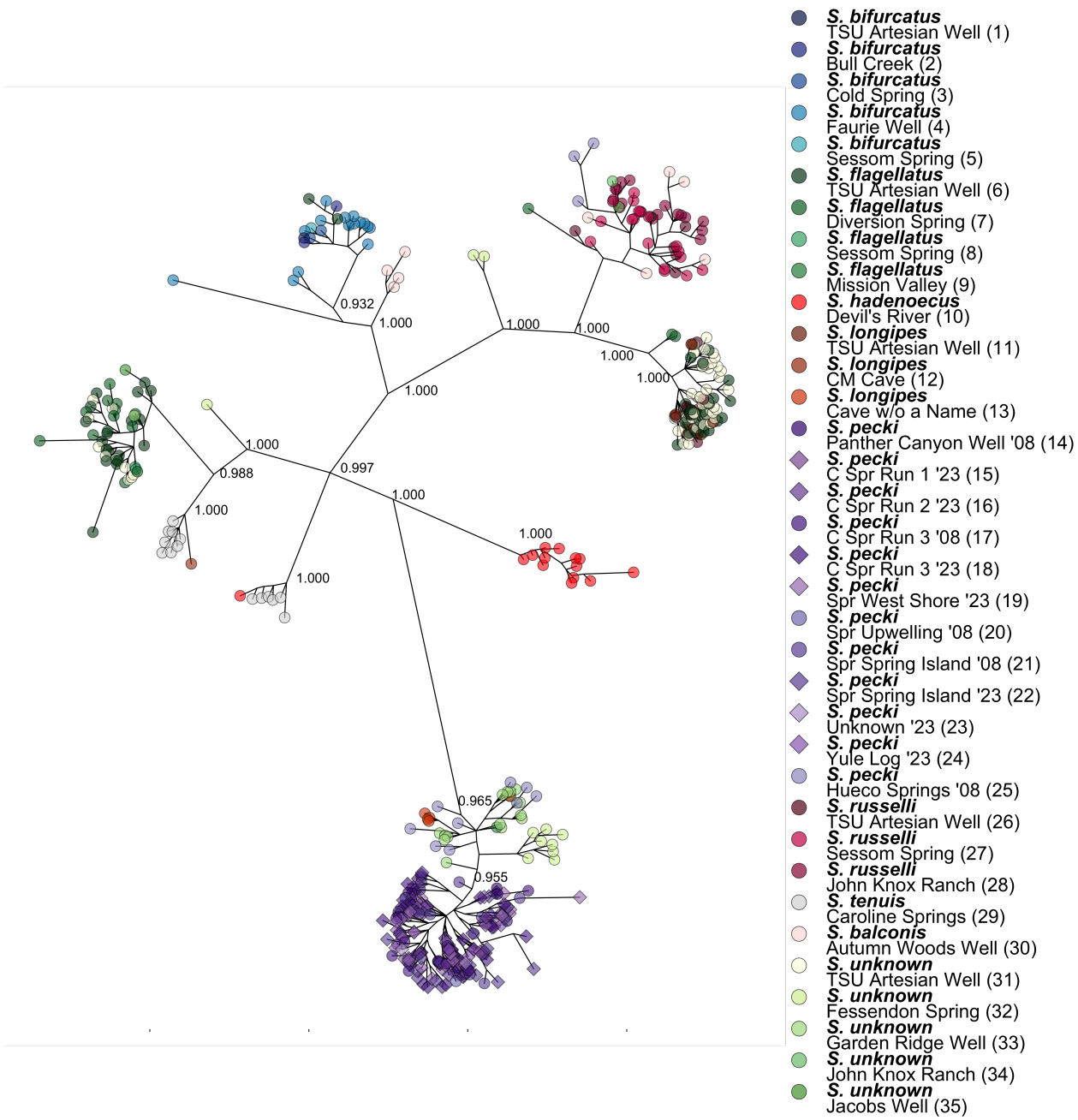

Figure 2: Phylogenetic tree of *Stygobromus* estimated using approximate maximum likelihood in FASTTREE (Price *et al.* 2010). We used the generalized time-reversible model with 10,000 resamples to estimate bootstrap values based on 23,103 single nucleotide polymorphisms (SNPs). Individuals are color coded by their sampling location and nominal species designation. Samples collected prior to 2016 are displayed with a circle, and individuals collected in 2023-2024 are displayed with a diamond (*S. pecki* only)

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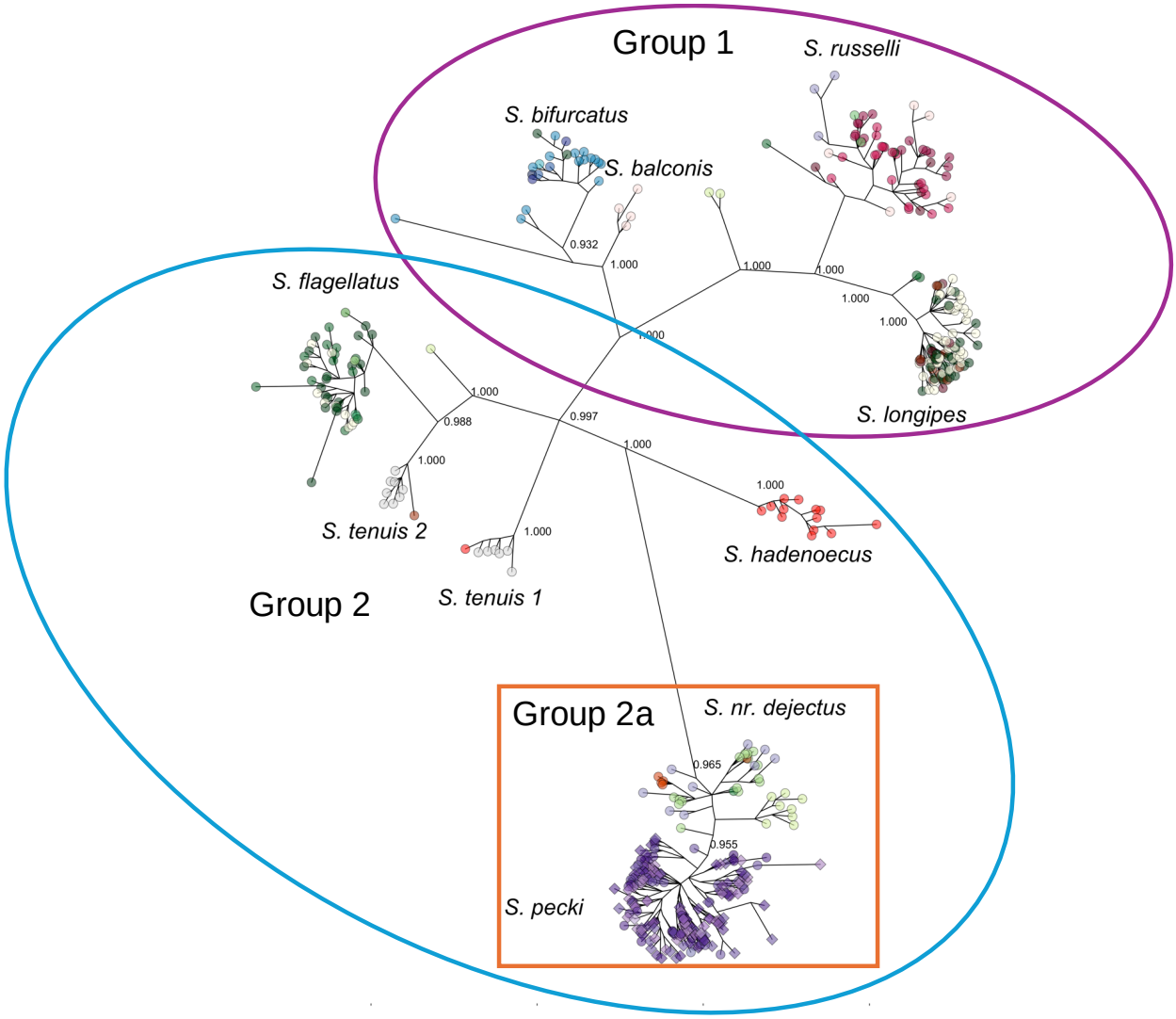

Figure 3: Phylogenetic tree of *Stygobromus* with ellipses denoting the subgroups representing partitions that were analyzed separately using population genetic methods.

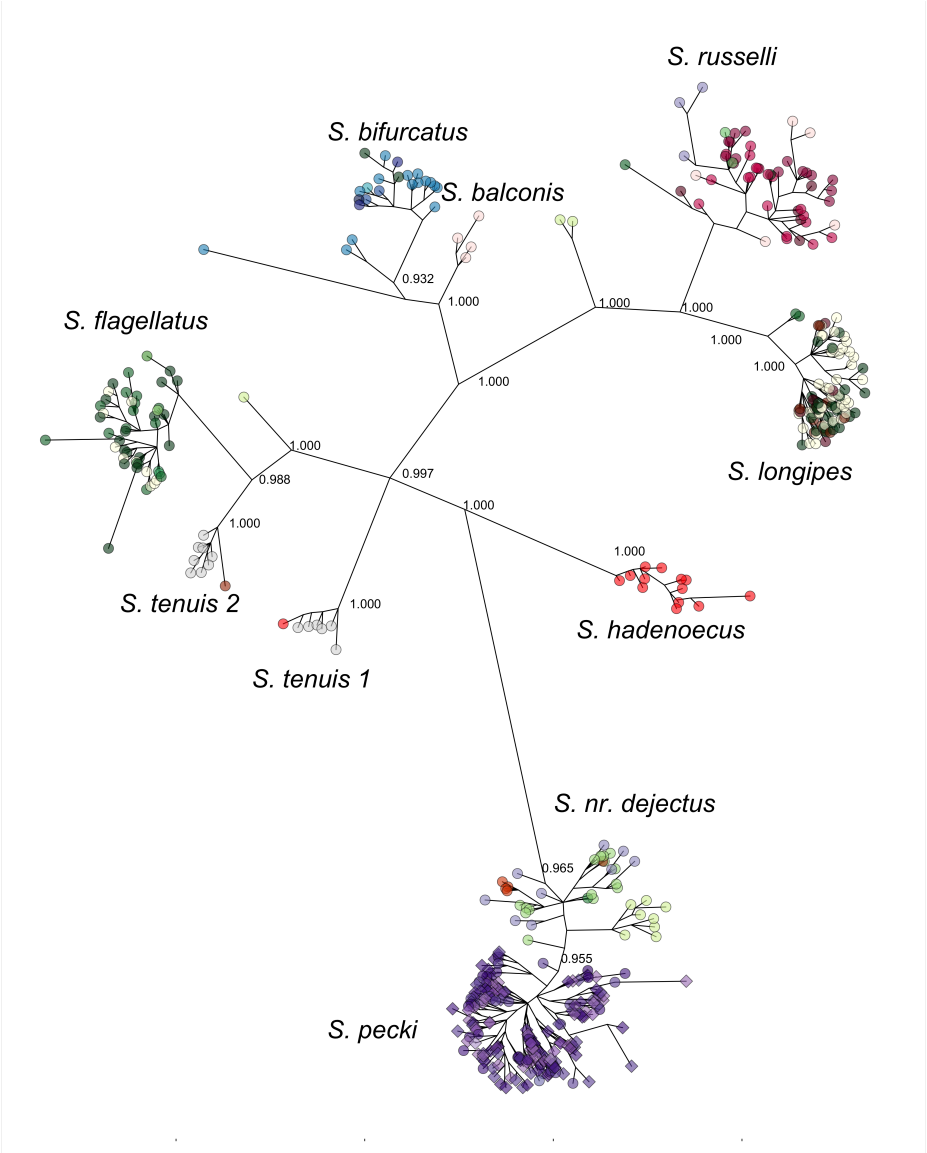

Figure 4: Phylogenetic tree of *Stygobromus* presenting our provisional hypothesis of the major lineages / species in the Edwards Plataeu region of central Texas.

Population Genetic Analyses of Sub-groups

For each of the three subgroups we re-did the assembly, alignment, and variant calling following the steps outlined above. Filtering steps were the same as above, but updated to reflect the number of individuals in each group. In addition to these steps we also only retained one SNP per contig to maintain independence between loci. This resulted in 5,823 SNPs for group 1, 5,171 SNPs for group 2, and 5,474 SNPs for group 3. For each group, genotype likelihoods from BCFTOOLS were updated in the Bayesian admixture model in ENTROPY (version 2) (Shastry *et al.* 2021) to estimate genotype probabilities and explore patterns of genetic differentiation among individuals. ENTROPY incorporates uncertainty in sequence reads stemming from several sources; variation in coverage, sequencing error, and alignment error (Falush *et al.* 2003, Gompert *et al.* 2014, Pritchard *et al.* 2000,

Shastry *et al.* 2021). The admixture model in ENTROPY incorporates an allele frequency-based prior (across K source populations). Therefore, each individual's genotype is estimated using a combination of sequence data in the form of genotype likelihoods, and estimates of allele frequencies from K source populations weighted by an individual's estimated admixture proportion (Gompert *et al.* 2014, Shastry *et al.* 2021). To obtain posterior probability distributions for parameters of interest, we used MCMC and ran the models for K=2 through K=15. Each model was run with 2 chains, for 105,000 steps, saving every 10th step, and a burn-in of 5,000 steps. We examined trace plots and estimated effective sample sizes and the Gelman-Rubin convergence diagnostic (**?**Gelman & Rubin 1992) with the package CODA version 0.19-1 in R Plummer *et al.* (2006), R Core Team (2022) to ensure that a stable sampling distribution had been reached. To examine patterns of genetic differentiation we conducted a principal component analysis (PCA) on centered (but not scaled) genotype probabilities in R. To visualize admixture proportions we used barplots. The PCA and barplots were made using GGPLOT2 (Wickham 2016).

We quantified differentiation among *S. pecki* sample sites and closely related groups (localities 10,13,32, and 34. Table 1) by calculating genome-average Nei's G_{ST} (Nei 1973). G_{ST} is an analog of the standard measure of population genetic differenetiation, F_{ST} G_{ST} is considered appropriate for pairwise comparisons of population samples, and calculated as $G_{ST} = \frac{\frac{1}{n}\sum_n(H_T - H_S)}{\frac{1}{n}\sum_n(H_T)}$ for all pairwise combinations of sites, which we refer to as F_{ST} hereafter. F_{ST} was calculated as the average across all loci and 1000 bootstrap resamples were used to calculate 95% confidence intervals.

We estimated Watterson's θ (based on the number of segregating sites), and Tajima's π (nucleotide diversity of heterozygosity) using ANGSD (Korneliussen *et al.* 2014, Nater *et al.* 2017) for all *S. pecki* sampling localities with ≥ 3 and closely related localities based on our phylogenetic tree (10,13,32, and 33). Estimates of θ and π were based on the maximum likelihood of the Site Frequency Spectra (SFS) for each population calculated using the realSFS function in ANGSD for each contig. Estimates were normalized by dividing the estimate by the number of sites in each contig. We summarized results using a boxplot for each population, plotted using GGPLOT2 in R (R Core Team 2022, Wickham 2016).

Results

The 36 individuals that were removed from the phylogenetic matrix due to excessive missing data including all of the sampled individuals from Oak Springs in Big Bend NP (36). It is possible that amphipods belonging to the *hubbsi* species group are very distantly related to the other amphipods in central Texas (Gibson *et al.* 2021) such that substantial evolution of restriction sites means that our genomic library preparation protocol is inappropriate for including very distantly related taxa in the same library and analyses.

Our phylogenetic inference identified several evolutionarily distinct lineages that were confirmed by population-level analyses of the subgroups. We describe the results of these various analyses from the context of a provisional systematic hypothesis (Table 1 and Figure 4):

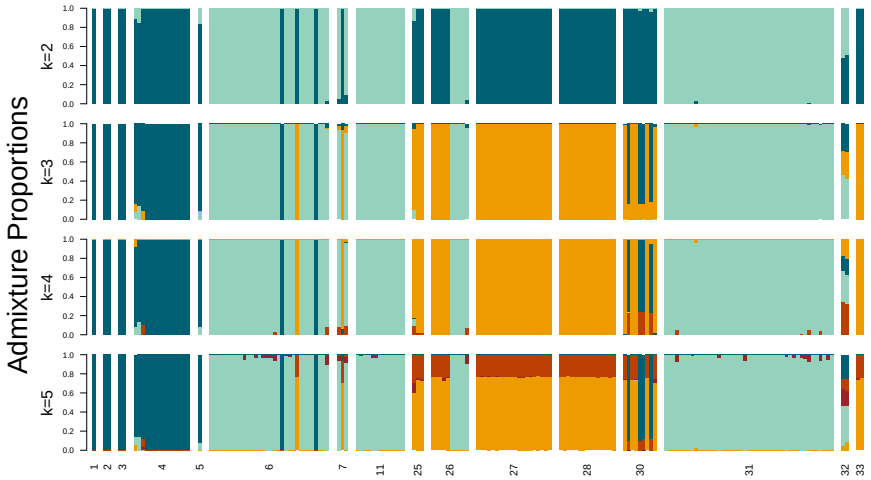

Figure 5: Admixture proportions for group 1 estimated using ENTROPY. Each bar represents one individuals admixture proportions for k = 2 through k=5 based on 5,823 single nucleotide polymorphisms (SNPs). Plots for k=6 through k=15 did not reveal biologically meaningful information so are not shown.

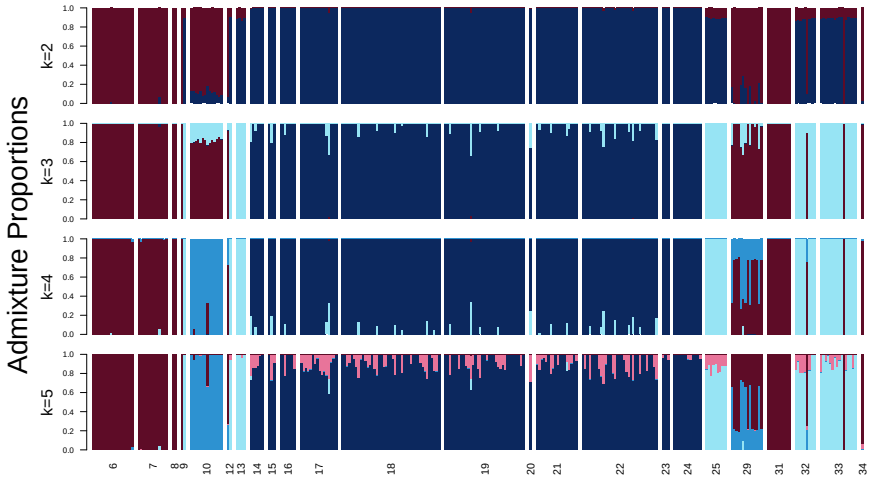

Figure 6: Admixture proportions for group 2 estimated using ENTROPY. Each bar represents one individuals admixture proportions for k = 2 through k=5 based on 5,823 single nucleotide polymorphisms (SNPs). Plots for k=6 through k=15 did not reveal biologically meaningful information so are not shown.

Figure 7: Admixture proportions for group 2a estimated using ENTROPY. Each bar represents one individuals admixture proportions for k = 2 through k=5 based on 5,823 single nucleotide polymorphisms (SNPs). Plots for k=6 through k=15 did not reveal biologically meaningful information so are not shown.

Stygobromus bifurcatis

Samples originally identified as *S. bifurcatus* (1-5) formed a monophyletic group in the phylogeny and we designate them as *S. bifurcatus*. This group also included two individuals from TSU Artesian Well (6) that were originally identified as *S. flagellatus* (Figure 4, Table 1).

Stygobromus flagellatus

Many samples originally identified as *S. flagellatus* from TSU Artesian Well (6), Diversion Spring (7), Sessom Spring (8) and Mission Valley (9) formed a monophyletic group we provisionally label *S. flagellatus* (Figure 4, Table 1). Also included in this group were 10 juveniles from TSU Artesian Well (31) and one unknown individual each from Fessenden Spring (32), Garden Ridge Well (33) and Jacobs Well (35). Other individuals originally identified as *S. flagellatus* grouped with other nominal taxa, see below and Table 1.

Stygobromus hadenoecus

Thirteen individuals sampled from the Devil's River (10) and identified as *S. hadenoecus* formed a monophyletic group that we identify as *S. hadenoecus* (Figure 4, Table 1). One individual sampled at the Devil's River Site grouped with samples from Caroline Springs (29).

Stygobromus longipes

Thirteen individuals identified as *S. longipes* sampled from TSU Artesian Well (11) formed a monophyletic group with individuals identified as *S. flagellatus* and juveniles from TSU Artesian Well (6, 26). We provisionally label this group *S. longipes* (Figure 4, Table 1). Other individuals originally identified as *S. longipes* from CM Cave and Cave Without A Name were found in other groups, see below and Table 1.

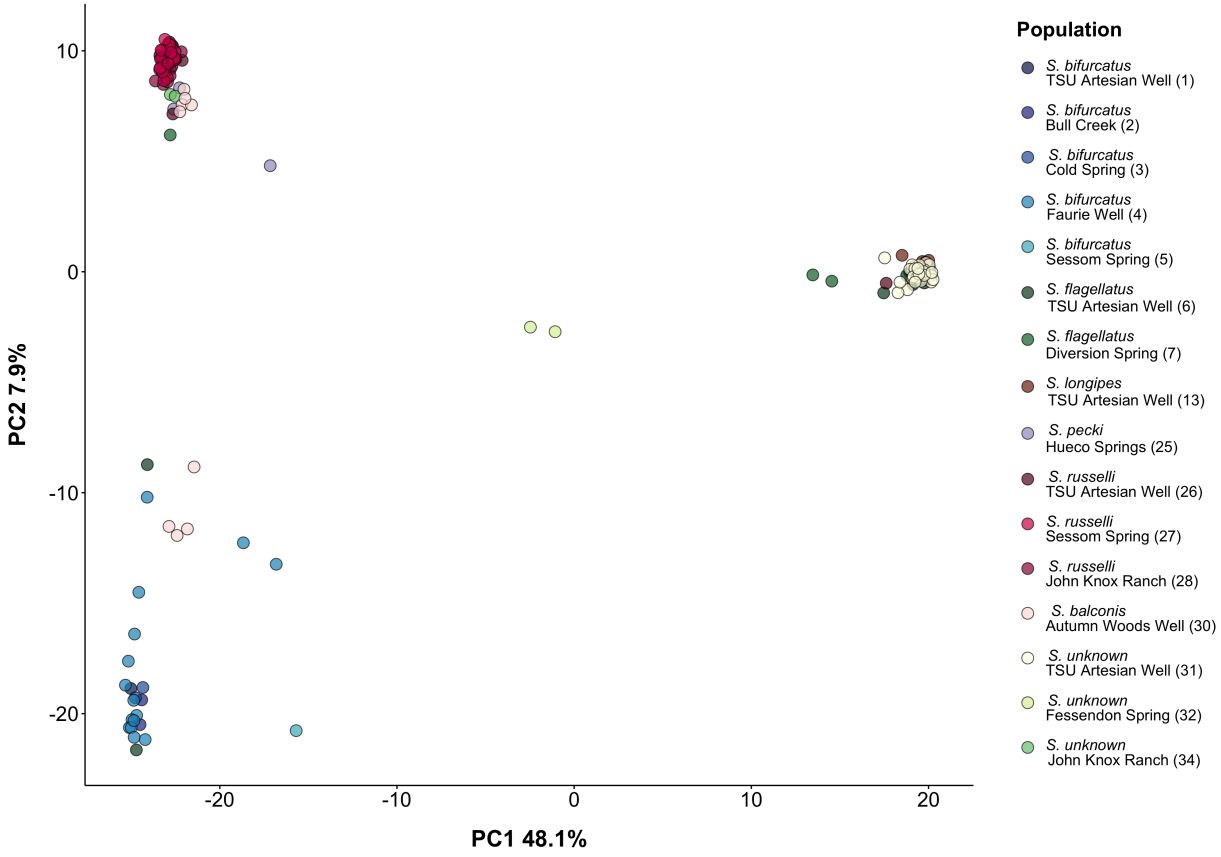

Figure 8: Principle component analysis (PCA) of genotype probabilities estimated from ENTROPY for group 1. Each data point represents one individuals genotype probabilities across on 5,171 single nucleotide polymorphisms (SNPs).

Stygobromus russelli

40 samples identified as *S. russelli* from TSU Artesian Well (26), Sessom Spring (27) and John Knox Ranch (28) formed a monophyletic group that we designate *S. russelli* (Figure 4, Table 1). One sample identified as *S. flagellatus* from TSU Artesian Well (6) and one identified as *S. flagellatus* from Diversion Spring (7) and three from Hueco Springs (25) were also included in this group (Table 1).

Stygobromus tenuis

Specimens identified as *S. tenuis* from Caroline Springs (29) formed two distinct (and polyphyletic) groups (Figure 4, Table 1). One group of six individuals from Caroline Springs (29) plus one individual from the Devil's River (10) we provisionally label *S. tenuis 1*. The other group included eight samples from Caroline Spring (29) plus one individual from CM Cave (12) which we label *S. tenuis 2* (Figure 4, Table 1).

Stygobromus balconis

Four individuals from Autumn Woods Well (30) that were initially identified as *S. balconis* formed a small, separate group in the phylogeny between *S. bifurcatus* and *S. russelli* (Figure 4, Table 1). We provisionally recognize this clade as *S. balconis*. The other five individuals sampled from Autumn Woods Well (30) were placed in the *S. russelli* group.

Stygobromus pecki

All individuals identified as *S. pecki* from Comal Springs (14-24) formed a monophyletic group (Figure 4, Table 1). Three samples from Hueco Springs (25) identified as *S. pecki* were found in the *S. russelli* clade and nine others are closely related to *S. pecki*. Indeed, this group that is closely allied with *S. pecki*, but appears to be differentiated from *S. pecki*, includes the nine individuals sampled from Hueco Springs (25), plus one individual from Mission Valley (9), one individual from CM Cave (12), eight individuals from Fessenden Spring (32) and 15 samples from Garden Ridge Well (33). While closely related to *S. pecki*, these samples appear to be distinct from *S. pecki* in both the phylogenetic analyses (though not monophyletic with respect to *S. pecki*) and in the clustering and Principal Coordinates analyses (Figures 7, 6, 10). We provisionally label this group as *S. nr. dejectus?* based on the notion that *S. dejectus* might be the sister taxon of *S. pecki* (Holsinger 1967), but this is extremely tentative and will require further investigation. This primarily *S. pecki* evolutionary clade appears as the most distinct lineage with the longest branch length in our tree (Figure 4).

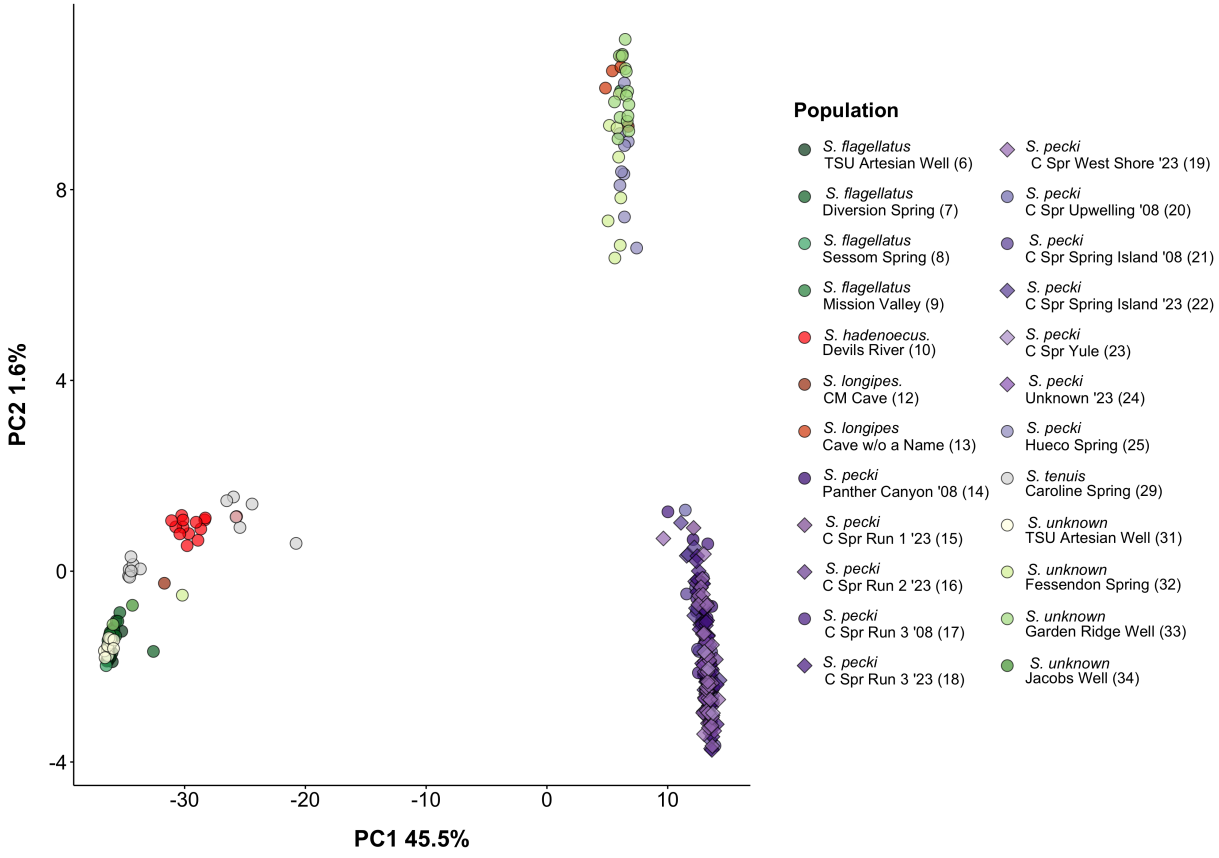

Figure 9: Principle component analysis (PCA) of genotype probabilities estimated from ENTROPY for group 2. Each data point represents one individuals genotype probabilities across on 5,474 single nucleotide polymorphisms (SNPs).

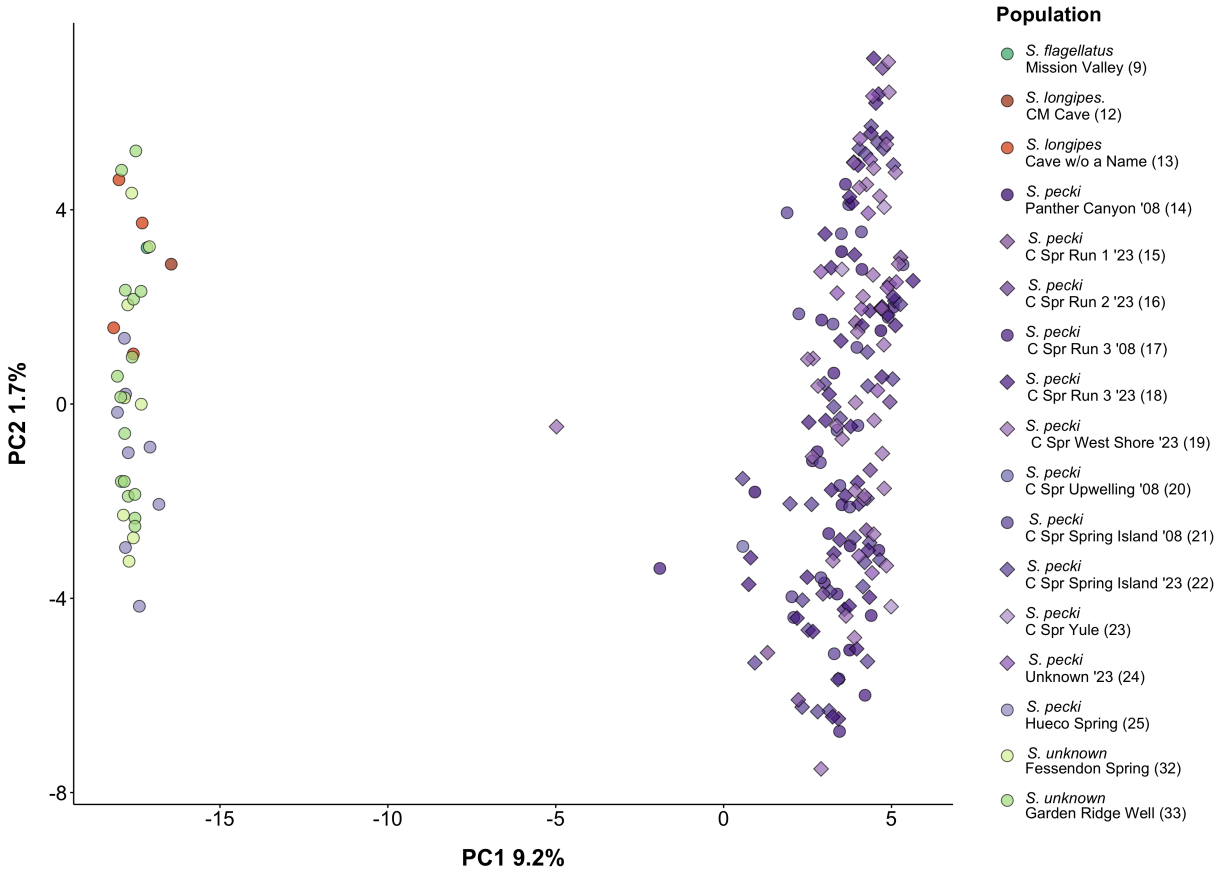

Figure 10: Principle component analysis (PCA) of genotype probabilities estimated from ENTROPY for group 2a. Each data point represents one individuals genotype probabilities across on 5,823 single nucleotide polymorphisms (SNPs).

Within *S. pecki*, there is very little genetic structure. Clustering analysis of group 2 and group 2a failed to uncover any further clustering among *S. pecki* localities or individuals (Figures 7, 6). There is also no structure evident in PCA plots (Figure 10). Pairwise F_{ST}'s are very low among *S. pecki* localities with a maximum of 0.037 and a mean of 0.0156 (Table 4), which are quite low levels of differentiation. Further, there were no discernible distinctions between individuals sampled from Comal Springs Spring Run 3 or Spring Island in 2008 vs. 2023, or among all individuals sampled before 2016 versus those sampled in 2023-2024, in the phylogenetic, clustering or PCA analyses (Figures 2, 7, 10). Values of pairwise F_{ST} between the temporal samples at Spring Run 3 and Spring Island were 0.005 (95% confidence interval: 0.005-0.005) for both temporal comparisons (Tables 2, 3). These values are comparable to pairwise F_{ST}'s reported by (Lucas *et al.* 2016) and reinforce their conclusion that gene flow appears to be relatively unrestricted across the range of *S. pecki*. Genetic diversity is relatively homogenous across Comal Springs (Figures 12 and 13). There also appears to have been no appreciable change in genetic diversity over time (Figures 12 and 13).

Table 2: Pairwise F_{ST} values among localities in Group 2a with n $\geq$ 3. These localities include all sampled *S. pecki* from Comal Springs (localities 14-24, Table 1) and closely related individuals from localities 25, 32 and 33 (Table 1).

	10	13	14	15	16	17	18	19	21	22	23	24	25	32	33
10	0.000														
13	0.153	0.000													
14	0.168	0.043	0.000												
15	0.179	0.053	0.026	0.000											
16	0.170	0.043	0.016	0.026	0.000										
17	0.162	0.037	0.012	0.022	0.011	0.000									
18	0.162	0.036	0.010	0.019	0.008	0.005	0.000								
19	0.162	0.036	0.010	0.020	0.009	0.005	0.003	0.000							
21	0.163	0.037	0.012	0.021	0.011	0.006	0.004	0.005	0.000						
22	0.160	0.036	0.010	0.019	0.009	0.005	0.003	0.003	0.005	0.000					
23	0.181	0.054	0.027	0.037	0.025	0.021	0.019	0.019	0.021	0.019	0.000				
24	0.167	0.040	0.014	0.023	0.012	0.009	0.006	0.006	0.008	0.006	0.022	0.000			
25	0.137	0.027	0.026	0.036	0.026	0.020	0.019	0.020	0.021	0.019	0.037	0.024	0.000		
32	0.141	0.032	0.032	0.042	0.032	0.026	0.025	0.025	0.027	0.025	0.043	0.030	0.019	0.000	
33	0.139	0.025	0.027	0.037	0.027	0.021	0.020	0.020	0.022	0.020	0.038	0.025	0.011	0.021	0.000

Table 3: 95% permutational confidence intervals for pairwise F_{ST} in Table 2. Upper interval limits are given above the diagonal, lower limits below the diagonal.

	10	13	14	15	16	17	18	19	21	22	23	24	25	32	33
10	0.000	0.160	0.175	0.186	0.177	0.169	0.169	0.169	0.170	0.167	0.188	0.174	0.144	0.147	0.145
13	0.146	0.000	0.046	0.056	0.045	0.039	0.038	0.038	0.039	0.038	0.057	0.042	0.028	0.034	0.026
14	0.162	0.041	0.000	0.027	0.017	0.013	0.011	0.011	0.013	0.011	0.028	0.015	0.027	0.034	0.028
15	0.172	0.051	0.025	0.000	0.024	0.021	0.020	0.020	0.020	0.018	0.038	0.024	0.034	0.044	0.039
16	0.163	0.041	0.015	0.027	0.000	0.011	0.009	0.009	0.010	0.008	0.026	0.012	0.025	0.034	0.029
17	0.155	0.036	0.012	0.023	0.012	0.000	0.005	0.005	0.007	0.005	0.022	0.010	0.021	0.028	0.023
18	0.155	0.034	0.010	0.018	0.008	0.005	0.000	0.003	0.004	0.003	0.020	0.006	0.018	0.027	0.022
19	0.155	0.034	0.010	0.019	0.008	0.005	0.002	0.000	0.004	0.003	0.020	0.006	0.018	0.027	0.022
21	0.157	0.035	0.011	0.021	0.011	0.006	0.004	0.005	0.000	0.005	0.022	0.008	0.023	0.029	0.023
22	0.154	0.034	0.010	0.020	0.009	0.005	0.003	0.003	0.005	0.000	0.020	0.007	0.018	0.027	0.021
23	0.174	0.052	0.026	0.035	0.024	0.020	0.018	0.018	0.020	0.018	0.000	0.021	0.035	0.045	0.040
24	0.160	0.038	0.013	0.022	0.011	0.009	0.006	0.006	0.007	0.006	0.023	0.000	0.023	0.032	0.026
25	0.131	0.026	0.024	0.038	0.028	0.019	0.021	0.021	0.020	0.020	0.039	0.025	0.000	0.021	0.012
32	0.134	0.030	0.030	0.040	0.030	0.024	0.023	0.024	0.025	0.023	0.041	0.028	0.018	0.000	0.022
33	0.132	0.024	0.025	0.035	0.026	0.020	0.019	0.019	0.021	0.019	0.036	0.023	0.011	0.019	0.000

Lastly, the Fessenden Spring (32) site contains some unusual diversity. Eight samples cluster with the *S. nr. dejectus?* group. One individual is located in the phylogeny by itself somewhat near the *S. flagellatus* (identified as *S. nr. flagellatus* in Table 1). Two other individuals form their own group between *S. balconis* and *S. russelli* (which we designate as *S. nr. russelli* in Table 1).

Discussion

Genome-wide SNP data were generated to quantify genetic variation across samples of *Stygobromus* cave amphipods from the Edwards Plateau region of central Texas. These data provide a view of the diversity of lineages and the structure of population genetic variation within lineages. Phylogenetic inference supports much of the nominal taxonomy (Holsinger 1967), though we also uncovered evidence of some cryptic lineages. The endangered *S. pecki* is one of the most distinct lineages in our data. However, many individuals from nearby localities appear to be closely related to *S. pecki*.

Table 4: Summary statistics for pairwise F_{ST} values among *S. pecki* sampling localities (localities 14-24 in Table 1.

Statistic	Value
Min	0.0030
1st Qu.	0.0080
Median	0.0160
Mean	0.0156
3rd Qu.	0.0210
Max	0.0370

There was very low levels of differentiation within *S. pecki* and little evidence of restricted gene flow within Comal Springs.

Samples from Oak Springs in Big Bend National Park, nominally *S. hubbsi* could not be included in the phylogenetic analysis because of extensive missing data for these individuals. The *hubbsi* species group is a western North American lineage and possibly quite differentiated from other *Stygobromus* in central Texas (Gibson *et al.* 2021). Failure to include these samples in the phylogenetic matrix could be due to poor quality of the specimens or the DNA extracted from them, but seems more likely due to their evolutionary distance from the other species included in the analysis. One of the limitations of the GBS approach is that it is based on the existence of homologous restriction sites in genomes of sampled individuals which are used to fragment genomic DNA prior to sequencing. Like many other features of genomic DNA, including standard nucleotide substitutions, divergence in restriction site sequences increases as time to common ancestor increases. In short, substitutions in restriction sites will destroy, or create new, restriction sites and this process increases over evolutionary time and therefore increasing the number of non-homologous restriction sites between lineages. We suspect that the species of the *S. hubbsi* group are distantly related to the other species included in this study and that restriction site evolution creates non-overlapping SNP sets and thus extensive missing data in the phylogenetic matrix.

The phylogenetic analyses support the recognition of nominal species corresponding to those identified by Holsinger (1967) for the region. These include: *S. bifurcatus*, *S. flagellatus*, *S. hadenoecus*, *S. longipes*, *S. pecki*, *S. russelli*, *S. balconis* and *S. tenuis*, though this last species appears to contain more diversity. However, while the nominal species are recognizable in the phylogenetic hypothesis, many of our original identifications of individuals based on morphology were incorrect (Table 1). This suggests either that the morphological characters that underlie the keys for *Stygobromus* can be variable within and between species, or those characters are not fully diagnostic of the species, or both.

These nominal species exhibit a great deal of range overlap with many localities harboring individuals from multiple lineages. As previously reported (Holsinger & Longley 1980, Hutchins *et al.* 2021), *S. bifurcatus*, *S. flagellatus*, *S. longipes*, and *S. russelli* were all detected at the TSU Artesian Well site. Other sites in San Marcos, Texas have overlapping species: Diversion Spring (7) in Spring Lake includes *S. flagellatus*, *S. longipes*, and *S. russelli*) and Sessom Spring (5, 8) is home to *S. flagellatus* and *S. bifurcatus*. Many other localities include individuals belonging to more than one nominal species (Table 1). It is possible that these co-ocurring species are not strictly sympatric and could occupy different habitats, possibly at different depths, within the aquifer. Their apparent sympatry might result from the samples being pushed to the surface and into drift nets during high flow events. Stable isotope analysis could be used to test the hypothesis of ecological differentiation

among these co-occurring species.

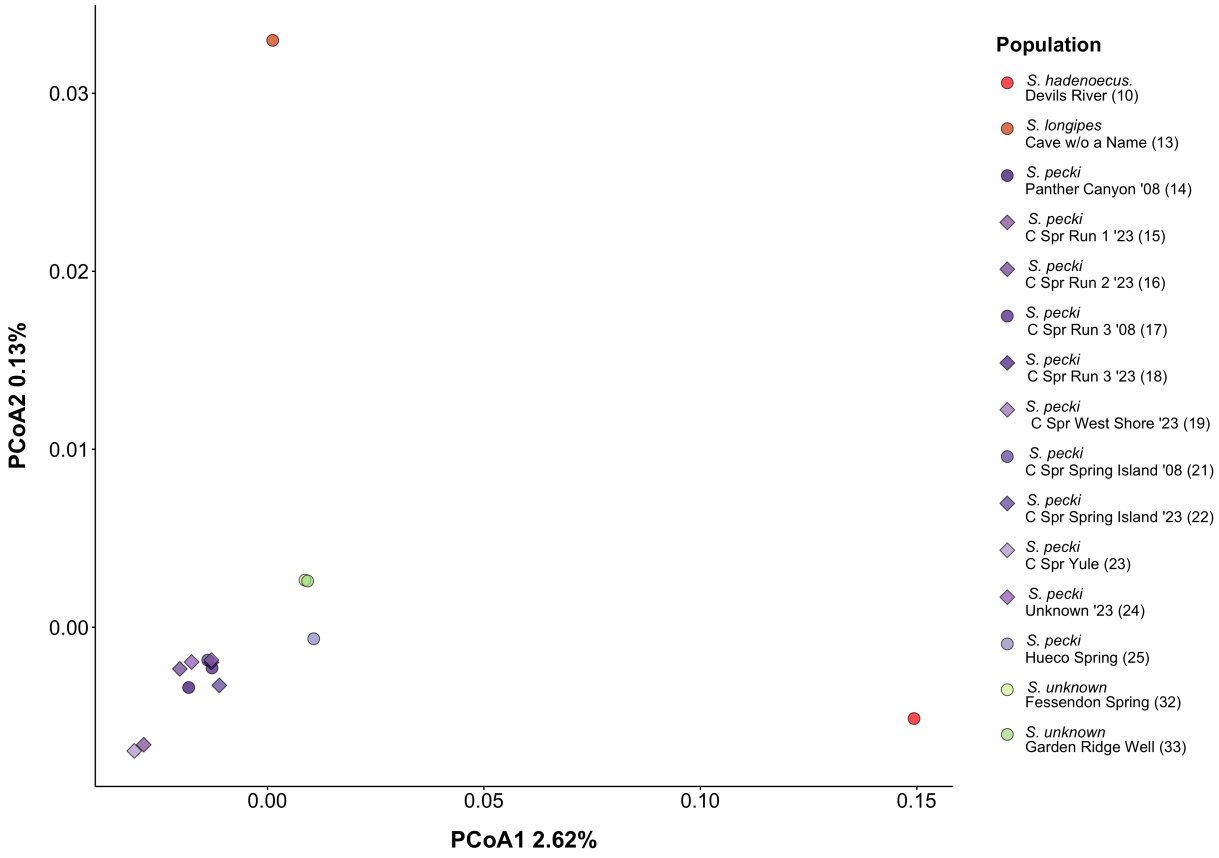

Figure 11: Principal Coordinates Analysis (PCoA) of genome-wide average pairwise Nei's F_{ST} for group 2a localities (14-25, 32, 33, Table 1).

There were some unexpected findings that suggest there is more diversity in Edwards Plateau *Stygobromus* than currently recognized. The nominal *S. tenuis* individuals sampled from Caroline Springs (29) comprise two distinct lineages that, while proximately located in the phylogeny, are not monophyletic. What we have provisionally labeled *S. tenuis 1*, includes six individuals from Caroline Springs (29) plus one individual from the Devil's River (10) site. The provisional *S. tenuis 2* includes eight individuals from Caroline Springs (29) and one individual from CM Cave in Comal county. More extensive sampling will be required to fully describe the range of these cryptic lineages within the nominal *S. tenuis*. Further morphological investigations will also be required to detect any variation that might be used to identify these lineages.

Samples collected from Fessenden Springs (32) also include some unexpected diversity. These samples were not readily identifiable to any of the nominal species *a priori*. Phylogenetic analysis places eight of the Fessenden Springs (32)individuals in the group that is closely related to *S. pecki* that we very tentatively label as *S. dejectus?* (see below). One individual occurs in isolation but near the *S. flagellatus* clade, and two individuals form their own group that is near, but distinct from, the *S. russselli* clade. This diversity spans a substantial portion of the entire tree, suggesting that there is considerable phylogenetic diversity that is sympatric at this site.

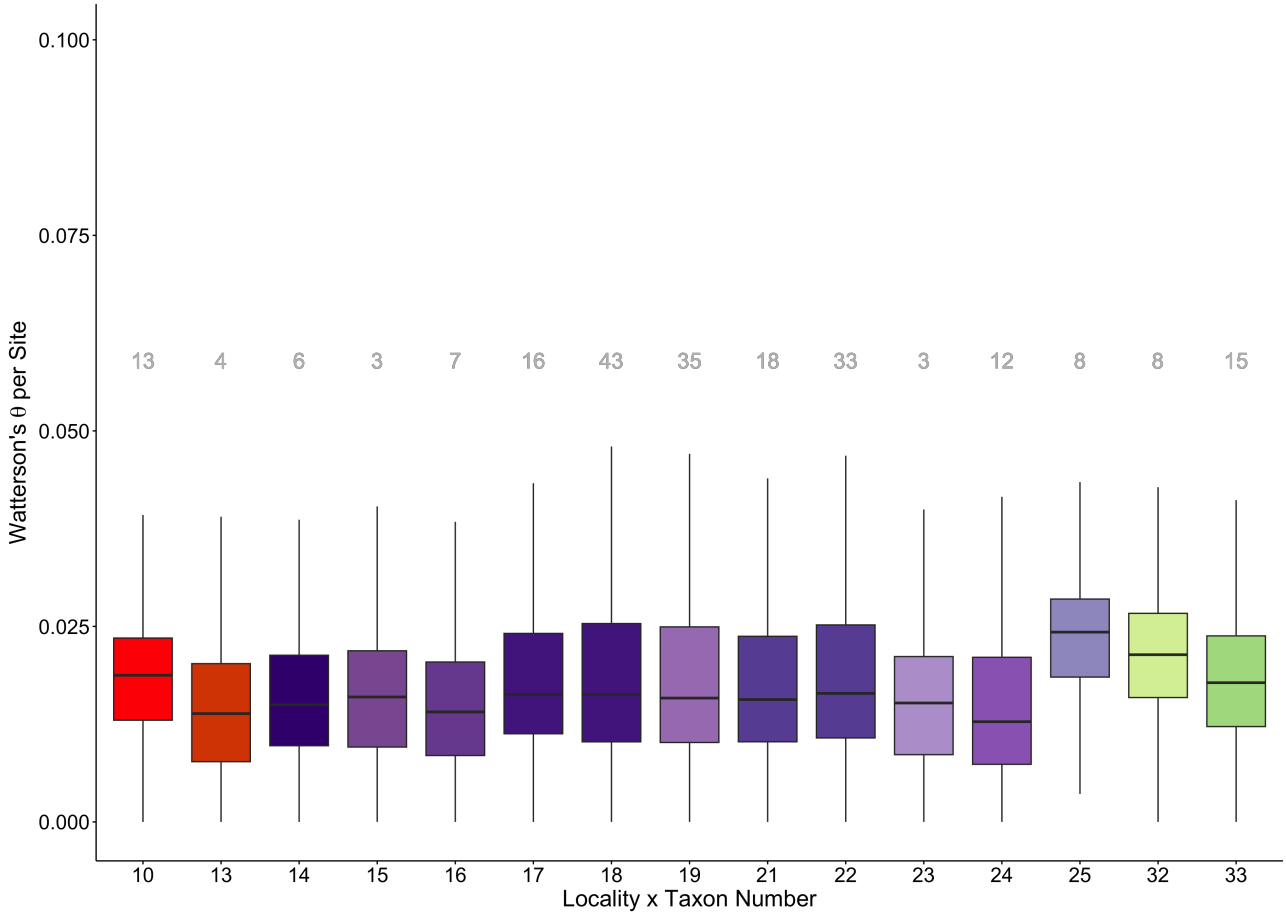

Figure 12: Estimates of Watterson's θ per site for group 2a localities (10, 14-25, 32, 33, Table 1). Sample size per sampling locality shown in grey above boxplots.

The sampled *S. pecki* from Comal Springs (14-24) form a distinct group in all analyses. All of the 181 individuals from eight sites and across two sampling events in 2008 and 2023-2024 appear as a monophyletic group in phylogenetic analyses (Figure 2 with high bootstrap support (0.955) (Figure 2. They also form distinct clusters in the ENTROPY analyses (Figures 6, 7) and in PCA plots (Figure 10. Indeed, ENTROPY models with higher numbers of clusters (k) fail to detect any substructure within the Comal Springs localities (14-24), both in space or time. *S. pecki* from Comal Springs (14-24) are surprisingly homogeneous with no apparent restrictions in gene flow. These sites also harbor very similar amounts of genetic diversity (Figures 12, 13). Further, there is no evidence of substantial differentiation (Table 2) or differences in diversity (Figures 12, 13) in temporal samples from Spring Run 3 (17, 18) and Spring Island (21,22). These observations suggest that effective population sizes at these sites are relatively large at these sites and have remained stable despite intervening periods of drought and reduced flows between 2008 and 2023-2024.

However, the picture of *S. pecki* is complicated by 38 individuals that are closely related to S. pecki that we label *S. nr. dejectus?* from Mission Valley Well (9), CM Cave (12), Cave Without A Name (13), Hueco Springs (25), Fessenden Springs (32) and Garden Ridge Well (33). The Hueco Springs (25) samples were originally diagnosed as *S. pecki* based on morphology. Three of these individuals are identified as *S. russelli* in our data and eight group with the other samples belonging to the *S. nr. dejectus?* group. This group is differentiated from S. pecki in all of our analyses

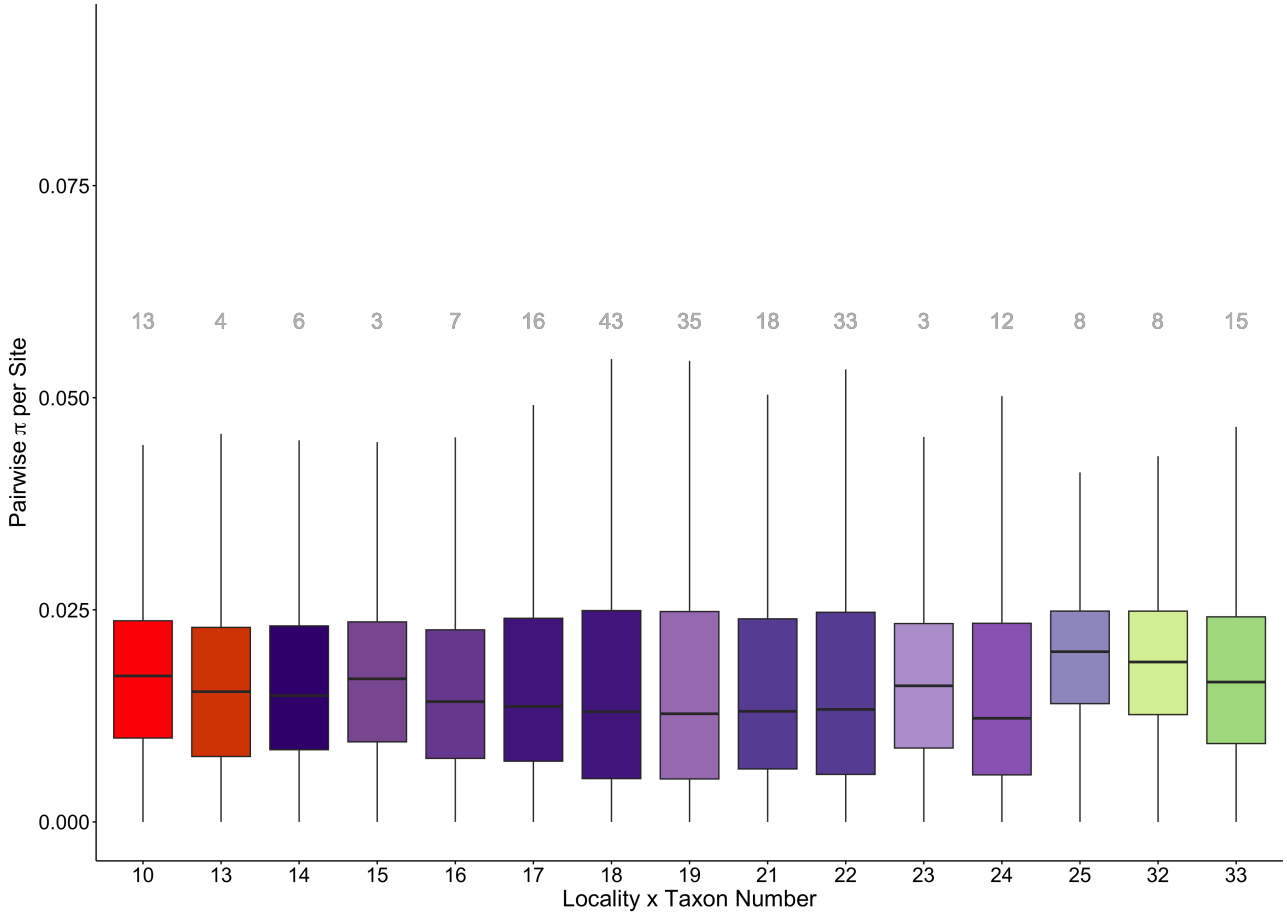

Figure 13: Estimates of Tajima's π (per site nucleotide diversity) for group 2a localities (10, 14-25, 32, 33, Table 1). Sample size per sampling locality shown in grey above boxplots.

(Figures 2, 6, 7, 9, 10, 11). However, we recommend caution in interpreting these findings for at least two reasons: 1) the *S. nr. dejectus?* group is not monophyletic with respect to *S. pecki* and indeed *S. pecki* makes the *S. nr. dejectus?* group paraphyletic. 2) all of the patterns described here, including the paraphyly of the *S. nr. dejectus?* group, could be caused by incomplete geographic sampling. It has been observed that incomplete geographic sampling of a continuously distributed population with a pattern of isolation-by-distance can create spurious clusters in clustering analyses and in PCAs (Wright 1943, Novembre & Stephens 2008, Frantz *et al.* 2009, Bradburd *et al.* 2018). Unfortunately, our current sampling does not allow us to resolve this "cline versus clusters" problem for *S. pecki*. This is a priority for future work.

They data reported here provide the best description of *Stygobromus* genomic diversity in the Edwards Plateau region of Texas to date. But that picture is incomplete. Our data provide support for the nominal taxa, but leave many questions unresolved. Despite genotyping 466 individuals from 36 unique localities and taxa (Table 1), we cannot precisely describe the ranges of most of the taxa examined. Further, our destructive sampling prevents a thorough retrospective examination of morphological variation. This was a tradeoff in the design of this study that we felt was necessary to gain a perspective on the dimensions of the species richness and genetic diversity in the group. And, again despite our sampling, we cannot adequately resolve the relationship between *S. pecki* and many closely related samples. Future work that includes even more extensive sampling for

genomic and morphological characterization will be required to address these fundamental aspects of *Stygobromus* biology in central Texas.

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Appendix F | **Establishing a developmental atlas and de novo transcriptome for *E. rathbuni, E. nana, and E. pterophila***

Establishing a developmental atlas and de novo transcriptome for *E. rathbuni, E. nana*, and *E. pterophila*

Ruben U. Tovar and David M. Hillis

INTRODUCTION

Courtship of the Texas blind salamander (*E. rathbuni*), San Marcos salamander (*E. nana*), and Fern Bank salamander (*E. pterophila*) has never been observed in the wild, however accounts have been noted in the captive colonies maintained at the U.S. Fish and Wildlife Service (FWS), San Marcos Aquatic Resource Center (SMARC). Further description of courtship and spermatophore deposition was also described by Bechler (1988). Courtship has been described as an elaborate tail-straddle walk similar to that seen by other species of plethodontid salamanders (Goricki et al. 2012). Furthermore, breeding seems to take place year-round in all the cave and spring dwelling *Eurycea* salamanders of Texas. Given the relatively consistent conditions of the Edwards-Trinity Aquifer (e.g., temperature, pH, and ambient light), and the aquifer's associated outflows, determining breeding cues for the *Eurycea* clade has been difficult. The paucity of potential breeding stimuli has made predictiable captive breeding difficult to consistently achieve.

Successful reproduction is contingent on a number of both endogenous and exogenous mechanisms. Exogenous-environmental cues (e.g., circadian rhythm, change in seasonal temperature, etc.) are perceived by an organism's sensory organs (eyes—phototransduction; olfactory bulb—chemosensory; skin—temperature), and are part of the initial signaling that indicates the ideal reproduction periods. The sensory phenotype associated with underground living (e.g., eye and pigment reduction, dorsal-ventrally compressed heads, elaborated lateral line and chemosensory, etc.) play a role in how these subterranean species perceive their environment relative to their ancestral state (surface). Having a fundamental grasp of these comparative

sensory systems may give us insight into which sensory modalities are selected and favored for breeding in each respective environment (subterranean vs. surface). Additionally, conserved genetic pathways underlie and work in concert with the endocrine system to produce successful oviposition and viability of offspring. Understanding the underlying expression profile associated with gravidity and spermatogenesis would help to disentangle what might be driving reproduction in these endangered species.

Question 1: What genes are important for reproductively active/gravid salamanders versus non-reproductive salamanders?

Question 2: What sensory organs (e.g., eye,ofactory epithelium, skin) are correlated to reproduction and how might this play a role in mating (specifically gravidity)?

SMARC has tested the induction of oviposition with hormones, separation, and light ques for both *E. nana* and *E. rathbuni* to induce reproductive activity and oviposition. Hormones were successful for *E. rathbuni*, but nothing has worked for San Marcos salamander (*E. nana*). For FWS, the ultimate goal of this study is to track the development of sensory organs responsible for communicating environmental ques that initiate reproduction, and to compare gene expression between different tissues during reproduction. Accomplishing this will inform the FWS about both organ development and the genetic underpinnings contributing to reproduction. To do this we have employed a novel microCT scanning protocol that allows us to both scan soft tissue and extract DNA post scanning. During 2023, we have managed to collect a developmental series for *E. rathbuni* and *E. nana*, microCT scanned them, and tested the DNA

extraction protocol on previously preserved and scanned embryos as proof of concept for utilizing the specimens collected for this study. We have now initiated an RNA-based reference for future comparative studies (e.g. comparing tissues from reproductive vs. non-reproductive salamanders). This future sequencing work will help the FWS identify genes associated with reproduction in *E. rathbuni* and *E. nana*.

Benefits to the Habitat Conservation Plan

Understanding how and what triggers reproduction and viable development is fundamental to future breeding and propagation objectives within the Edwards Aquifer Authorities (EAA) Habitat Conservation Plan. Herein we propose to establish a baseline understanding of the molecular underpinnings responsible for gravidity (females) and spermatogenesis (males), identify candidate genes and sensory pathways associated with reproductive state, and to establish a developmental series outlining proper development of each species (*E. nana*, *E. rathbuni*, and *E. pterophila*). This study fits well with objectives of the San Marcos Springs and River Ecosystem Adaptive Management Activities. This study is in line with the San Marcos Aquatic Resources Center, off-site refugia's adaptive management objectives to "*Determine life history characteristics (life span, tolerance to water quality changes, **reproduction**, food resources) and minimize impacts*." (Edwards Aquifer Recovery Implementation Program-6.4.4.3, 2012).

Benefits to the Edwards Aquifer Refugia Program

Anecdotal observations suggest *E. nana* oviposit on a seasonal schedule with peak oviposition occurring over the winter months, between November and January. Multiple studies at the Refugia have attempted to determine what the environmental cures are for triggering this seasonal oviposition behavior in order to trigger oviposition and courtship on demand. Light,

habitat configuration, separation of males and females, group composition, as well as hormone exposure have all been tested with little success. By comparing gene expression profiles and physical developmental stages, we can hope to determine what genetic pathways are triggered when salamanders are brooding eggs and as they develop into mature adults. This information will help guide future research instead of continuing to make shots in the dark and testing what we think may trigger oviposition. In this study, we hope the San Marcos salamander genetics will point us in the right direction and where to focus research efforts.

MATERIALS AND METHODS

Specimen and Tissue Collection

All animal manipulations were approved by The University of Texas at Austin Institutional Animal Care and Use Committee (IACUC) under AUP-2021-00090. Here our goal was to use an embryonic series to track and describe developmental progression and corresponding RNA expression using PAXgene fixation and RNA-isolation protocol (Green et al. 2017), and high throughput sequencing. Importantly, this fixation method allows us to isolate RNA for downstream sequencing post diceCT scanning. We identified four similar embryonic stages for both *E. nana* and *E. rathbuni* using morphological feature (e.g. head and tail bud, somite count, hyomandibular folds; Fig.3&4). Embryos were allowed to develop in an environmental chamber with controlled temperature (21.1^{o}C) and lighting, and checked daily for morphological progression. Once the embryos reached the desired stage we followed the PAXgene fixation and scanning method described below. We also obtained a series of post embryonic developmental stages to track differences in soft tissue development, and focused on the eyes for this study.

Specimen's were collected, euthanized, and immediately fixed overnight using PAXgene Tissue FIX (Qiagen, PreAnalytics, Cat. No. 765312), washed fifteen minutes in PAXgene Stabilizing Solution (Qiagen, PreAnalytics, Cat. No. 765512), and placed in fresh stabilizing solution before storage at -80^{o}C. Specimens remained in -80^{o}C until staining. As a variation on the standard diceCT protocol, Green et al. 2017 demonstrated that an iodine-contrast agent using PAXgene Stabilizing Solution as the base solvent (e.g., instead of ethanol) preserves both biomolecules and morphologies. We mimicked this approach by preparing a 2% weight-to-volume mixture of PAXgene Stabilizing Solution and iodine metal (Gignac et al. 2016) to stain most of our samples (i.e., those older than one-month post-oviposition). For specimens just one-month post oviposition, which are especially small, we instead prepared a lower-concentration, 1% weight-to-volume staining solution. As with high concentrations of ethanol (Gignac et al. 2016), iodine crystals dissolve readily into PAXgene Stabilizing Solution. To ensure complete dissolution, the solute was thoroughly mixed by hand and left overnight before first use (Green et al. 2016).

All tissues were imaged at The University of Texas High-Resolution X-ray Computed Tomography Facility (UTCT) and were scanned at room temperature using an Xradia MicroXCT 400 (Zeiss). Because biological specimens are expected to have slight variation in staining, each specimen was individually optimized at reconstruction to utilize the full 16-bit dynamic range of the detector (Veith et al. 2020). Scan settings for heads representing the developmental series were as follows: 4X objective, 70 kilovolts (kV), 8.5 watts (W), 0.25–0.5 second (s) acquisition time, detector position at 10.002 millimeter (mm). Scan settings for heads representing the adult stage were as follows: flat panel, 70 kV, 8.5 W, 0.06 s, 5 frames per view, detector position at 155.707 mm. Voxels were isometric, ranging from 4.00 x 4.00 x 4.00 micrometers (µm) for

specimens in the developmental series to 7.00 x 7.00 x 7.00 µm for adults. Following scanning specimens were returned to a 15-mL collection tube filled with fresh PAXgene Stabilizing Solution and stored in -80^{o}C where the iodine was allowed to passively diffuse.

DiceCT Scans

DiceCT data was reconstructed by Xradia Reconstructor. Sixteen-bit Tagged Image File Format (TIFF) series were imported, rendered, and soft tissues segmented using Dragonfly software, version 2020.2 (Object Research Systems, ORS Inc.). Soft-tissue segmentation of the lens and retina was accomplished using the magic wand feature, then detailed using the automated tool, finally the volumes for each segmentation were recorded. Most datasets were analyzed and figures composed using R Statistical Software (R Core Software, 2022).

RNA Sequencing Pipeline

We used the entire embryo to sequence using a paired end read length (PE) of 300 base pairs (bp) on a MiSeqV3 to sequence RNA expression globally. The same fixative was used (PAXgene fixation) and RNA-isolation protocol was used for the postembryonic stages. Post embryonic (PE) specimens one to five months' post oviposition (mpo) and all adults were micro dissected, leading to the RNA isolation of eye, olfactory, and skin tissues. We sequenced these individuals using a Tagged-based RNA sequencing (Tag-seq) (Meyer et al., 2011). Tag-seq platform is ideal for comparative expression, and cost effective. Therefore, we were able to incorporate a number of species to gain the most comprehensive data set between species and phenotypes to understand eye development with the *Paedomolge* clade. RNA quality was determined using an RNA 6000 Nano Assay with BioAnalyzer, processed samples had an average RNA Integrity Score (RIN score) of ~2.5 (Sup. Fig. 6) with specimens of the comparative eye development (n=324). RIN score for the comparative gonad sequencing ranged from 2.5-7.6 (n=45) (Sup. Fig. 7). RNA concentration was determined with a Qubit RNA High

Sensitivity assay kit (Thermo Fisher Scientific, Cat. No. Q32855). In total, 45 samples were submitted for RNA-seq 300PE. These samples consisted of both embryos and comparative adult gonad and sensory organ tissues. A total of 324 samples were submitted for Tag-seq consisting consisting of six species, six developmental stages, three individuals per stage and three tissues per individual (eyes, olfactory epithelium, and skin). All sequences were submitted and processed at the Genome Sequence and Analysis Facility at The University of Texas at Austin for Tag-based and 300PE RNA sequencing.

RNA Sequencing Analysis

Raw reads were processed and mapped to an in house de novo *Eurycea paludicola* transcriptome that was annotated using *Xenopus tropicalis* and *Homo sapien* reference genomes (Ensemble 2024, BioMart). Annotation with *Homo sapien* resulted in >3.5 million reads within the *E. paludicola* de nova transcriptome and >5.5 million reads identified post annotation with the *Xenopus tropicalis* genome. Mapping the raw-count read data (using tx2gene) of the first batch (n=263) resulted in >4.7 million Trinity-genes identified. We then normalized counts using DESeq. We mapped all tissue samples (eyes, olfactory epithelium, skin). We filtered the data set by tissue and identified the optimal filtering criteria would be <10 reads. Mapped sites with less that 10 reads would be filtered out of the dataset (see Sup. Fig.'s 3-5, for filtering iterations). This resulted in a more normal distribution of the data (Fig.'s 7-13, A's). We focused on eye tissue only, and will continue to explore the other tissue types (olfactory epithelium and skin). It's important to note that every filtering iteration results in less overall genes and a reduced dataset. There are 6,102 genes represented in the eye tissue among the specimens (n=90) post filtering (<10). We ran principal component analysis (PCA) to identify trends associated with

variation within the data set. We applied a metadata excel sheet with categorical data; phenotype (either subterranean or surface), and developmental stage (1-5mpo, and adults).

RESULTS

RNA Sequencing

We report initial quality scores at several critical steps during the RNA-isolation, library prep for high throughput sequencing, and reads (post sequencing) for each sequencing platform, Tag-seq and 300PE respectively. Qubit scores for most of the Tag-seq, gonad, and whole embryo (developmental atlas) were relatively low from .05-27ng/ul (Sup. Table. 1). This is to be expected given the type and amount of tissue. Samples were then Bio Analyzed to estimate RNA quality by way of the RIN score. RIN scores also ranged in quality, and for the most part reflected lower scores. A past test batch of similarly fixed and isolated RNA samples worked, even in the face of relatively low Qubit and RIN scores. Therefore, we felt confident moving forward with sequencing. The first batch (batch#1) of sequences consisted of 264 samples, fourteen samples did not pass library prep (likely due to poor quality RNA). This resulted in 250 samples being sequenced using a Tag-seq approach. One tissue sample did not result in a good quality score (*E. rathbuni* 1.1_skin). After receiving sequences from the Genome Sequencing GSAF, we ran a MultiQC with all Fasta files. The results indicated that the above tissues sample, although having successfully passed library prep, resulted in poor quality sequencing. We resubmitted this sample along with the fourteen others that did not initially pass library preparation in a second batch. The second batch (batch#2) consisted of 95 samples, of both the remaining developmental tissues and those that did not pass library prep. The majority of those that did not pass library prep the first time, once again showed unfavorable readings and did not result in usable sequences (after observing sequence quality in a MultiQC, see Sup Fig. 2).

Results of eye expression are shown here (Fig. 6), and results from additional adult tissues are expanded on below. We have found that the greatest amount of variation is due to developmental stage 1mpo vs. all other stages (PC1 56%). This is followed by PC2 (9%) accounts for the difference in phenotype (subterranean vs. surface, Fig. 6).

The gonad RNA sequencing batch (batch_gonad) consisted of both gonad tissue and four whole embryo stages each for *E. rathbuni* and *E. nana* (n=8). The gonad tissues consisted of three gravid female *E. rathbuni* and *E. nana*. We submitted four tissues for each of the three females, including eye, skin, olfactory, and gonad tissues that were dissected and flash frozen, resulting in twenty-four tissue samples between the two species. We were limited on the number of non-gravid females, but were able to obtain the four tissues including the gonads of one representative for each species, and would allow us to compare genetic and expression differences between gravid and non-gravid females. Moreover, a sexually mature male representative for each species (*E. ratbhuni* and *E. nana*) was sacrificed, and the above four tissues were obtained including gonad tissue (testis) to establish an expression and genetic baseline for gonadal male sexual maturity. All of these tissues were sequenced using a MiSeq V3 300PE (as mentioned above), and together resulted in forty-five samples sequenced for the gonad batch. Three skin tissues (*E. rathbuni*_non-gravid, *E. rathbuni*_male_rattlesnake cave, and *E. rathbuni*_gravid) did not make it past library prep and reduced the number of samples to forty two.

DISSCUSSION

Understanding the genetic and environmental cues responsible for sexual maturity and gravidity is essential to ongoing conservation work of endangered species. This information is

particularly useful when the goal is to establish a successfully reproductive refugia population intended to for potential reintroduction. Salamanders of the genus *Eurycea*, and within the clade *Paedomolge* from central Texas are excellent examples of such a need. Some salamander species from this clade are federally listed (*E. rathbuni*, *E. nana*, etc.), and are hard to study given their limited and difficult to reach range (deep regions of the Edwards-Trinity Aquifer). This has resulted in the paucity of fundamental components of their biology including factors that contribute to their reproduction (e.g. seasonality, courtship, sexual maturity, etc.). Captive husbandry has helped with this conundrum, but lacks in the natural settings some of these species inhabit (e.g. complete darkness). What is less likely to deviate are the conserved-genetic and molecular underpinnings responsible for sexual and reproductive maturation. Herein, we set out to establish a genetic foundation for sexual maturity and gravidity. Because these salamanders have a relatively translucent underbelly, observing oocytes to determine gravidity, or the dorsally compressed dark black lines of mature testis is relatively easy and the way we categorized specimens and tissues. Moreover, we sequence the adult sensory tissues (eyes, olfactory epithelium, and skin) that may play an intermediate role between environmental cues and initiation of sexual maturation (eye tissue through development), and an active role during reproduction of sexually mature individuals (gravid vs. non-gravid). A compounding factor of the *Paedomolge* salamander clade is their genome size (~10x the size of the human genome). With such a massive genome, it's extremely hard to fully understand all of the genetic and molecular variables contributing to sexually mature individuals (e.g. non-coding regulatory regions). However, we can obtain a snapshot in time and tissue to identify genetic patterns that are being "turned on" or "off" to understand the conserved genes that are playing a role in gravidity and sexual maturity

To understand genes that are being expressed we composed a de novo transcriptome of the closest related ancestor to the *Paedomolge* clade, Western dwarf salamander (*E. paludicola*). This species represents a biphasic (completes metamorphosis to reach sexual maturity) outgroup, and a likely representative of the ancestral genome to the *Paedomolge* clade. By isolating the RNA, we are capturing the genes being expressed in a particular tissue at that moment in time. Therefore, by isolating and sequencing the RNA from gonadal tissue we aim to test for differential gene expression and identify genes responsible for gravidity (i.e. ***Question 1*** above). Similarly, by isolating and sequencing RNA from eyes, olfactory epethilium, and skin of gravid and non-gravid females we can test for differences within thes sensory tissue's given gravidity (i.e. ***Question 2*** above). Moreover, we use a similar approach for male gonadal tissue (i.e. testis) and compare gene expression between male and female expression. We were able to identify differential expression between tissue state (gravid vs. non-gravid; Fig. 8), and in this way show compelling evidence that there are underlying genetic mechanisms at work that contribute to female gravidity (Table 1). We observe species level differences among all comparison of all tissues, but observe no difference between gravidity state (gravid vs non-gravid) within tissue types (eyes; Fig. 9*B*, olfactory; Fig. 10*B*, skin; Fig. 11*B*). Therefore, we can not reject the null hypothsis for that there are no differences within tissue types (eyes, skin, olfactory) between gravidity states.

To make comparisons of different tissues we normalization and filtered using DESseq threshold of genes with a 50 or less total count (Sup. Fig. 3). For example, this resulted in 18,972 total annotated genes and 558 unique genes among all of the gonad project tissues (eyes, skin, olfactory epithelium, gravid oviduct, non-gravid oviduct, testis). The greatest amount of variation observed largely divides gonadal tissues and all other tissue types (Fig. 7 PC1 31%).

This result was compelling because it suggests that gonadal tissue has a unique expression profile and is largely different in this expression even when considering other tissue types. The second greatest amount of variation is associated with species level differences (Fig. 7, PC2 6%).

Excitingly, when comparing gonadal tissues (oviduct and oocytes) of gravid vs. non-gravid female *E. rathbuni* and *E. nana* we found the greatest amount of variance is between gravid and non-gravid females (Fig. 8, PC1 42%), and the second greatest amount of variation seems to account for species level differences (Fig.8, PC2 23%). Normalization and filtering using a threshold of genes with 10 or less total count (Sup. Fig. 4), resulted in 2,432 total annotated genes and 304 unique genes among the gonad-gravidity tissues. It appears that two of the three *E. nana* gravid females fall out towards the non-gravid female controls. This was an interesting observation, but after further exploration we identified that these females, although superficially exhibiting one or two oocytes (relatively minimal) compared to ten or more large oocytes, seem to represent an intermediate stage of gravidity. Unsurprisingly, these semi-gravid females express a repertoire of genes that falls out somewhere between gravid and non-gravid (Fig. 8). Our serendipitous identification of a transitionary state between gravid and non-gravid *E. nana* females is a welcomed opportunity to further classify genes associated with reaching a mature gravid state (likely indicated by an abundance of large oocytes), and will provide the bases for future studies.

Herein, we identify differntialy expressed genes between gravid and non-gravid female *E rathbuni* and *E. nana* (Fig. 8). We found seven significantly differentially expressed genes (Table. 1). These genes are upregulated in non-gravid females, which can also be interpreted as down regulation in gravid females (Sup. Fig. 8-14). These genes are relatively ubiquitous, and their roles in gonad tissue and oocyt developmental have yet to be fully explored. Most of them

are implicated in maintaining homeostasis in amphibians and other organisms. Some examples include roles in ion transport (*clcc1*; Campbell et al. 2013), mitochondrial function (*miga1*; Hong et al. 2024), protein syhtnesis (*usp38*; Ross et al. 2021), cilia formation (*mks1*; Cui et al. 2013), and gene expression and regulation (*asxl3*; Bainbridge et al. 2013).

Four whole embryonic stages for each species were sequenced and represent the first window into understanding differential expression between the early development of two different species of endangered *Paedomolge* species. Normalization and filtering using a threshold of genes with 10 or less total count (Sup. Fig.5), resulted in 8,040 total annotated genes and 1,005 unique genes among the eight embryos. The greatest amount of variation is largely associated between species, *E. rathbuni* and *E. nana*, respectively (Fig. 13, PC1 28%). Interestingly, all four *E. nana* embryos cluster together and PC2 18% largely separates two early *E. rathbuni* stages and all other samples.

Eyes can be an important sensory modality when considering the reproductive biology of salamanders, for example kin recognition and circadian rhythms both have been implicated as cues for amphibian reproduction (Sinervo et al . 2001, and Wilczynski et al. 2005) . However, several species including the federally listed *E. rathbuni* have invaded subterranean habitats resulting in reduced non-functioning eyes. How much of a role do eyes play through development leading to sexual maturity? We have sequenced eye expression through development (1-5mpo) and in adults, and after normalization and filtering using a threshold of genes with 10 or less total count (Fig.6) resulted in 4,113,327 total annotated genes and 37,057 unique genes among the dataset of n=111. We have found that the greatest amount of variation is largely due to developmental stage early development vs. later stages (Fig. 6 PC1 56%). Interestingly, PC2 (9%) accounts for the difference in phenotype (Fig. 6). Interestingly, when

considering gravidity, eye expression does not appear to differ (Fig. 9). This might be because of relative vestigel state of the subterranean species eye (Fig. 5). What is interesting is the lack of compensation from other sensory modalities given gravidity (in *E. rathbuni* and *E. nana*). There might be more to gain from targeting the transcriptome profile of the respective brain regions assocaietd with each of these sensory modalities (optic lobe, olfactory bulb, etc.), as sensory tissue may play a more transient role, perhaps in the moment(s) leading to mating and courtship.

Differences in gene expression between male and female gonad tissues were also identified. Male gonad tissues are seprated by PC1 43% (Fig. 12 *B*) and fall out with female non-gravid gonad tissue. However, male gonad tissue appears to seprate from non-gravid female gonad tissue in PC2 20% (Fig. 12 *C*). We tested for differtially expressed genes (Fig. 12 *D*) and found 297 (Sup. Table. 2) differentially expressed genes between female and male gonad tissues. This is an extensive list that likley represents two drastically different sex linked tissue types.

Together these transcriptome datasets represent the most comprehensive transcriptome data for the Texas *Eurycea* group, and an invaluable toolset for future research. The above comparative analysis provides a platform for thinking about and asking questions regarding reproduction, sexual maturity, and tissue specific differential expression associated with species and development within an evolutionary framework. The millions of genes represented within these datasets can also be mined to identify species specific genes of interest. This is powerful for future investigators as they will not need to rely on a model system from a distantly related taxa (e.g. *Xenopus,* or *Axolotl*) to obtain an *E. rathbuni* or *E. nana* specific sequence. These genes can be used to create species specific assays and primers for downstream use in environmental DNA (eDNA), quantitative PCR, or high throughput sequencing platforms.

FIGURES AND TABLES

***E. nana* (stages 21-31)**

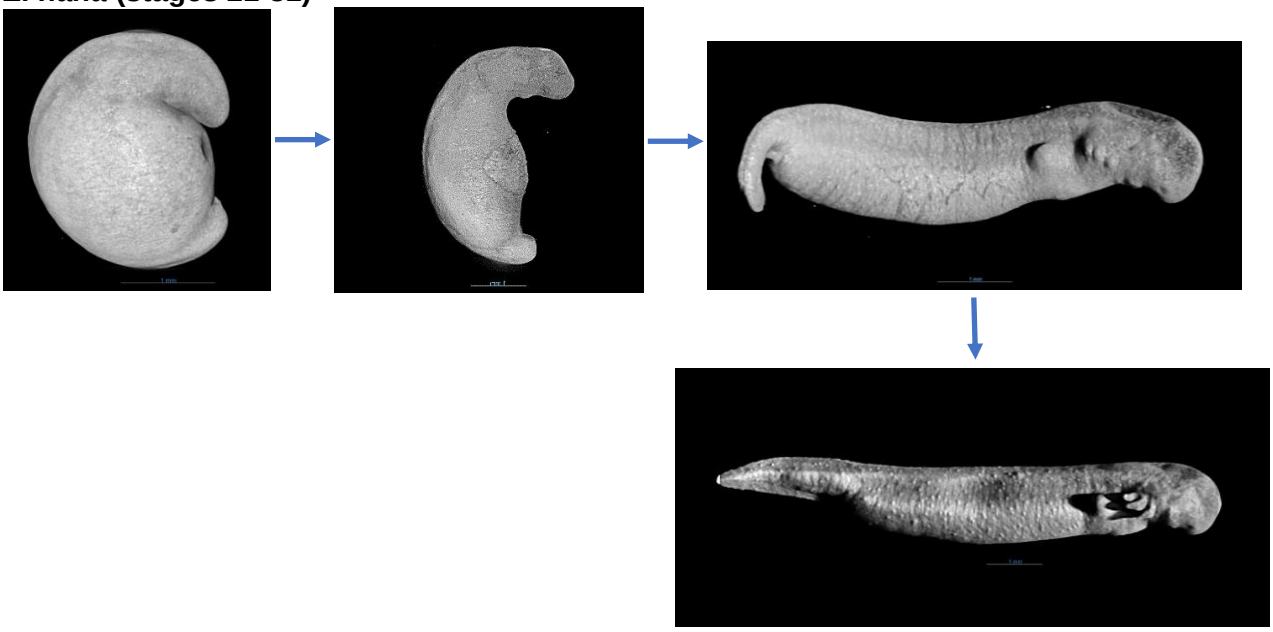


Figure 1. *E. nana* flow chart of developmental series from stage 21-40.

***E. rathbuni* (stages 21-31)**

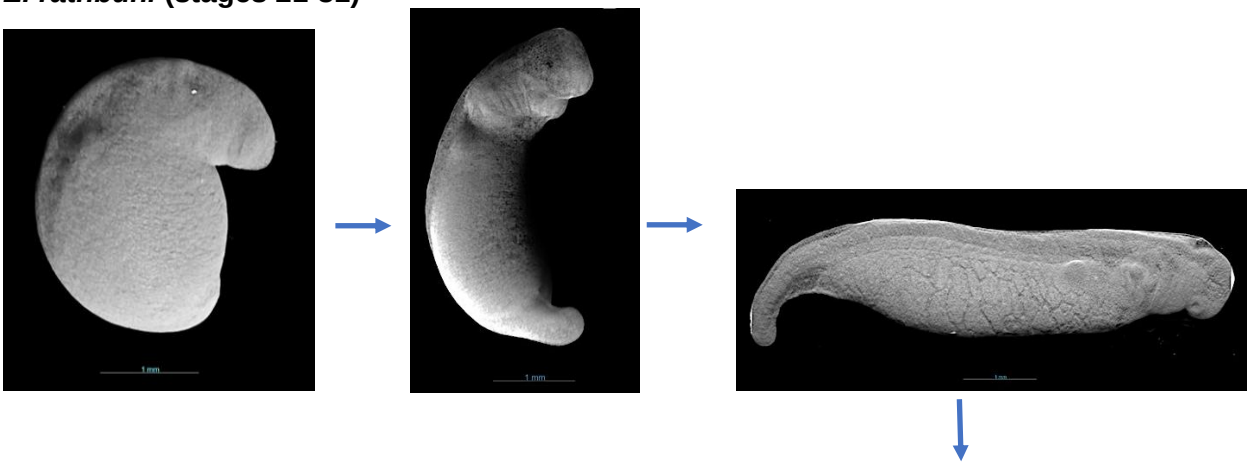


Figure 2. *E. rathbuni* flow chart of developmental series from stage 21-40.

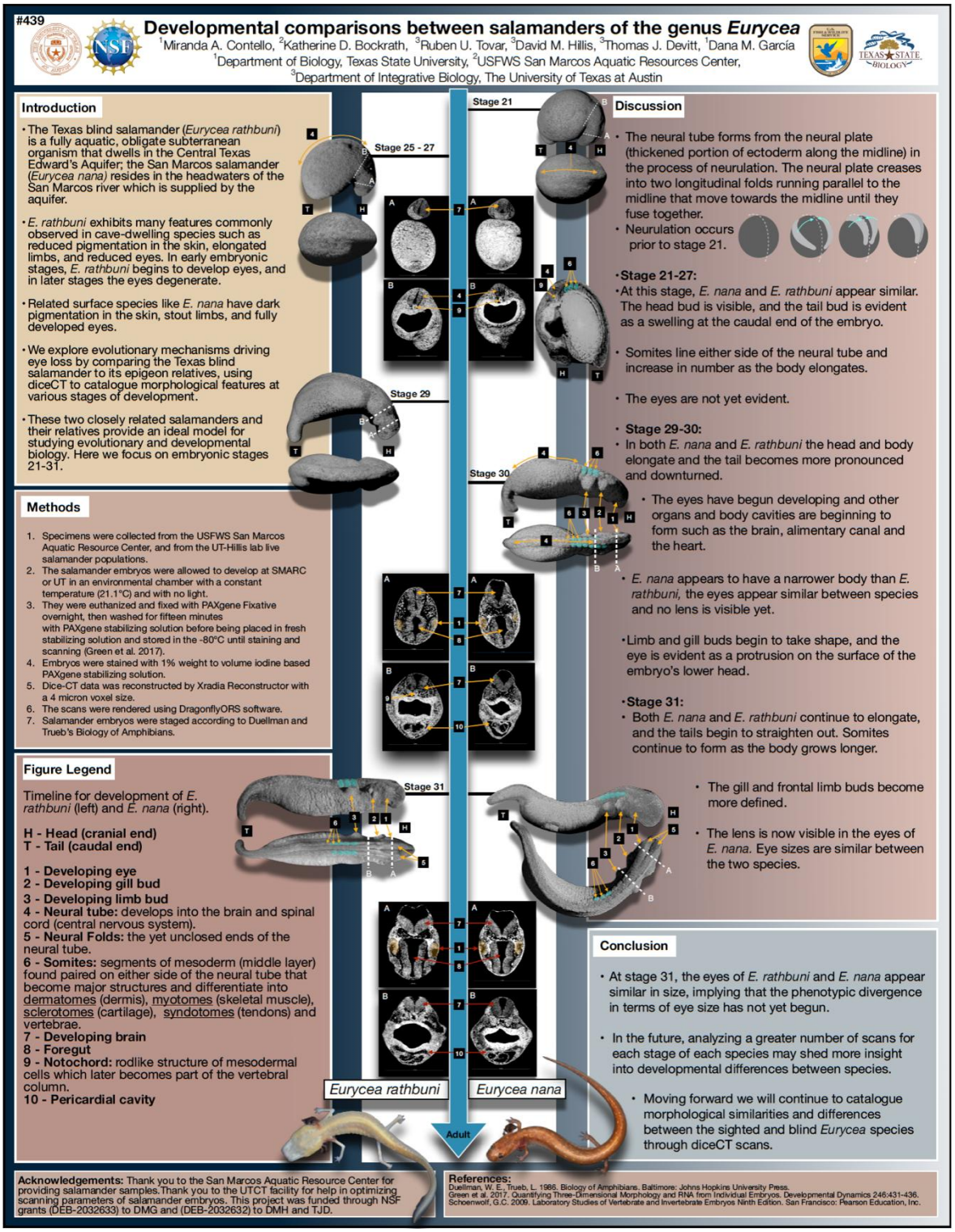


Figure 3. The culmination of the developmental atlas description. We are working on drafting a manuscript of this work.

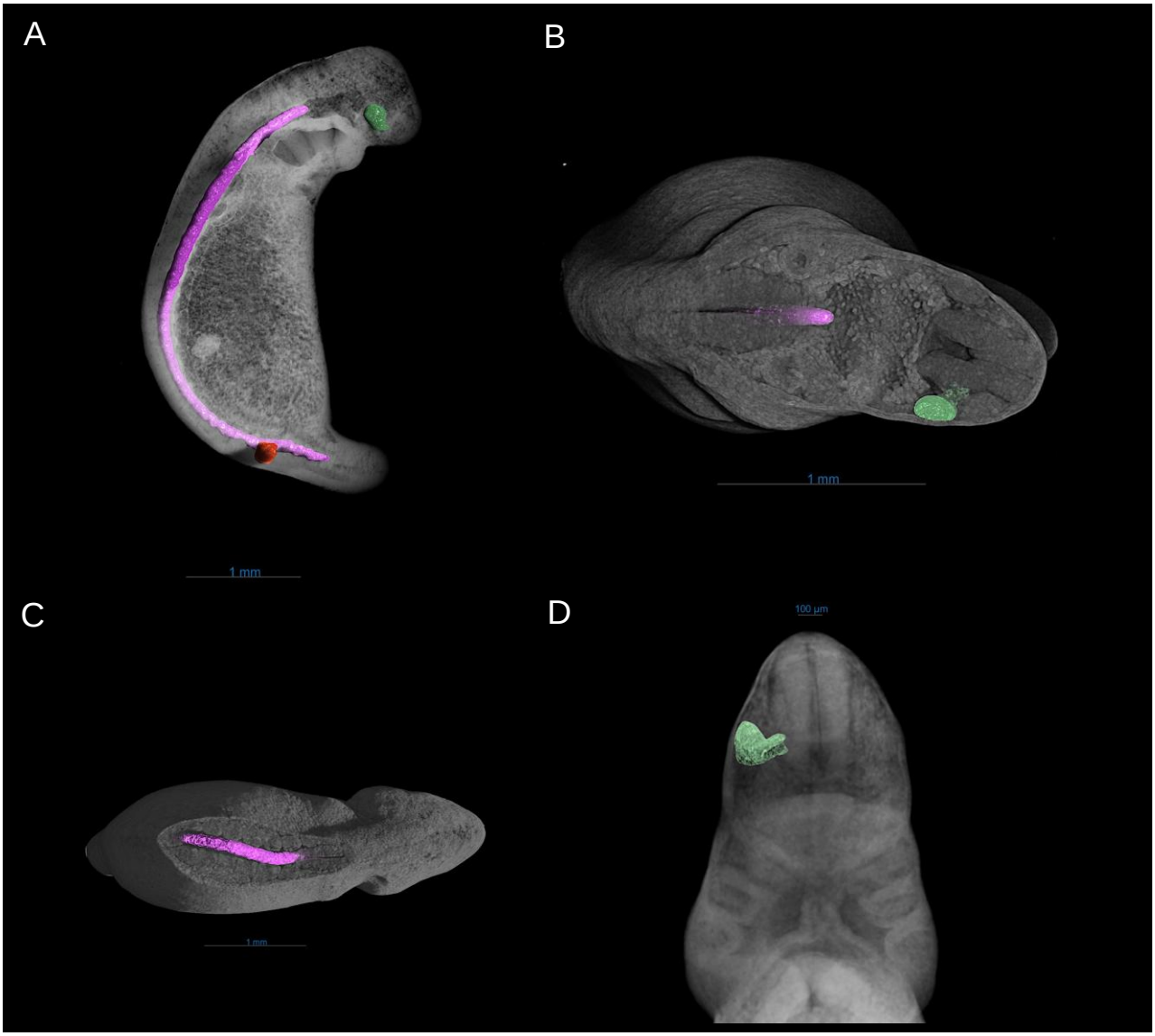
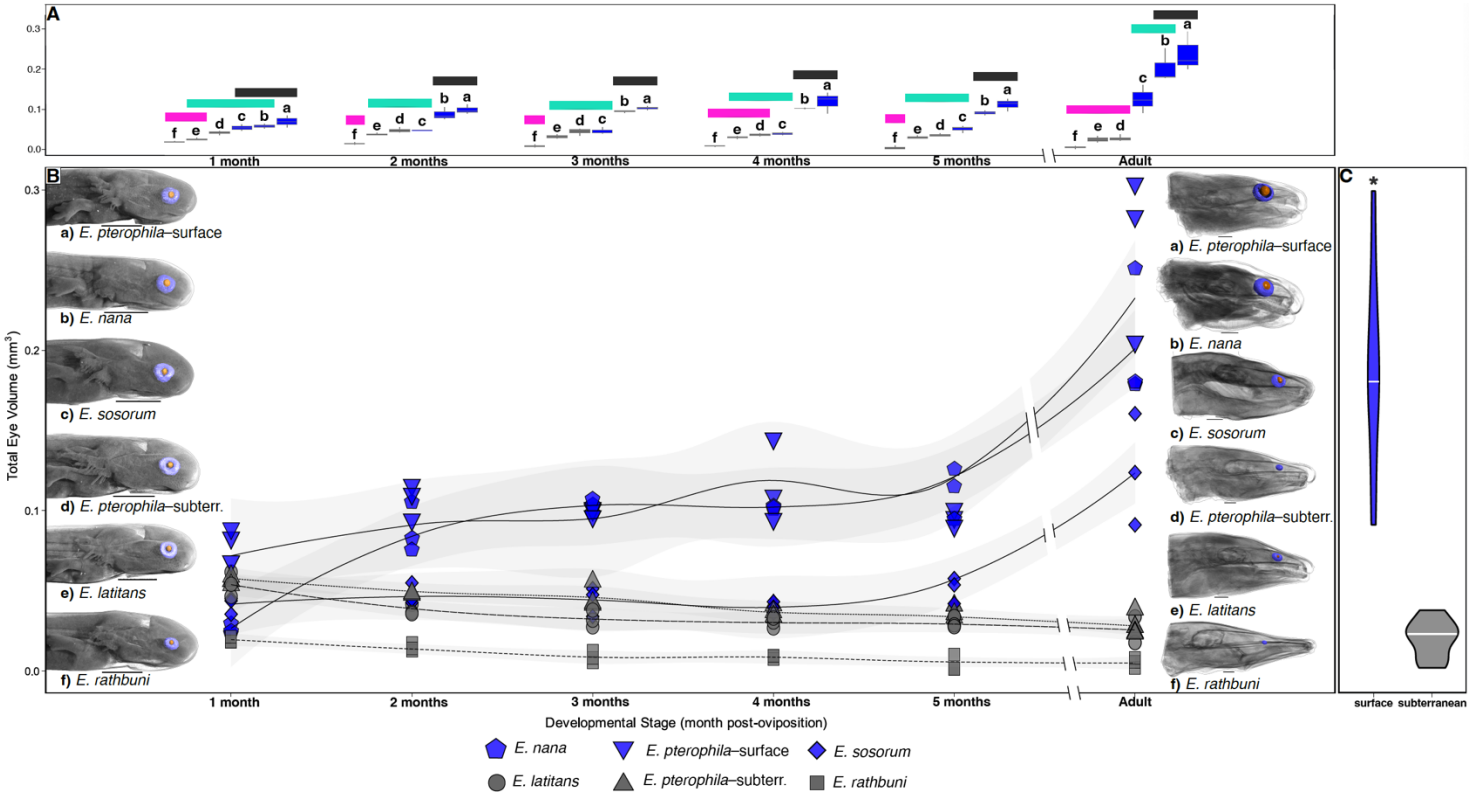


Figure 4. Segmented soft tissue of *E. rathbuni* at stage 31. Segmented components of the central nervous system can be identified here including, the neural tube (purple), somite (A, red), and optic stalk and cup (green). At stage 31 in *E. rathbuni* several cutaway angles are provided including transverse (A), and coronals at different planes (B-C). A figure with increased opacity is also presented to illustrate the typical morphology of the optic stalk and cup formation (D, in green) relative to other features of the developing head.

Figure 5. (A) Boxplots show mean volume distribution for every developmental stage



and colored bars (black, turquoise, and pink) represent significance groupings from a Tukey's post-hoc. (B) Generalized linear regression was used to measure and plot the developmental trajectories of eye volume (the retina in blue and lens in orange) through a developmental series of six taxa of *Eurycea*. Taxa include three surface populations (a: *E. pterophila*– surface; Comal Springs, upside down blue triangle; b: *E. nana*, blue pentagon; c: *E. sosorum*, blue diamond) and three subterranean populations (d: *E. pterophila*– subterranean; Preserve Cave, gray triangle; *E. latitans*–Honey Creek Cave, gray circle; f: *E. rathbuni*, gray square). Eye volumes are plotted for each developmental stage (1-5 months post-oviposition and adults; n=3 for each stage and taxon). (C) Standardized adult eye volumes between surface and subterranean species are significantly different. Scale bars indicate 1 mm.

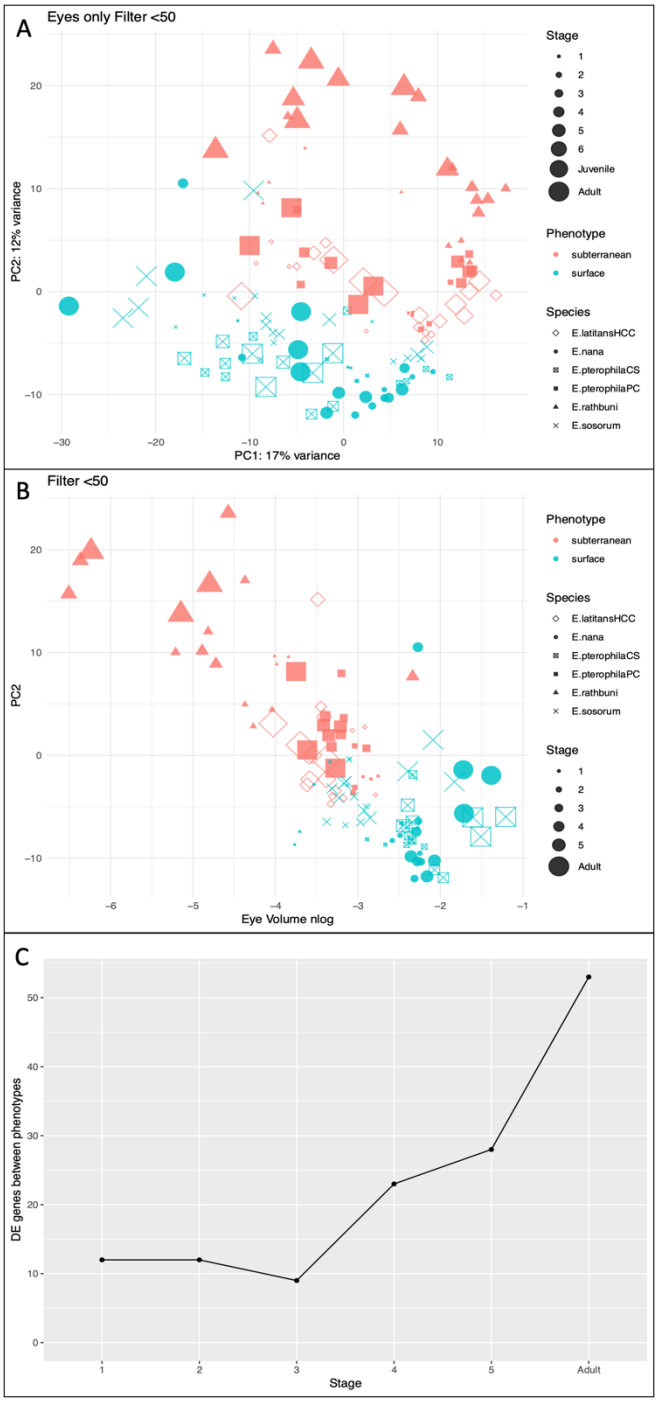


Figure 6. After normalization the expression data for eye tissue through development is plotted using a PCA (A), illustrating grouping largely by phenotype (subaterranean is salmon color, and surface is blue), and mostly accounted for by PC2 12%. Species are represented by different shapes and the age of by shape size (1-5mpo, anD adults). Eye volume gathered from diceCT is plotted as a linear regression against PC2 (B) and shows broad trends in expression based on phenotype to eye size given developmental stage. The number of differentially expressed (DE) genes are plotted for 1-5mpo and adults (C), illustrating an increase of eye DE genes through development.

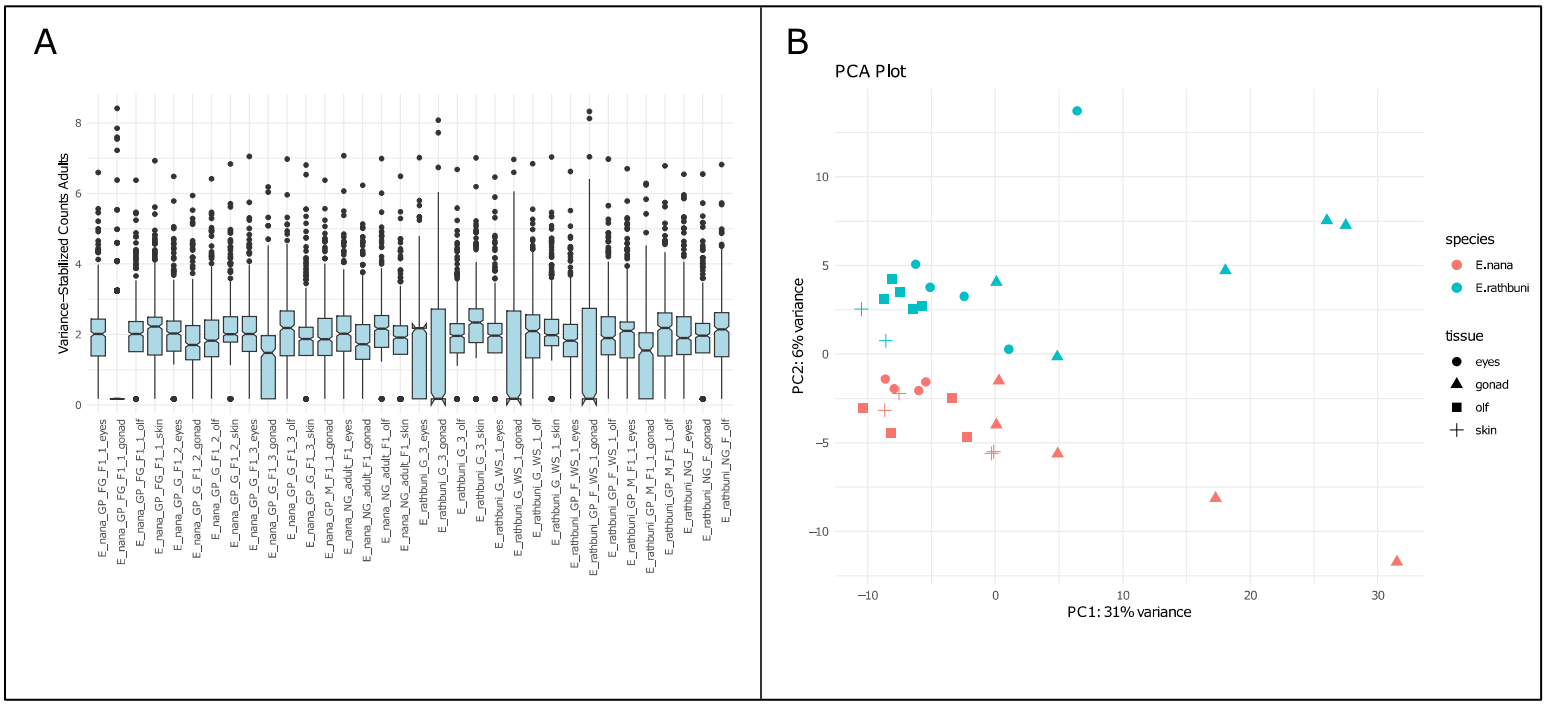


Figure 7. Normalized RNA expression data (A) of all adult tissues for the gonad project with a PCA (B) identifying variation mostly associated with tissue type (gonads vs. all other tissues) (PC1 31%) and species (PC2 6%).

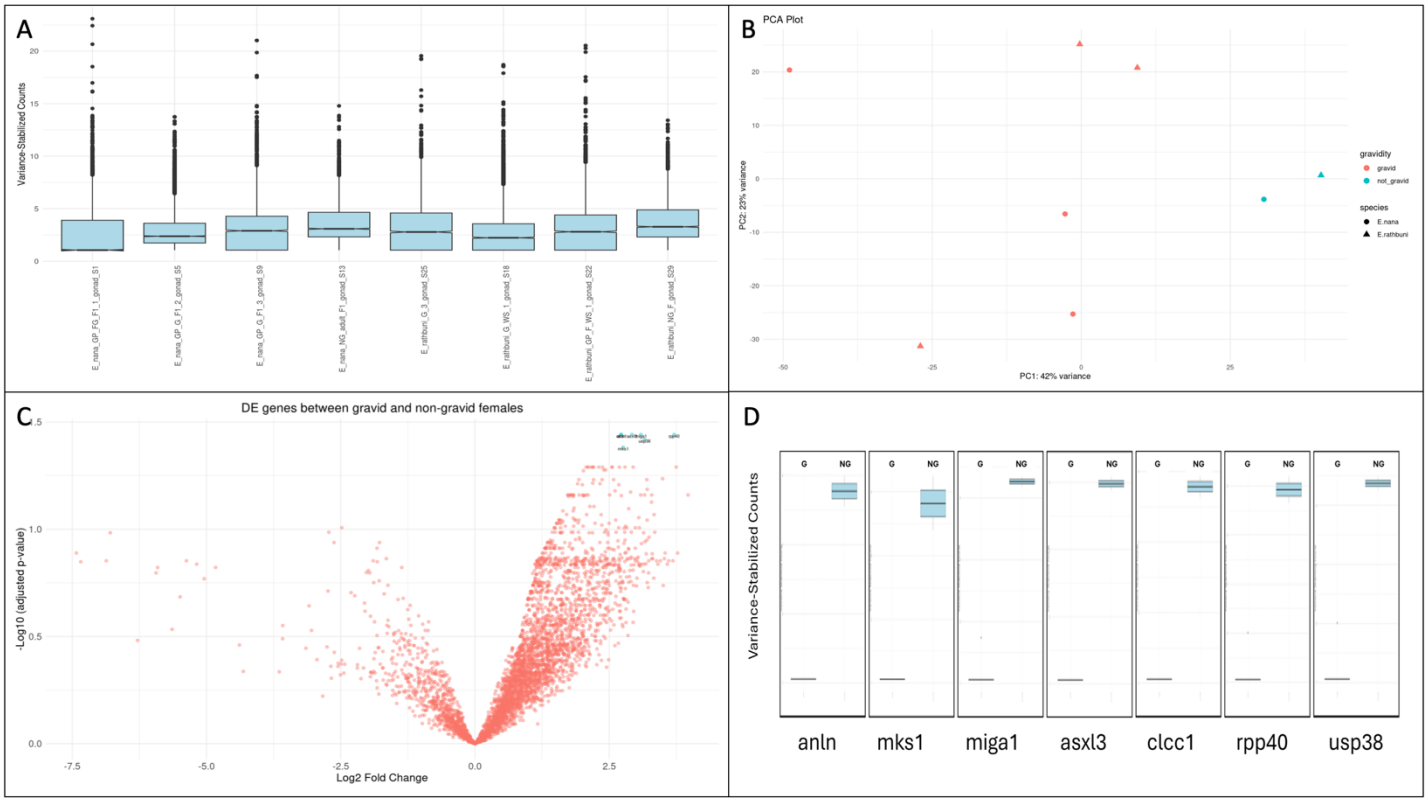


Figure 8. Gonad tissue of gravid and non-gravid female tissue. Normalized RNA expression data (A) of all female gonad tissue. The gonad tissues are divided into gravid (pink) or non-gravid (blue) and by species using shape; *E. rathbuni* (triangle) and *E. nana* (circle) (B). A PCA (B) identifies variation mostly associated with gravidity type (gravid vs. non-gravid) (PC1 42%) and then species (PC2 23%) (B). Differential genes (in light blue) being expressed are shown with a volcano plot along with all other genes (salmon color) captured (C, see Table 1 for more detail). Individual gene box plot distributions illustrate the observed gene variation (D) between gravid (labled, G) and non-gravid (labled, NG).

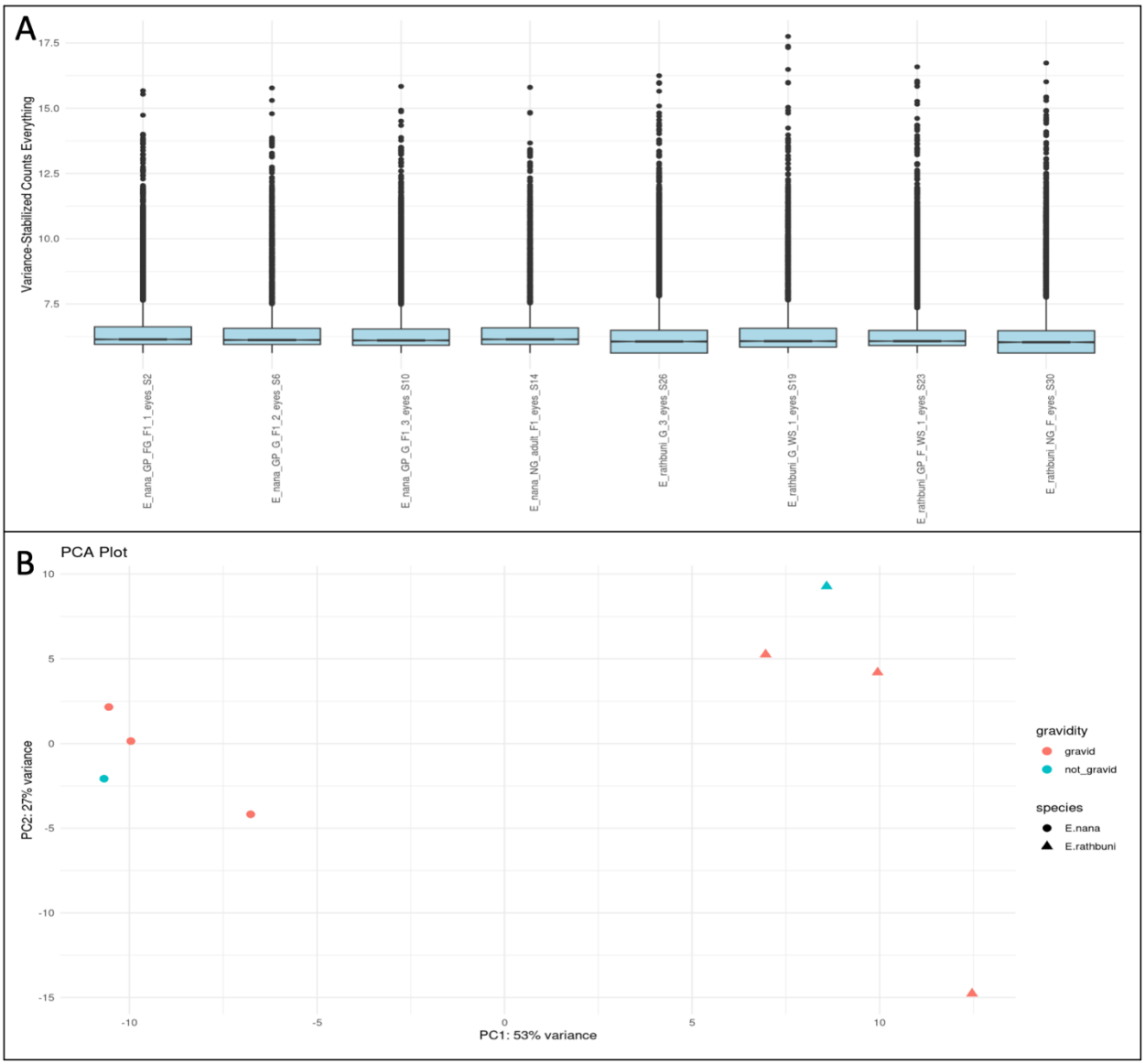


Figure 9. Eye tissue of gravid vs non-gravid females. Normalized RNA expression data (A, excluding genes with a ten or less read count) of adult eye tissue for the gonad project with a PCA (B) identifying variation associated with species level differences (PC1 53%).

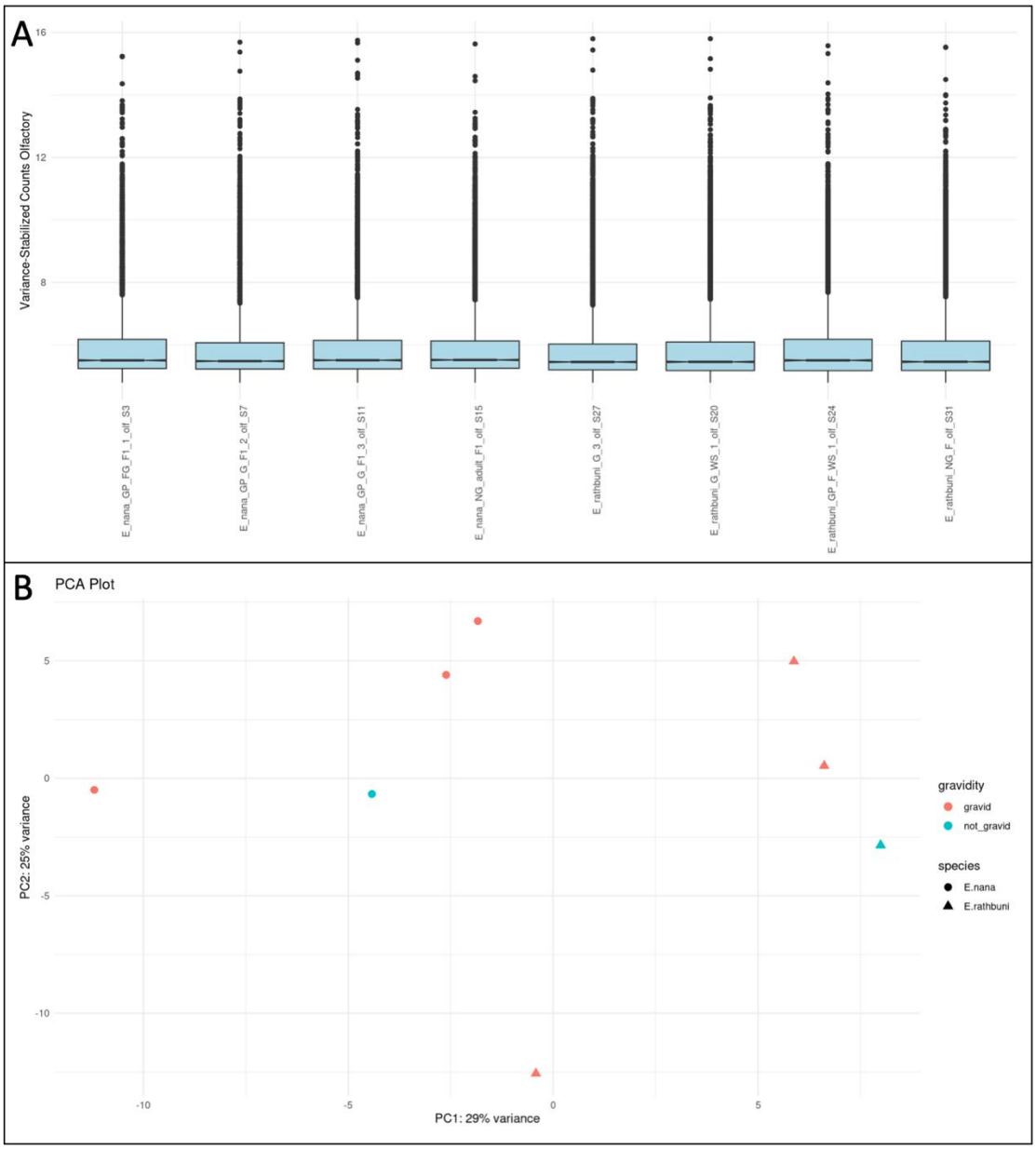


Figure 10. Olfactory tissue of gravid vs non-gravid females. Normalized RNA expression data (A, excluding genes with a ten or less read count) of adult eye tissue for the gonad project with a PCA (B) identifying variation mostly associated with species level differences (PC1 29%).

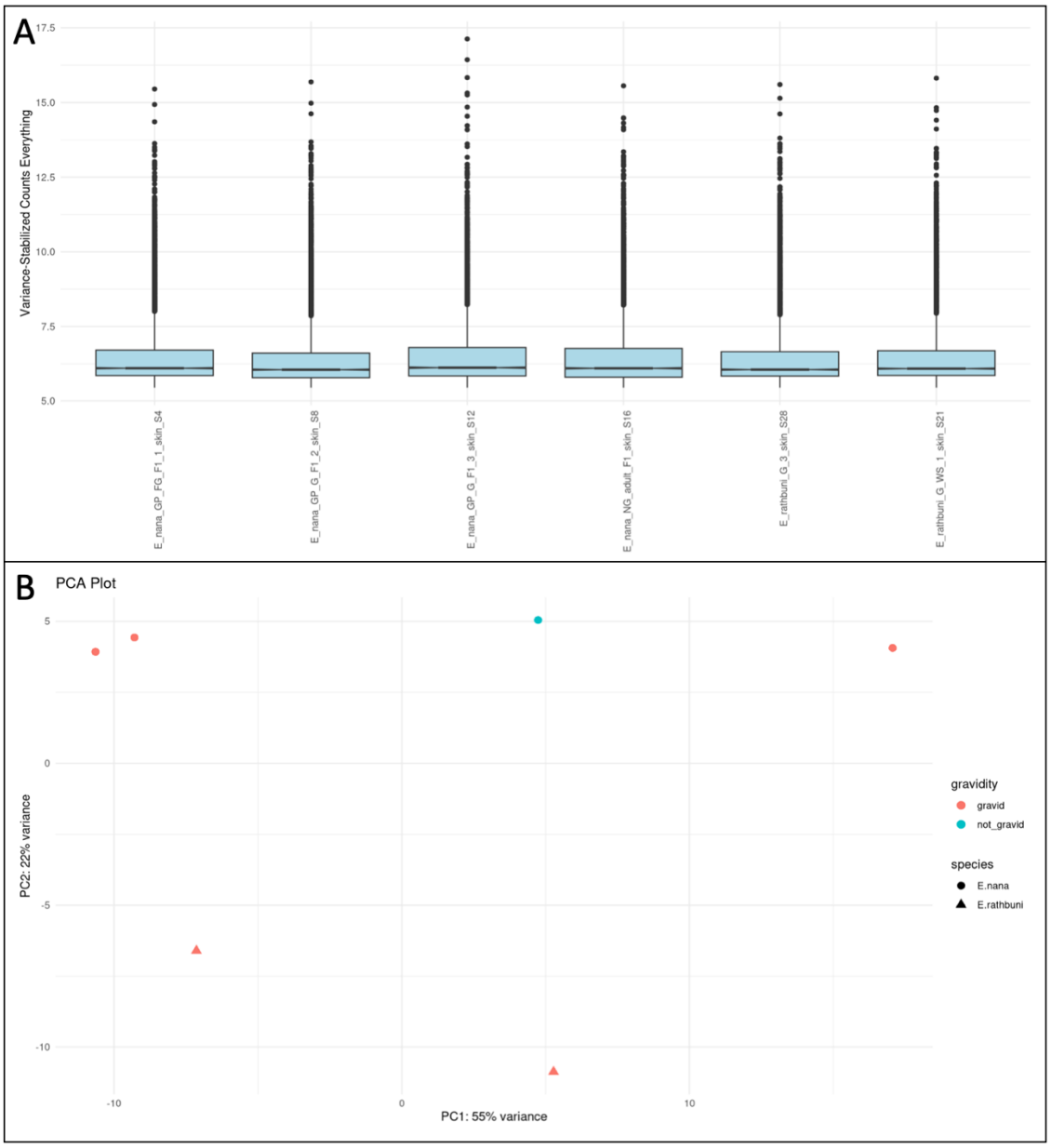


Figure 11. Olfactory tissue of gravid vs non-gravid females. Normalized RNA expression data (A, excluding genes with a ten or less read count) of adult eye tissue for the gonad project with a PCA (B) identifying variation mostly associated with species level differences (PC2 22%).

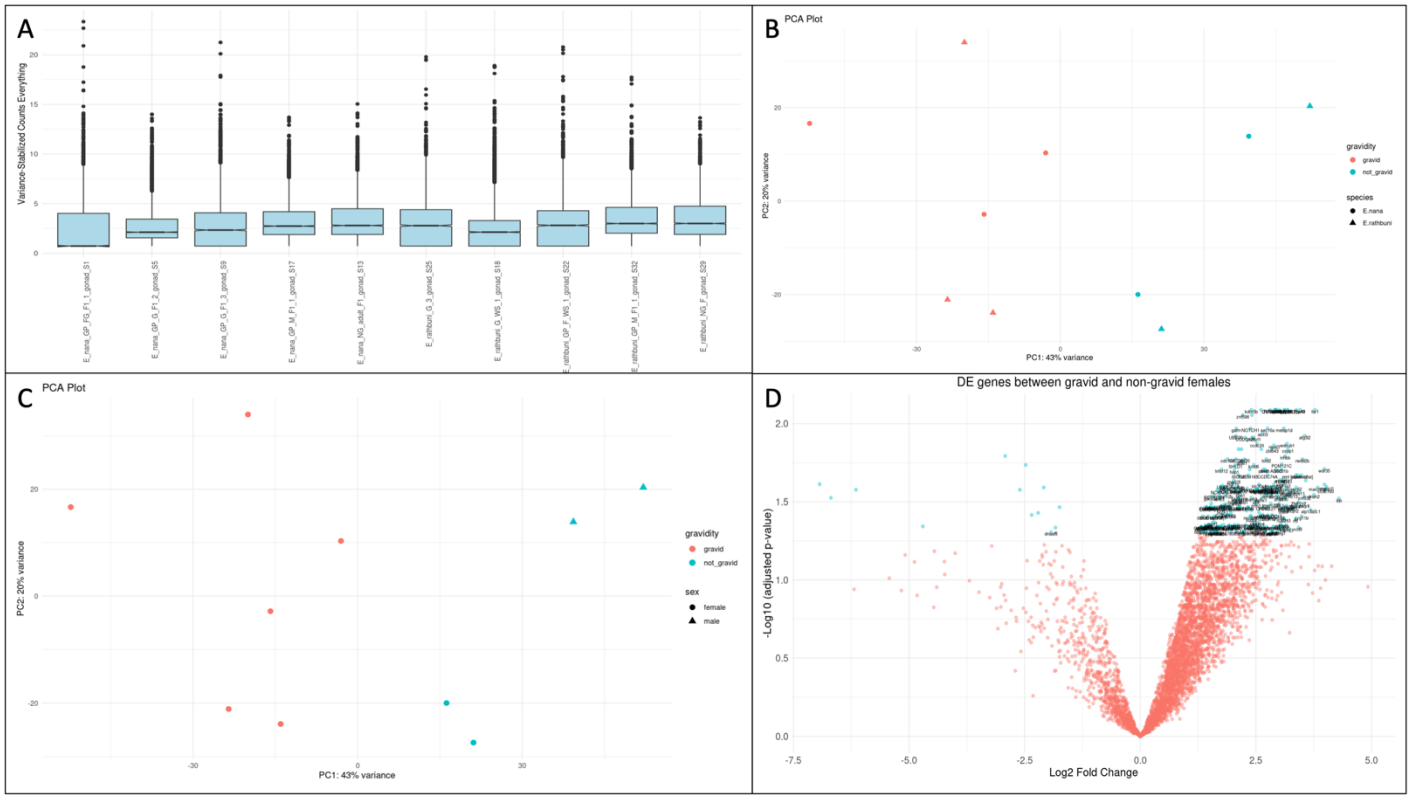


Figure 12. Normalized RNA expression data (A, excluding genes with a ten or less read count) of all female and male gonad tissue. The gonad tissues are divided into gravid (pink) or non-gravid/male testis (blue) and by species using shape; *E. rathbuni* (triangle) and *E. nana* (circle) (B). A PCA (B) identifies variation mostly associated with gravidity type (gravid vs. non-gravid) (PC1 43%) and then species (PC2 20%) (B). The two upper blue triangle and circle in panel-(B) represent the males, the male gonads are further distinguished in panel-(C). Differential gene expression is illustrated with a volcano plot (D), identifying 297 differentially expressed genes between males and females (please see Sup. Table 2 for a list of these genes).

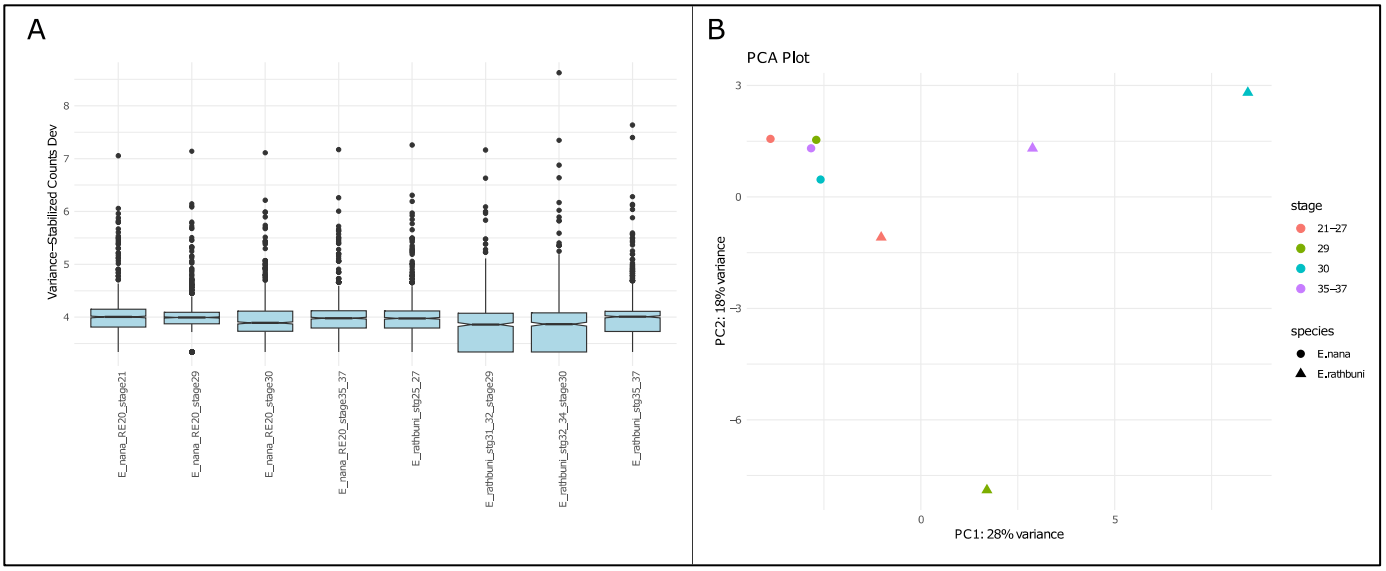


Figure 13. Normalized RNA expression data (A) of only whole embryo tissue. The embryos are categorized by developmental stage 21-27 (pink), 29 (green), 30 (blue), and 35-37 (purple). Shapes separate species *E. rathbuni* (triangle) and *E. nana* (circle). Expression data is plotted using a PCA (B) identifying variation mostly associated with species (PC1 28%) and then stage (PC2 18%).

Table 1. Differentially expressed genes between gravid and non-gravid gonad tissue from female *E. rathbuni* and *E. nana* (as identified in Fig. 8).

Gene Esemble ID	Log FC	Avg. Exp.	P. Value	Adj. P. Value	B	Gene Name
ENSXETG00000005228	2.727232	1.740401	3.54E-05	0.03612149	2.65173	clcc1
ENSXETG00000010339	2.760198	1.748642	6.67E-05	0.04142608	2.110445	mks1
ENSXETG00000013134	3.091637	1.944622	2.94E-05	0.03612149	2.807589	miga1
ENSXETG00000017515	3.157711	2.006587	5.26E-05	0.03811851	2.315026	usp38
ENSXETG00000017617	3.70756	2.14405	1.93E-05	0.03612149	3.154819	rpp40
ENSXETG00000020547	2.922239	1.789152	1.95E-05	0.03612149	3.144556	asxl3

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SUPPLIMENTARY MATERIAL

Sup. Table 1. An example table of tissue samples from batch #2 and their corresponding Qubit concentrations (ng/ul).

Sample Type: RNA_Batch#2

Sample Name	Volume (μL)	Concentration (ng/μL)
E_rath_J_1_Eyes	20.0	1.2
E_rath_J_1_Skin	20.0	16.5
E_rath_J_1_Olf	20.0	1.7
E_rath_3_3_Eyes	20.0	1.0
E_rath_3_3_Skin	20.0	7.3
E_rath_3_3_Olf	20.0	1.0
E_rath_5_2_Eyes	20.0	1.0
E_rath_5_2_Skin	20.0	8.2
E_rath_5_2_Olf	20.0	1.5
E_nana_4_2_Eyes	20.0	2.2
E_nana_4_2_Skin	20.0	4.2
E_nana_4_2_Olf	20.0	1.3
E_nana_5_1_Eyes	20.0	1.5
E_nana_5_1_Skin	20.0	8.2
E_nana_5_1_Olf	20.0	2.3
E_nana_5_2_Eyes	20.0	1.5
E_nana_5_2_Skin	20.0	5.6
E_nana_5_2_Olf	20.0	1.0
E_rathbuni_Adult_1_Eyes	20.0	1.0
E_rathbuni_adult_1_Skin	20.0	36.1
E_rathbuni_Adult_1_Olf	20.0	5.6
E_rathbuni_Adult_2_Eyes	20.0	2.0
E_rathbuni_adult_2_Skin	20.0	2.5
E_rathbuni_Adult_2_Olf	20.0	9.5
E_rathbuni_Adult_3_Eyes	20.0	1.0
E_rathbuni_adult_3_Skin	20.0	24.9
E_rathbuni_Adult_3_Olf	20.0	6.44
E_nana_Adult_1_Eyes	20.0	6.33
E_nana_Adult_1_Skin	20.0	35.4
E_nana_Adult_1_Olf	20.0	2.7
E_nana_Adult_2_Eyes	20.0	11.4
E_nana_Adult_2_Skin	20.0	47.1
E_nana_Adult_2_Olf	20.0	1.5
E_nana_Adult_3_Eyes	20.0	10.5
E_nana_Adult_3_Skin	20.0	31.5
E_nana_Adult_3_Olf	20.0	11.3
E_sosorum_Adult_1_Eyes	20.0	8.71
E_sosorum_Adult_1_Skin	20.0	39.6
E_sosorum_Adult_1_Olf	20.0	14.3
E_sosorum_Adult_2_Eyes	20.0	6.96
E_sosorum_Adult_2_Skin	20.0	36.7
E_sosorum_Adult_2_Olf	20.0	17.8
E_sosorum_Adult_3_Eyes	20.0	4.06
E_sosorum_Adult_3_Skin	20.0	33.2
E_sosorum_Adult_3_Olf	20.0	10.5
E_latitansHCC_Adult_1_Eyes	20.0	2.0
E_latitansHCC_Adult_1_Skin	20.0	30.2
E_latitansHCC_Adult_1_Olf	20.0	9.6
E_latitansHCC_Adult_2_Eyes	20.0	2.0
E_latitansHCC_Adult_2_Skin	20.0	44.5
E_latitansHCC_Adult_2_Olf	20.0	12.0
E_latitansHCC_Adult_3_Eyes	20.0	2.0
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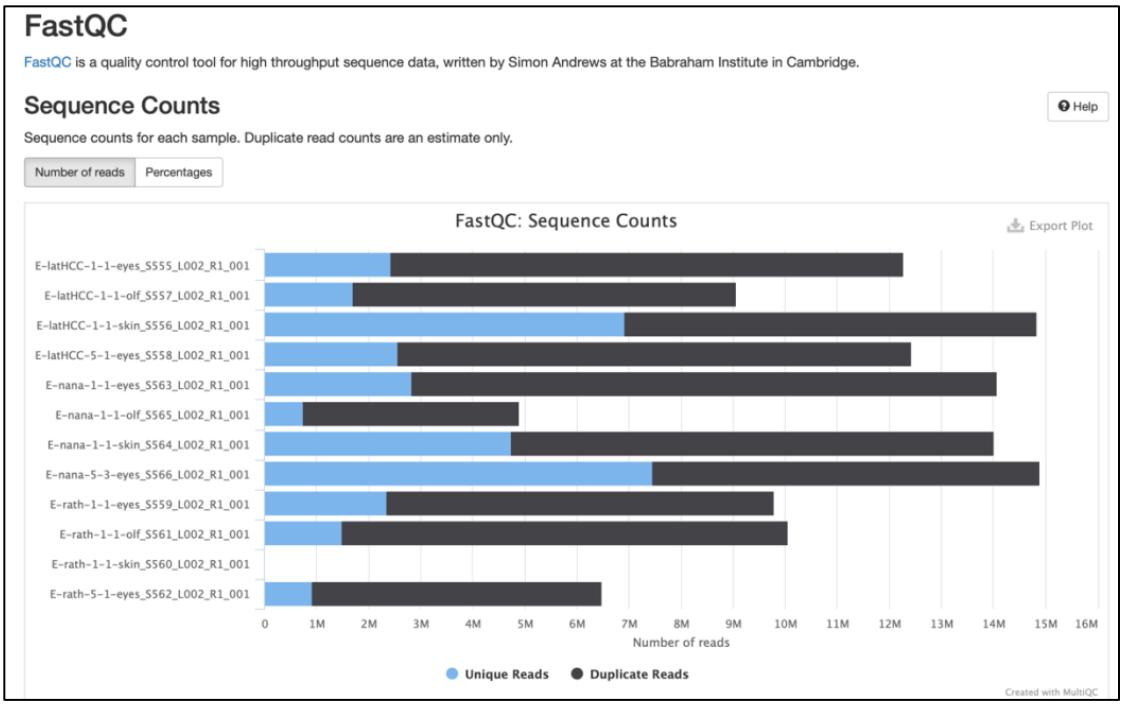
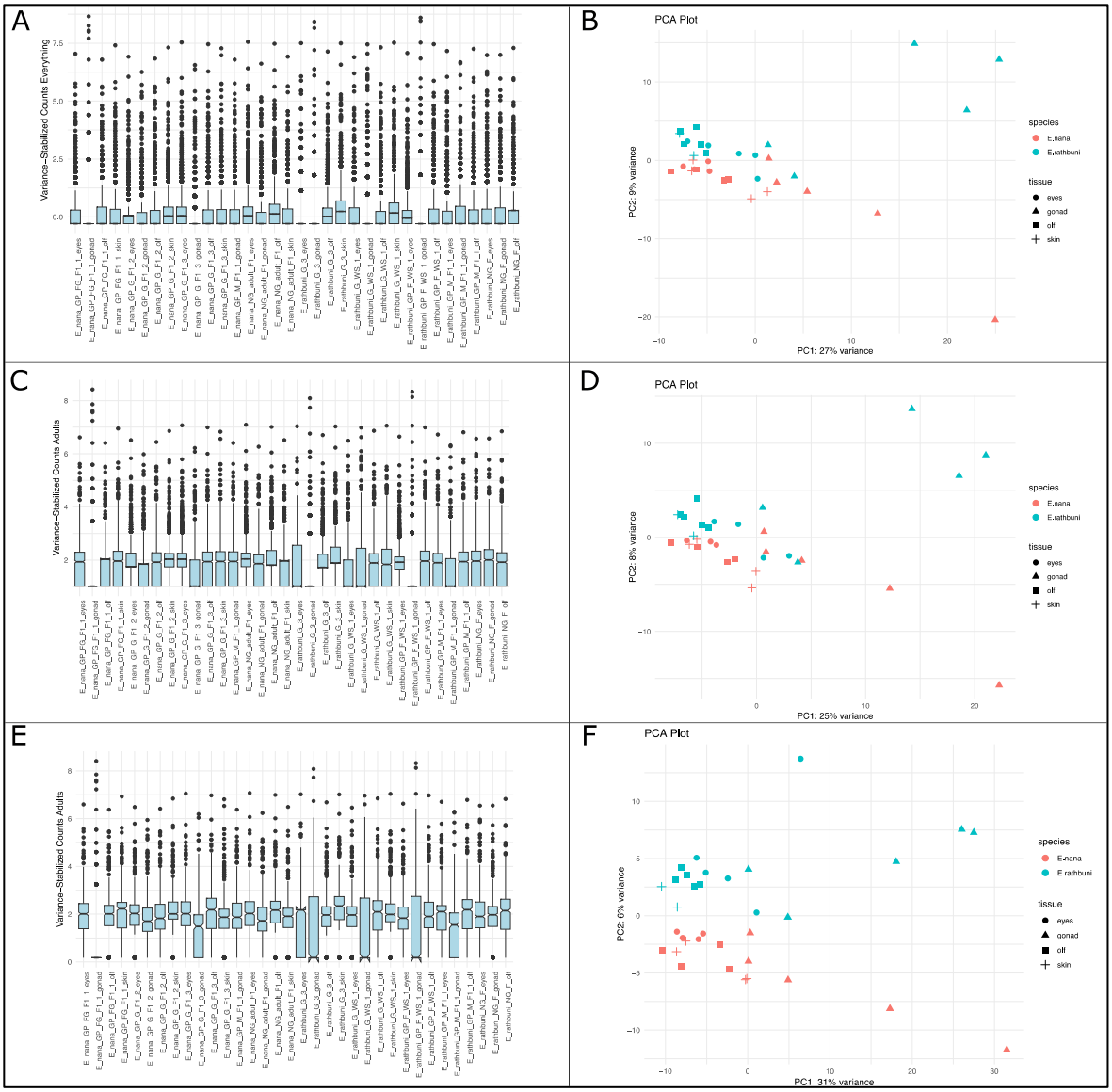
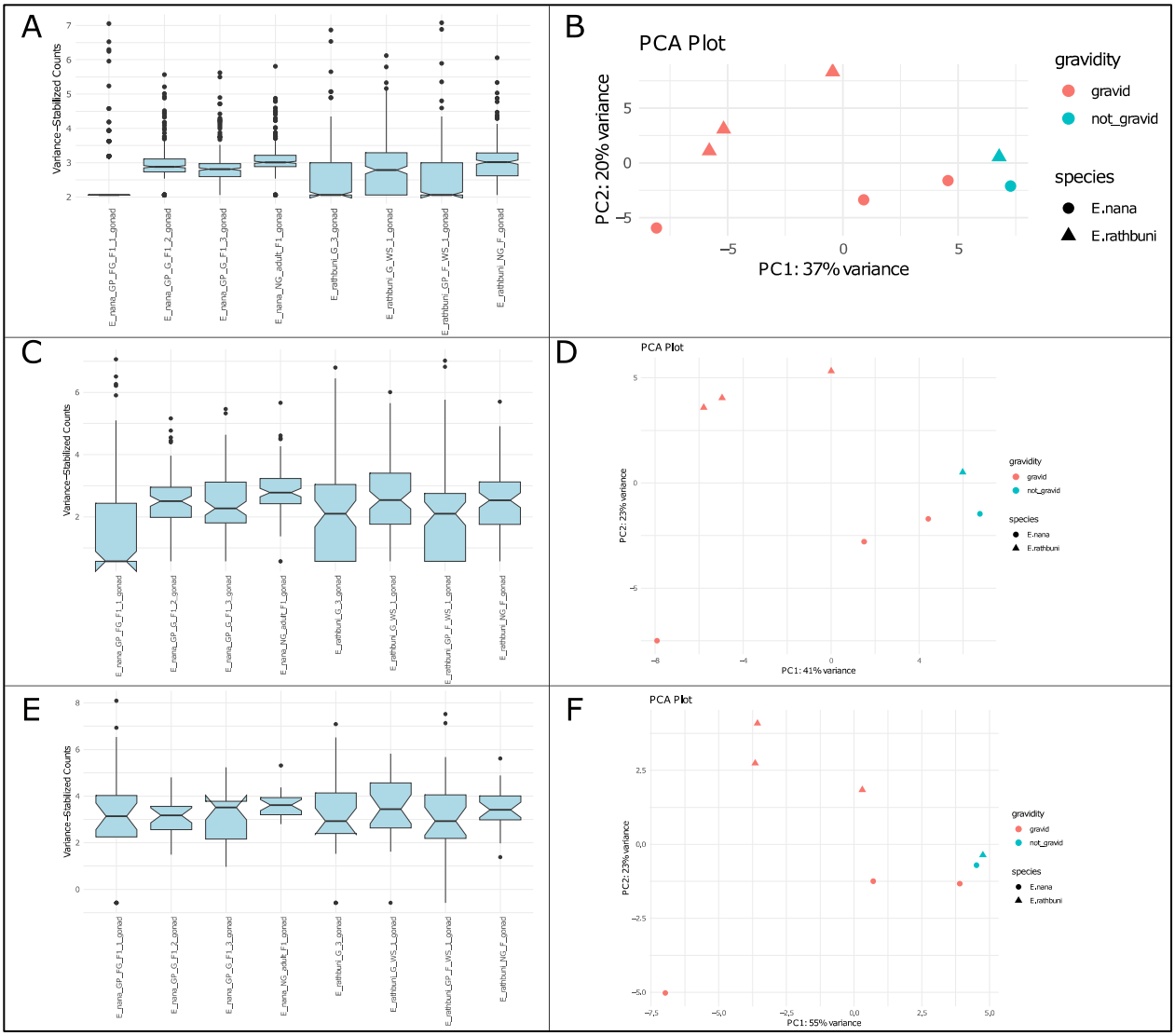
E_latitansHCC_Adult_3_Olf	20.0	3.5



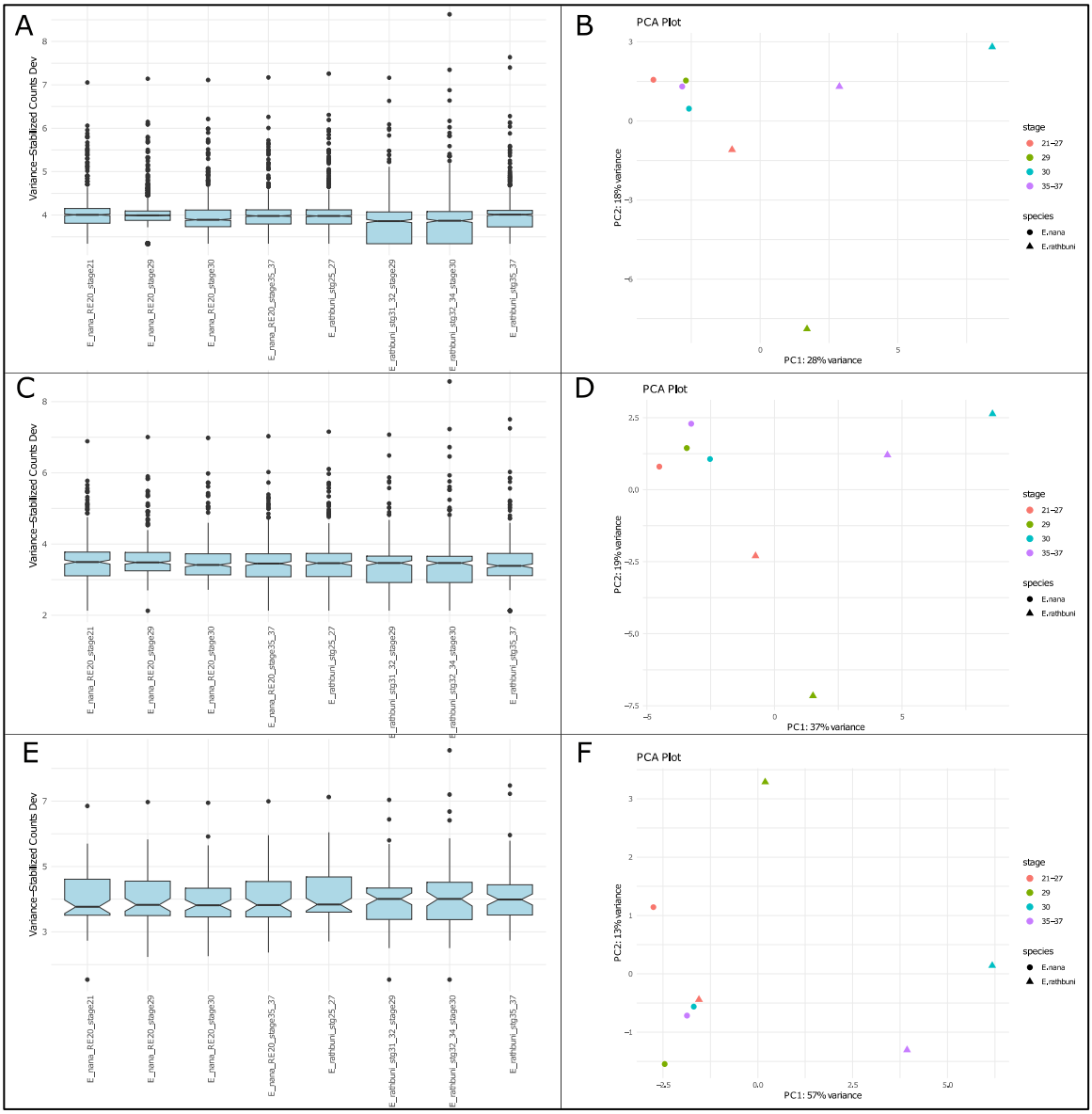
Sup. Figure 2. A portion of the MultiQC report post-sequencing. A parameter of unique vs. duplicate reads in the sequence data. Unique reads are favored over duplicates in that they are typically more informative in downstream analysis. Note, the *E. rathbuni* 1-1 skin sample. Although this sample made it through library prep, it will not be informative for our downstream analysis.



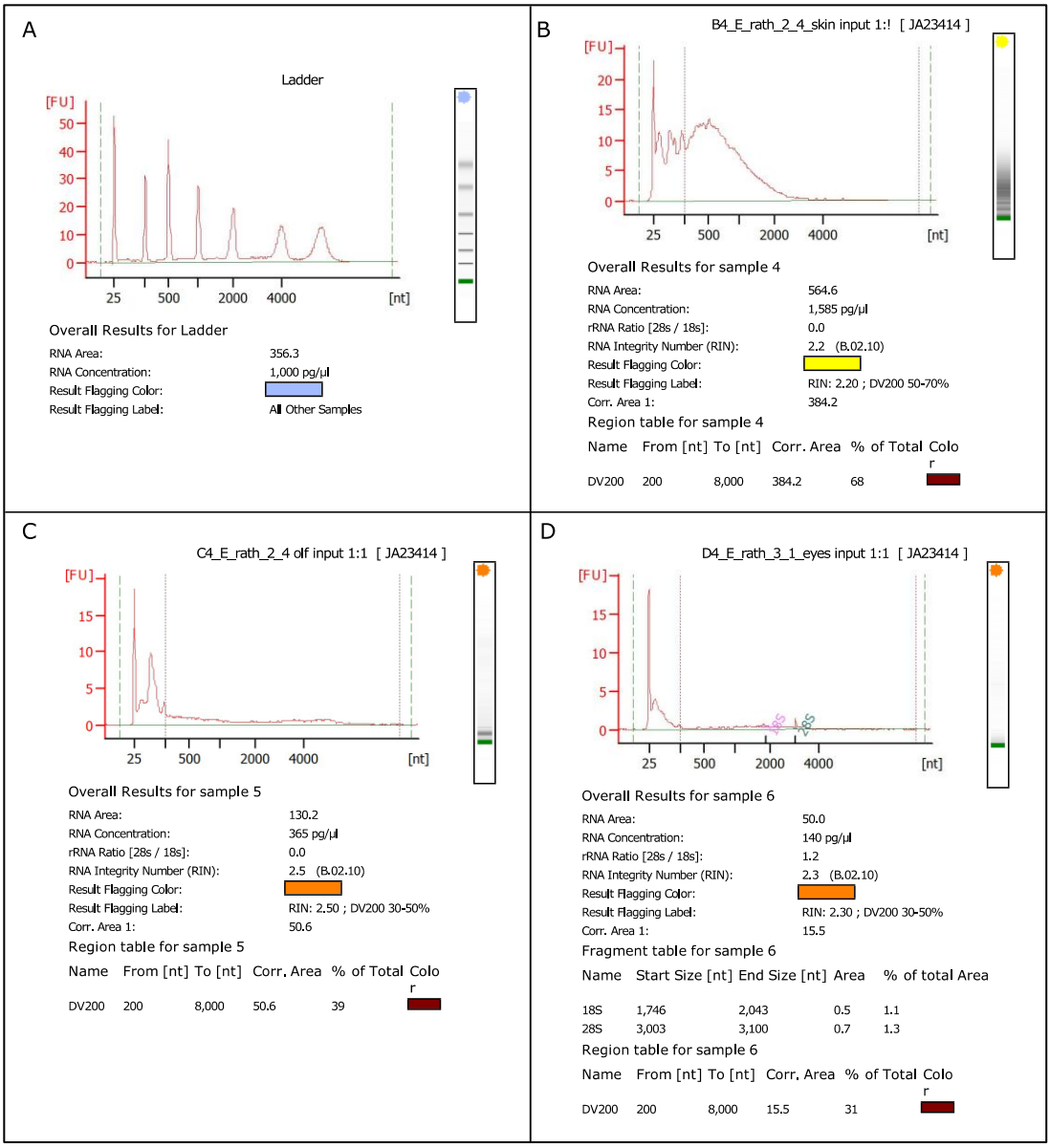
Sup. Figure 3. Exploring different normalization plots by adjusting filtration parameters and corresponding PCA plots; <10 (A) and PCA plot (B), <20 (C) and PCA plot (D), <50 (E) and PCA plot (F).



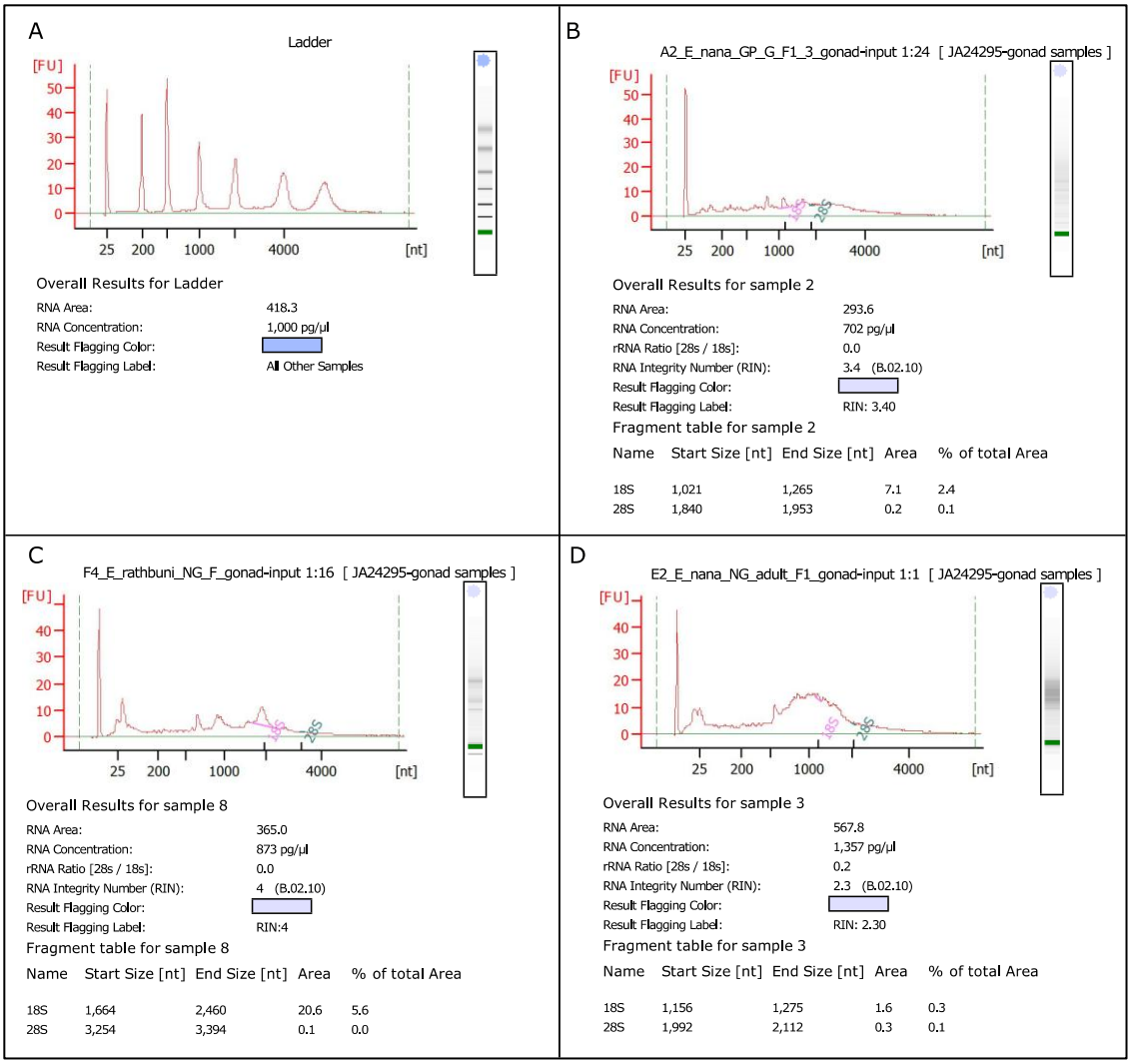
Sup. Figure 4 . Exploring different normalization plots by adjusting filtration parameters and corresponding PCA plots; <10 (A) and PCA plot (B), <20 (C) and PCA plot (D), <50 (E) and PCA plot (F).



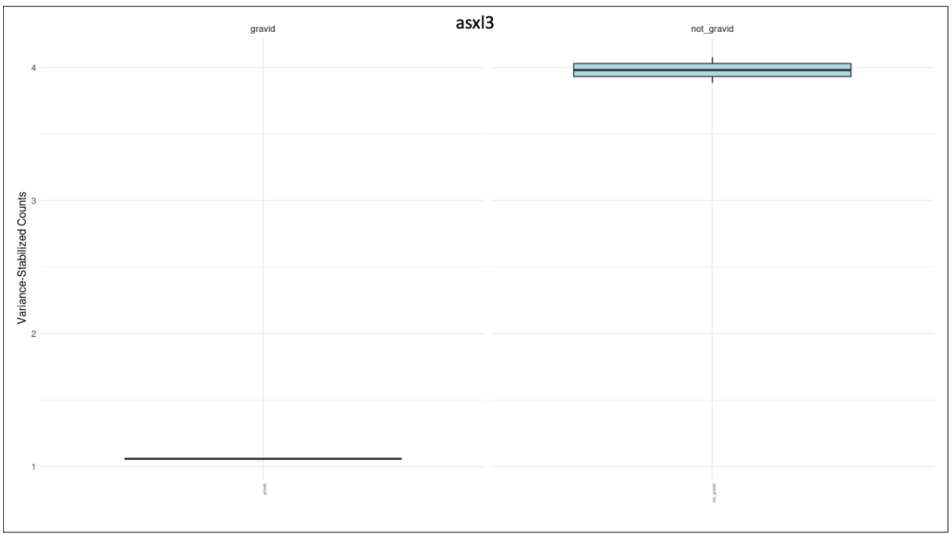
Sup. Figure 5 . Exploring different normalization plots by adjusting filtration parameters and corresponding PCA plots; <10 (A) and PCA plot (B), <20 (C) and PCA plot (D), <50 (E) and PCA plot (F).



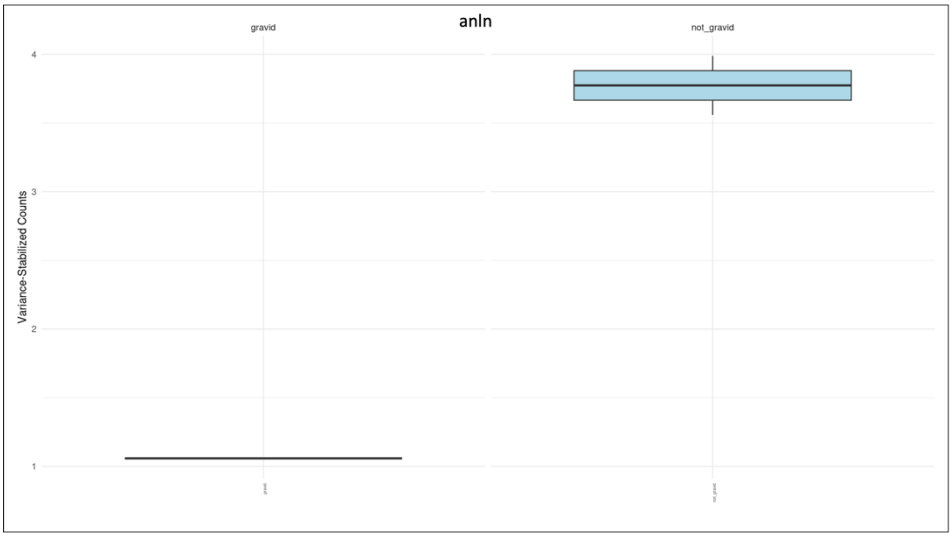
Sup. Figure 6 . Exemplar RNA quality reads from the Bioanalyzer of the comparative development project. A ladder sets the standards for the run (A). Several tissue examples from E. rathbuni at two different developmental stage and include skin (B), olfactory epithelium (C), and eyes (D).



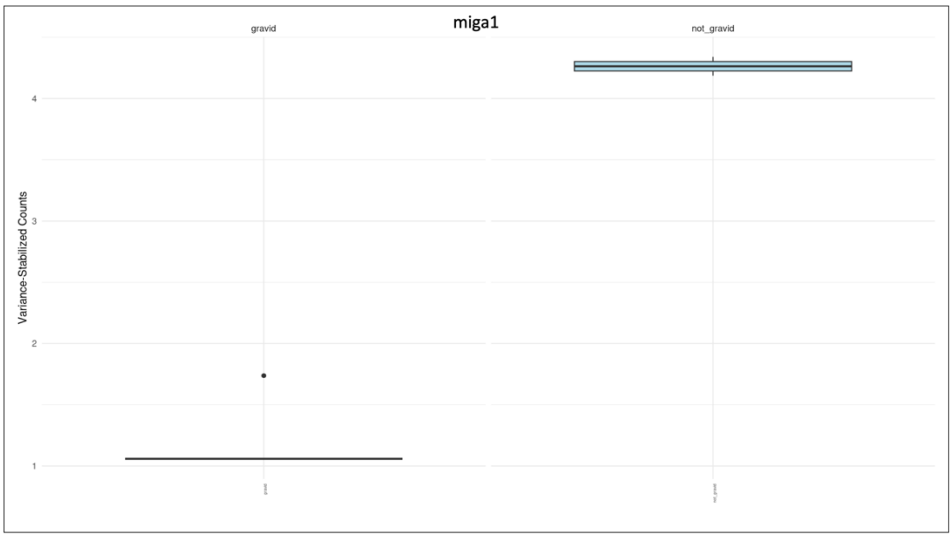
Sup. Figure 7 . Exemplar RNA quality reads from the Bioanalyzer of the gonad project. A ladder sets the standards for the run (A). Several tissue examples from *E. rathbuni* and *E. nana*, including the *E. nana* gravid female #3 (B), *E. rathbuni* non-gravid female (C), and an *E. nana* non-gravid female (D).



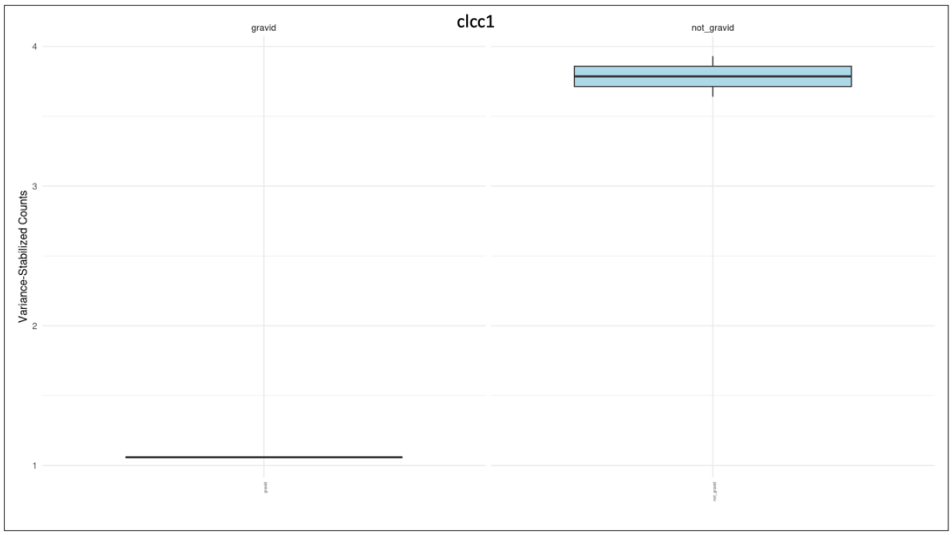
Sup. Figure 8. *asxl3* gene box plot with count distributions illustrated between gravid (labled, G in Fig.8*C*) and not_gravid (labled, NG in Fig.8*C*).



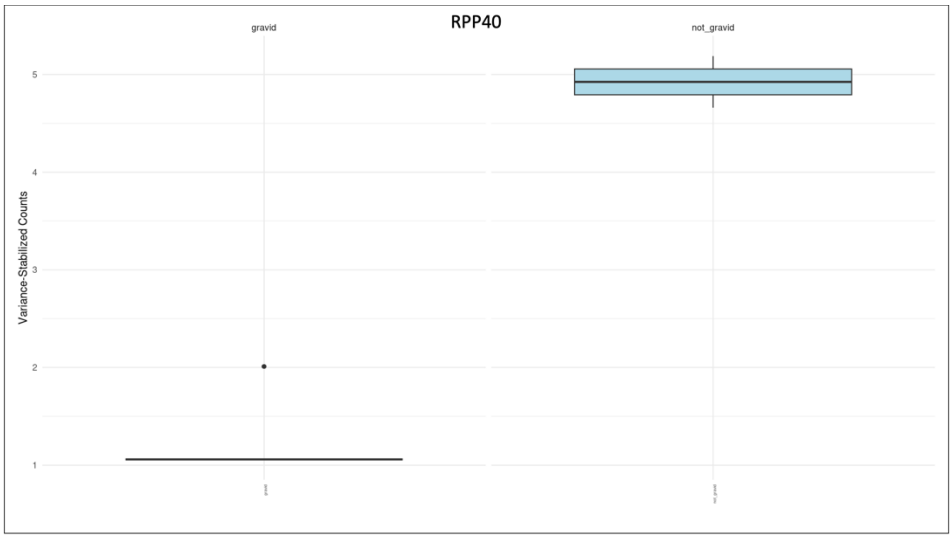
Sup. Figure 10. *anln* gene box plot with count distributions illustrated between gravid (labled, G in Fig.8*C*) and not_gravid (labled, NG in Fig.8*C*).



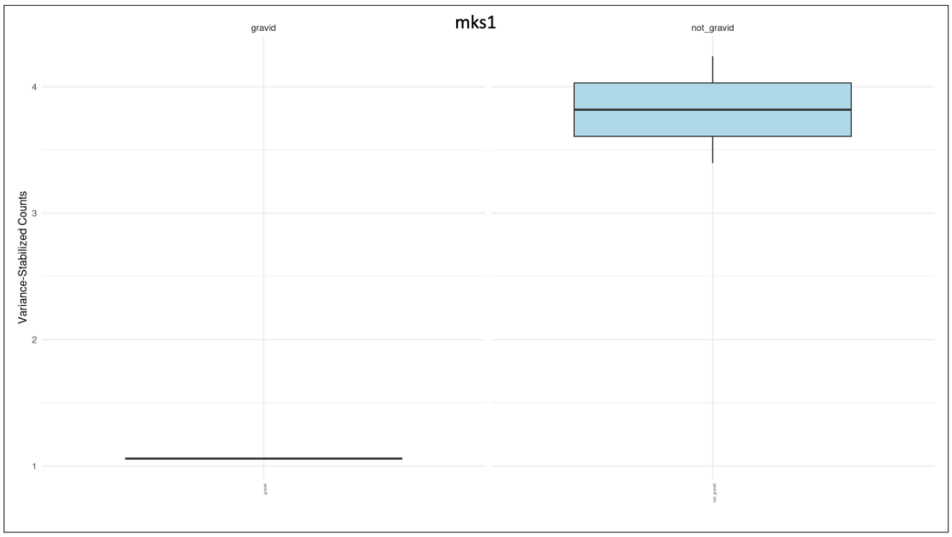
Sup. Figure 9. *miga1* gene box plot with count distributions illustrated between gravid (labled, G in Fig.8*C*) and not_gravid (labled, NG in Fig.8*C*).



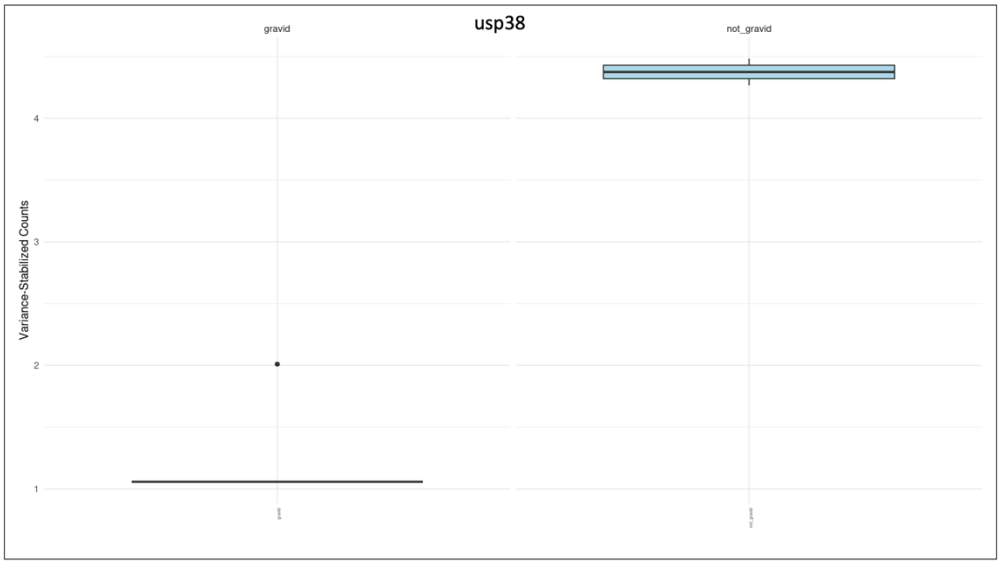
Sup. Figure 11. *clcc1* gene box plot with count distributions illustrated between gravid (labled, G in Fig.8*C*) and not_gravid (labled, NG in Fig.8*C*).



Sup. Figure 12. *rpp40* gene box plot with count distributions illustrated between gravid (labled, G in Fig.8*C*) and not_gravid (labled, NG in Fig.8*C*).



Sup. Figure 13. *mks1* gene box plot with count distributions illustrated between gravid (labled, G in Fig.8*C*) and not_gravid (labled, NG in Fig.8*C*).



Sup. Figure 14. *usp38* gene box plot with count distributions illustrated between gravid (labled, G in Fig.8*C*) and not_gravid (labled, NG in Fig.8*C*).

Appendix G | **Genetic assessment of Comal Springs riffle beetle in Landa Lake** (Final Report)

Genetics Assessment of the Comal Springs Riffle Beetle in the Comal Springs System

2024 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Dr. Katie Bockrath

San Marcos Aquatic Resources Center

U.S. Fish and Wildlife Service

Table of Contents

Table of Contents **1**
Introduction **2**
Objectives **3**
Methods **3**

Field Collections 3
DNA Extraction 6
Library Preparation and Sequencing 6
Data Analysis 7

Results **9**

Lab Work 9
Data Analysis 10
Entropy 10
F_{ST} 11
Tajima's D 11
Effective Population Sie (N_e) 12

Discussion **13**
Investigator Responsibilities **14**
Works Cited **14**

Introduction

Groundwater species are sensitive to fluctuations in environmental conditions. Drought events and water usage put immense pressure on ground water availability in the Edwards Aquifer and can lead to low flow and high temperature conditions. The Edwards Aquifer Refugia Program (EARP) serves to develop a functional refugia for endemic species dependent on the Edwards Aquifer. In the event of a catastrophe, these endemic species will be brought into the EARP Refugia until they can be reintroduced. To ensure the population is accurately reflected in the Refugia, it is critical to understand how populations are structured across a species range and where individuals should be sampled to capture a representative collection of their genetic diversity. Here, we aim to assess the genetic diversity of the Comal Springs riffle beetle (CSRB) found in spring upwellings across the Comal Spring System (Spring Runs 1-3, Spring Island, Western Shore, and Upper Spring Run).

Gonzales (2008) and Colman (2021) found distinct spatial genetic structure among riffle beetle species across central Texas, as expected. Their data also showed genetic separation between the Comal Springs and San Marcos Springs populations of CSRB. When assessed at a finer scale, both Gonzales (2008) and Colman (2021) showed distinct clustering of subpopulations across the Comal Springs System with one of the studies suggesting distinct genetic lineages among spring runs (Colman 2021, unpublished).

The goal of this study is to conduct collections of CSRBs at individual spring openings across Landa Lake and collect a genome-wide genetic dataset from a subset of individuals sampled to survey genetic diversity within and amongst spring openings as well as investigate evidence of population subdivision or isolation amongst the Comal Springs System. The genetic data gathered will inform Refugia collection needs, genetic management plans and reintroduction strategies by ensuring the total genetic diversity of this population is reflected in the Refugia and individuals are properly reintroduced into the habitat if reintroduction efforts were required.

2022 research investigated DNA extraction procedures and focused on procuring equipment and supplies for the greater study. In 2023-2024, EARP staff focused on the collection of Comal Springs riffle beetles for genetic material across spring openings and across seasons. 2024 efforts also focused on DNA extraction, sequencing, and data analysis.

Objectives

Assess the genetic diversity of the CSRB across the Comal Springs system to inform Refugia collections, genetic management plans and CSRB reintroduction strategies.

Methods

Field Collections

Field collections occurred in coordination and in tandem with BIO-WEST biomonitoring efforts and a BIO-WEST led occurrence study. BIO-WEST placed poly-cotton lures, from here on called lures, at 80 spring openings across Spring Run 1, Spring Run 3, Spring Island, Western Shore and Upper Spring Run, New Braunfels, Texas (Figure 1). Staff from the Edwards Aquifer Refugia Program placed lures in Spring Run 2. GPS locations were collected for each of the 80 sampled spring openings and repeat sampling was conducted over a 2-year span, occurring in May, August and November of 2023 and May and June of 2024. During each collection event, lures were placed in or on spring openings, held down with a rock, and left for 4-6 weeks for biofilm to be generated and beetles to be attracted to the lures. After 4-6 weeks, lures were collected and inspected for the presence of CSRB larvae and adults. *Microcylloepus pusillus* is a co-occurring Elmid riffle beetle that looks very similar to CSRB with minor differences in body size, grooves in the thorax and overall shape of the abdomen. *M. pusillus* can be easily misidentified as CSRB if not inspected for key differences in physical features under a dissecting scope. All adults and larvae were identified to species using a dissecting scope in the field and at time of collection.

Because the collections for the genetic assessment and the occurrence model were co-occurring, a subset of adult individuals were retained for the genetic assessment and the remaining adults were returned to the spring openings from where they were collected. All larvae CSRB were retained for the genetic assessment. A subcollection scheme was developed to make collections across lures consistent while allowing for enough adults to be retained for the genetic assessment and releasing the majority of individuals collected back to the springs for the occurrence study. If a lure had less than 5 adult CSRB, a single adult was retained. If a lure had 5-8 adult CSRB, 2 adults were retained. If a lure had 8 or greater adult CSRB, 4 adults were retained. All retained CSRB adults and larvae were preserved in 95-100% molecular grade non-denatured ethanol and lure ID and collection date were recorded. We aimed to collect 30 beetles from each location across all lures within each location.

Figure 1. Sampling locations across the Comal Spring System, New Braunfels, Texas. Spring Run 1 (Yellow), Spring Run 2 (Red), Spring Run 3 (Green), Western Shore (Purple), Spring Island (Blue), and Upper Spring Run (Orange).

Table 1. Adult and larval Comal Spring riffle beetle (CSRB) collection numbers as well as the total number of individuals collected from each sampling location in the Comal Spring System (2023-2024).

Site/Location	Adult CSRB	Larval CSRB	Total
Spring Run 1	3	7	10
Spring Run 2	6	11	17
Spring Run 3	28	27	55
Western Shore	40	23	63
Spring Island	46	51	97
Upper Spring Run	0	0	0
Total	123	119	242

DNA Extraction

DNA was extracted from all adult and larval CSRB using the gMax Mini Genomic DNA extraction kit (IBI Scientific IB47282) following manufacturers recommendations with the exception of the initial cell lyses step where samples were allowed to lyse overnight. DNA was eluted using 100 ul of elution buffer. DNA was quantified using a Qubit 4 fluorometer (Invitrogen Q33238) using the High Sensitivity dsDNA reagent and standards (Invitrogen Q33230). Low quantity samples were concentrated using a SpinVac at the U.S. Fish and Wildlife Service Abernathy Fish Technology Center Genetics Lab. Concentrated samples were rehydrated using 10ul of nuclease free water.

Library Preparation and Sequencing

Library preparation followed Gompert et al (2014). The library size distribution and quantity was measured using a D1000 High Sensitivity ScreenTape and reagents (Agilent 5067-5584 and 5067-5585, respectively) on a TapeStation 4200 (Agilent G2991BA).

Samples were pooled and sent to the U.S. Fish and Wildlife Service Conservation Genetics lab at Auburn University for size selection.

Libraries were size selected to 300-450 bps using a BluePippin. 30 ul of pooled library was loaded into 5 lanes so that a total 150 ul of library was size selected. Final size selected library was pooled, and library size was confirmed using a 2% agarose gel and a D1000 High Sensitivity ScreenTape and reagents (Agilent 5067-5584 and 5067-5585, respecivitly) on a TapeStation 4200 (Agilent G2991BA). Final library concentration was quantified using the D1000 ScreenTape on the TapeStation 4200 and a Qubit 4 fluorometer using the High Sensitivity dsDNA assay. The final library was sent to the U.S. Fish and Wildlife Service Midwest Fisheries Center, Whitney Genetics Lab for sequencing. The library was single end sequenced twice using a NextSeq 1000 sequencer and a NextSeq 1000/2000 P2 XLEAP-SBS Reagent Kit (100 Cycles) (Illumina 20100987) at 100 bps. Following Gombert et al. (2014), sequencing consisted of 100 bp single end reads.

Data Analysis

Sequence data was analyzed following Gombert et al (2014). Barcode sequences attached to individual samples during library preparation were used to separate the pooled sequence data into separate sample files. Barcodes and low-quality sequences were then removed. All samples were then assembled to generate a “reference genome”. Samples were individually compared to this reference genome to identify Single Nucleotide Polymorphisms (SNPs), or individual base pair differences between the reference and the sample. The SNPs were filtered to removed very rare and very abundant SNPs, both of which are non-informative for population genetic assessments. The double filtered SNPs were saved in a Variant Call Format (VCF) file and were analyzed using ENTROPY and statistical packages in R.

Entropy is a program that statically assigns individuals to ancestral genetic lineages (or genetic variation) represented in a metapopulation. This type of analysis can be compared to commercial genetic ancestry testing such as 23andMe or Embark. By assigning individuals to sampling locations, the user can assess how ancestral genetic lineages are distributed within and across locations/populations. This analysis provides insights into the degree of isolation between populations and if there have been recent reductions or expansions in population sizes. Bayesian MCMC models were used to assign ancestral genetic lineages to individuals using a pre-specified number of genetic lineages (K values). K values ranged from 2-7. Because we are using nuclear data, which is diploid (2 distinct copies), the minimum K value is 2 and Entropy results would return a K=2 if all populations were genetically homogenous. If all populations are distinct and share no genetic variation, the maximum K value is 7; a K for each sampling location/population plus 1 for diploid genome.

Pegas and custom scripts in R were used to gather additional population genetic measures. F_{ST}, or the measure of geneflow among populations, was measured by assessing the genetic variation present within locations/subpopulations (S) in relation to the total genetic variation of the total population (T) using the following calculation:

$$F_{ST} = \frac{H_T - \overline{H_S}}{H_T}$$

Where, H_T is the heterozygosity (genetic diversity) of the total population (all sampling locations), $\overline{H_S}$ is the average heterozygosity of each subpopulation (individual sampling location, i.e. Spring Island). F_{ST} is a measure that ranges from 0-1. An F_{ST} of 0 means there is open geneflow/migration among populations, thus there is no population structure. An F_{ST} of 1 means there is no geneflow/migration among populations, the populations are isolated and have strong population structure.

Tajima's D was estimated for the total population by calculating the number of segregating sites (S) and the average nucleotide differences between paired samples (π). Tajima's D is a value that runs between 0-1, where a value of 0 means there is not a lot of change in population size or genetic selection pressure; the population is existing neutrally. A value below 0 indicates strong genetic selection or population expansion after a large reduction

in population size. A value above 0 indicates a sudden population contraction/loss or balancing selection (rare genetic diversity is underrepresented by expectations).

The number of individuals contributing to the next generation (N_e), was estimated by calculating the number of segregating sites (θ) across all samples and using a standard rate of mutation ($\mu = 2.5^{-8}$ mutations/generation). The following equation was used to estimate effective population size (N_e):

$$N_e = \frac{\theta}{4\mu}$$

Results

Lab Work

DNA was successfully extracted from all adult and larval CSRB (n=242). DNA concentrations were lower than ideal for downstream double enzymatic digest steps. DNA was concentrated by evaporative centrifugation at the US Fish and Wildlife Service Abernathy Fish Technology Center. This process removes liquid from the DNA extractions with minimal loss of DNA and allows for rehydration at a lower volume to increase overall DNA concentrations.

Library preparation, size selection and sequencing was successful for all 242 samples. Sequences were of the highest quality (>Q30) for over 95% of the sequences generated. Over 42 Giga base pairs, or 420,000,000 sequences, were generated. This resulted in over 2 million sequences per individual sample.

Data Analysis

Entropy

Bayesian MCMC showed equal support for K=3 and K=4. Entropy assigned individuals to ancestral genetic linages and those individuals were organized relative to their sampling location. Ancestorial lineages were not equally represented across sampling locations and not all sampling locations had the same ancestral lineages represented. Assessments indicate that genetic diversity (ancestral lineages) located at Spring Island and Western Shore is distinct from Spring Run 2 and Spring Run 3. Additionally, genetic diversity represented in Spring Runs 2 and 3 is absent from Spring Island and Western Shore, and vice versa (Figure 2). The representation of a unique genetic lineage and relative uniformity in Spring Runs 2 and 3 indicates unique subpopulations relative to the main river channel and the potential of a reduction in genetic diversity due to reduction in population size (bottleneck). Spring Island and Western Shore showed less significant evidence of reductions in population size.

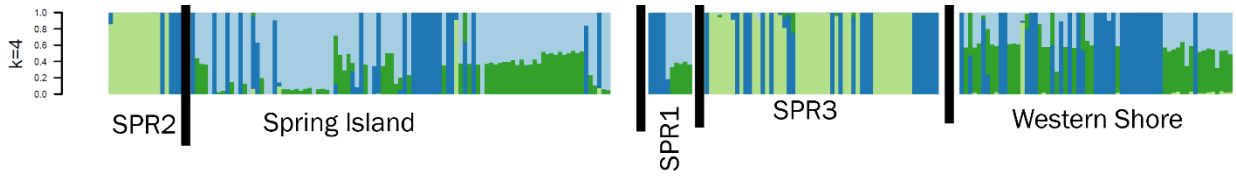


Figure 2. Population Genetic Structure bar plot. Vertical bars represent an individual included in the study. Individuals are grouped by sampling location (population). The different colors represent genetic lineages. Each individual (vertical bar plot) is assigned to a genetic lineage (color). Those with a single color were 100% assigned to that genetic lineage. Those with multiple colors were assigned to multiple lineages, indicative of retained ancestral genetic diversity.

F_{ST}

F_{ST} comparisons among populations showed significant population structure (lack of gene flow and migration) between Spring Island and Spring Run 2 (11.24%, Table 2). Additionally, Spring Run 2 showed strong but less significant population structure between Spring Run 1 and Western Shore (5.96% and 6%, respectively). This suggests that little migration and geneflow occurs between Spring Run 2 and other locations, apart from Spring Run 3. There is almost no population structure between Spring Run 2 and Spring Run 3, suggesting geneflow and migration regularly occurs between these two populations.

Table 2. F_{ST} values across CSRB sampling locations in the Comal Spring System. Below the diagonal is the F_{ST} value which ranges between 0-1. Above the diagonal is the F_{ST} value represented as a percentage. Significant F_{ST} values are bolded.

	Spring Run 2	**Spring Island**	**Spring Run 1**	**Spring Run 3**	**Western Shore**
Spring Run 2	0	**11.24%**	5.95%	0.76%	6%
Spring Island	**0.1124**	0	3.48%	7.27%	1.78%
Spring Run 1	0.0595	0.0348	0	3.54%	3.76%
Spring Run 3	0.0076	0.0727	0.0354	0	3.84%
Western Shore	0.0681	0.0178	0.0376	0.0384	0

Tajima's D

All populations exhibited a negative Tajima's D value, with Spring Run 2 having the most negative Tajima's D value (Table 3). Negative Tajima's D values indicate a population expansion after a significant loss in the number of individuals in that population. Here, all sampled populations showed an expansion in population from the few individuals remaining after a significant reduction in population size.

Table 3. Tajima's D for CSRB at each sampling location in the Comal Spring System. Metrics used to calculate Tajima's D are reported. Metrics include. Segregating Sites (S) and average differing nucleotides between pairwise comparisons (π). Tajima's D of 0 suggests populations are under neutral selection and are not impacted by environmental changes or other factors that would cause a deviation from Hardy-Weinberg Equilibrium. Tajima's D above 1 indicate a sudden population contraction or balancing selection where rare genetic variants are maintained in the population at rates above expectation. A value below 0 indicates strong genetic selection or population expansion after a large reduction in population size. The sample size for Spring Run 1 was too small for analysis and was excluded.

Population	Segregating Sites (S)	π	Tajima's D
Spring Run 2	1,472.41	0.2246744	-0.8353022
Spring Run 3	1,069.78	0.2246744	-0.350743
Western Shore	1,096.97	0.1439468	-0.3140079
Spring Island	1,062.65	0.1562964	-0.2983454
Total Population	1,091.47	0.0167799	-0.2371682

Effective Population Sie (N_e)

The effective population size for the Comal Springs Riffle Beetles in the Comal Springs system is 416,363 breeding individuals. This estimate includes adults and larvae, which introduces potential error due to overlapping generations.

Discussion

Overall, these results suggest that Comal Springs riffle beetles are sensitive to habitat loss, low flows, and do not retreat into the aquifer as far as previously hoped. The reducing in ancestral lineages at Spring Runs 2 and 3, relative to Spring Island and Western Shore, combined with a significant FST value at Spring Run 3, and negative Tajima's D value across all locations show the Comal Spring riffle beetle has undergone significant population loss at these locations and that Spring Run s 2 and 3 are significantly isolated from Spring Island and Western Shore. Spring Run is the first location to go dry during low flow events, followed by Spring Run 2 and then Spring Run 3. This anecdotal observation suggests that the Spring Runs are sequentially connected and dry in succession, potentially in relation to relative elevation. The results presented here suggest otherwise and that Spring Run 2 and 3 are connected but isolated from the other sampling locations, most importantly Spring Island and Western Shore, which could have served as a source population to replenish Spring Run 2 and 3 after those populations declined due to habitat loss. Ancestorial genetic lineages in Spring Run 2 and 3 are very reduced relative to the other locations, which reduces these populations adaptability and leaves them vulnerable to future environmental stresses.

The effective population size for the Comal Spring System (N_e = 416,363) is significantly different and larger than what Gonzales (2008) estimated for Western Shore (N_e= 64,435) and Spring Island (N_e= 52,256). This can be due to the inclusion of additional populations, the inclusion of larvae with adults, resulting in overlapping populations, and a larger sample size included in this studies' estimate of effective population size (N=242 vs N=50 in Gonzales 2008). Additionally, estimates of Linkage Disequilibrium should be used to more robustly estimate effective population size. To see how populations have changed over time and relative to severe drought years, the data analysed in this study needs to be assessed in relation to historical collections. Future studies should be done to partner with those who possess preserved individuals from 2008 to current and the analyses reported here repeated.

These results show that the Comal Spring riffle beetle population is not as robust to drought and low flow conditions as previously assumed. Spring flows in all the Spring Runs must be maintained at a sufficient minimum flow rate to prevent the Spring Runs from drying to prevent further reductions in population size, the loss of unique genetic (and thus adaptive) diversity, and potential local species extirpation from habitat loss. Spring Runs 2 and 3 are connected and individuals move between these locations, but are isolated from Spring Run 1, Spring Island and Western Shore. This suggests that the more stable Spring Island and Western Shore locations are not serving as source populations (or refuges) to the Spring Runs and will not replenish depleted populations after the Spring Runs dry.

Investigator Responsibilities

Experimental design and oversight: Dr. Katie Bockrath
Experimental execution: Dr. Katie Bockrath
Data analysis: Dr. Katie Bockrath
Experimental write up: Dr. Katie Bockrath
Document Review and Oversight: Dr. Jennifer Howeth and Dr. David Britton

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Appendix H | **Genetic assessment of San Marcos salamander** (Interim Report)

Genetics Assessments of Wild and Captive San Marcos Salamanders

2024 Interim Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Dr. Katie Bockrath

Contents

Background **3**
Objectives **4**
Methods **5**

Lab Work 5
Data Analysis 6

Interim Results **6**
Works Cited **7**

Background

San Marcos salamanders (*Eurycea nana*) are endemic to the headwaters of the San Marcos River. Due to their limited range and high potential of habitat loss, the San Marcos salamander is listed as Threatened under the Endangered Species Act and by Texas Parks and Wildlife. The San Marcos salamander is a priority species of conservation concern under the Edwards Aquifer Habitat Conservation Plan (EAHCP) and captive assurance populations are held under the Edwards Aquifer Refugia Program located at the San Marcos Aquatic Resources Center and the Uvalde National Fish Hatchery. The captive assurance populations serve as emergency populations for reintroduction if a catastrophic event, such as a loss of flow or a chemical spill, were to decimate the available habitat in which these species occur.

To have an effective captive assurance population, regular genetic assessments of the wild population and the captive assurance population are required to ensure the wild population is accurately reflected in the captive assurance population. Additionally, regular genetic assessments of the wild population inform how many individuals from each subpopulation are required to accurately capture the wild genetic diversity. Last, with the help of p-chip transponder tags, genetic assessments of captive individuals inform responsible captive propagation and reintroduction efforts. Although reintroducing wild caught individuals is preferred, there is a possibility that it may take many years for the habitat to recover after a catastrophic event. By assigning each individual San Marcos salamander with a genetic profile that can be tracked with p-Chip transponders, the Refugia Program would be able to pair salamanders during captive propagation to maintain relative levels of wild genetic diversity in offspring that may be used during reintroduction events.

There have been limited genetic assessments of the San Marcos salamander. Lucas (2009) used multiple genetic markers to assess the genetic diversity and spatial distribution of that diversity in wild San Marcos salamander populations and compared wild genetic diversity to those genotypes maintained in captivity. Lucas (2009) found similar levels of genetic diversity as other *Eurycea* species and no evidence of spatial population structure across three sampling locations at Spring Lake, San Marcos, Texas (Hotel, Diversion, and Eastern Spillway). This study did reveal a minor reduction of genetic diversity in the captive population when compared to the wild. This may be due to the limited number of captive born individuals included (N=26) in that aspect of the study but may also be due to loss of unique genetic profiles that were present in the wild but not in the captive population. Lucas (2009) recommended reassessing the diversity of the captive population every few years. It has been almost 15 years since the Lucas (2009) study was completed, thus it is time to reassess the wild populations and the captive assurance population. This time, individual genetic profiles for each captive individual will be retained and tracked throughout each salamander's lifetime.

Single Nucleotide Polymorphisms (SNPs) allow for genetic assessment of diversity by generating 10-100s of individual data points (loci) across the genome.

Objectives

- Assess the genetic diversity of wild San Marcos Salamanders
    - Determine if genetic structure exists within and among sampling/monitoring sites.
    - Determine minimum number of individuals needed to represent wild genetic diversity of the species.
- Assess the genetic diversity of captive San Marcos Salamanders
    - Identify haplotype information for captive individuals to inform reintroduction and captive propagation strategies.

Methods

Lab Work

Tail clips collected from the 2023 San Marcos salamander p-Chip mark-recapture study and were used to conduct this population genetic assessment of wild individuals across three regularly monitored and sampled sites. The three sites include Hotel, Diversion, and Eastern Spillway. Tail clips were preserved in 95-100% ethanol. DNA extracted using a Qiagen DNeasy Blood and Tissue DNA extraction Kit. A negative extraction control was included in all DNA extraction sets. Extracted DNA was quantified using a Qubit fluorometer and low quantity DNA samples were concentrated using a DNA precipitation protocol (Qiagen) where the DNA is concentrated into a pellet and the supernatant is decanted and dried away from the DNA pellet. DNA was rehydrated with 10ul sterile DI water so that all DNA samples were within recommended starting concentrations for double enzyme digest (20ng/ul). All DNA samples went through Double Digest RadSeq library preparation protocol following Gompert (2014). The pooled library was size selected between 350-400bps using a PippinBlue at the USFWS Conservation Genetics Lab at Auburn University. The pooled library quality, fragment length and quantity was measured using a D100 ScreenTape on an Agilent TapeStation 4200. Library quantity was confirmed using dsDNA reagents on a Qubit fluorometer. Libraries were sequenced twice, single-end and 100 bps, on an Illumina NextSeq 1000 high through-put sequencer at the US Fish and Wildlife Service Whitney Genetics Laboratory using a P2 XLEAP-SBS Reagent Kit (100 Cycles) (Illumina 20100987).

Data Analysis

Data analysis will conclude in 2025. Sequence data will be analyzed following Gompert (2014). Barcode sequences attached to individual samples during library preparation will be used to separate the pooled sequence data into separate sample files. Barcodes and low-quality sequences will then be removed. All samples will be assembled to generate a “reference genome”. Samples will be individually compered to this reference genome to identify Single Nucleotide Polymorphisms (SNPs), or individual base pair differences between the reference and the sample. The SNPs will then be filtered to remove low and high coverage SNPs. This removes very common SNPs that are less informative as well as very rare SNPs that may be sequencing error. Samples and their SNPs will be correlated to their sampling locations/type and Entropy will be used to cluster samples into “populations”.

Interim Results

DNA was successfully extracted from all 453 San Marcos salamander tail clips. The pooled library was successfully size selected to the desired base pair length range and quantities were sufficient for sequencing. The library of samples was sequenced twice on a NextSeq 1000 at 100 bps lengths in a single direction. Data was successfully retrieved from the partnered sequencing lab and data analysis will be conducted in 2025.

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Appendix I | **Genetic assessment of Texas blind salamander** (Interim Report)

Genetics Assessments of Wild and Captive Texas Blind Salamanders

2024 Interim report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Dr. Katie Bockrath

Contents

Background 3
Objectives 4
Methods 5

Lab Work 5
Data Analysis 6

Works Cited 6

Background

Texas blind salamanders (*Eurycea rathbuni*) are endemic to the Edwards Aquifer. Due to their limited range and high potential of habitat loss, the Texas blind salamander is listed as Endangered under the Endangered Species Act and by Texas Parks and Wildlife. The Texas blind salamander is a priority species of conservation concern under the Edwards Aquifer Habitat Conservation Plan (EAHCP) and captive assurance populations are held under the Edwards Aquifer Refugia Program located at the San Marcos Aquatic Resources Center and the Uvalde National Fish Hatchery. The captive assurance populations serve as emergency backup populations for reintroduction if a catastrophic event, such as a loss of flow or a chemical spill, were to decimate the available habitat in which these species occur.

In order to have an effective captive assurance population, regular genetic assessments of the wild population and the captive assurance population are required to ensure the genetic diversity of the wild population is accurately reflected in the captive assurance population. Additionally, regular genetic assessments of the wild population inform how many individuals from each subpopulation are required to accurately capture the wild genetic diversity. Last, with the aid of p-chip transponder tags, genetic assessments of captive individuals inform responsible captive propagation and reintroduction efforts. Although reintroducing wild caught individuals is preferred, there is a possibility that it may take many years for the habitat to recover after a catastrophic event. By assigning each individual Texas blind salamander a genetic profile that can be tracked with p-Chip transponders, the Refugia Program would be able to pair (mate) salamanders during captive propagation to maintain relative levels of wild genetic diversity in offspring that may be used during reintroduction events.

There have been limited genetic assessments of the Texas blind salamander. Chippindale (2009) completed a genetic assessment of the Texas blind salamander by sequencing a mitochondrial marker (CytB) and four microsatellite markers for 70 individuals. Chippindale (2009) found low Fst values, suggesting high gene flow across sampling locations. Microsatellite allelic frequency was different between to phylogenetic clades (or groups) of Texas blind salamanders. Chippendale (2009) also found evidence of potential introgression between species (or incomplete linage sorting), which should be further investigated using faster evolving genes or nuclear data, such as Single Nucleotide Polymorphisms (SNP) data.

It has been almost 15 years since the Chippindale (2009) study was completed, thus it is time to reassess the wild populations and the captive assurance population. This time, individual genetic profiles for each captive individual will be retained and tracked throughout each salamander's lifetime. Additionally, SNP data will provide a larger dataset with loci under variable evolutionary clocks, which may provide some additional information on the distribution of genetic diversity for the Texas blind salamander.

Objectives

- Assess the genetic diversity of wild Texas blind salamanders.
    - Determine if genetic structure exists within and among sampling/monitoring sites.
    - Determine minimum number of individuals needed to represent wild genetic diversity.
- Assess the genetic diversity of captive Texas blind salamanders.
    - Identify haplotype information for captive individuals to inform reintroduction and captive propagation strategies.

Methods

Lab Work

Over the last few years, tail clips were collected from all Texas blind salamanders encountered at Primer's Well, Johnson's Well, and Rattlesnake Cave. Tail clips were preserved in 95-100% ethanol and will be included as the "Wild" population for this study. All refugia individuals will be p-Chip tagged and tail clips will be collected. These tail clips will represent the "Refugia" population for this study. In 2024 a total 172 wild stock and Fx Texas blind salamander tails clips were collected. 60 of those clips were from live individuals in the Refugia. The other 112 were preserved mortalities. Prior to DNA extraction, the tail clips will be exposed to the air at room temperature for 30-60 minutes to let the ethanol evaporate. DNA will be extracted using a Qiagen DNeasy Blood and Tissue DNA extraction Kit. A negative extraction control will be included in all DNA extraction sets. Extracted DNA will be quantified using a Qubit fluorometer and normalized to working input concentrations for restriction digest reactions. Single Nucleotide Polymorphisms (SNPs) will be collected from all individuals using a Restriction site Associated DNA sequencing (RADseq) method. SNPs allow for genetic assessment of diversity by generating 10-100s of individual datapoints (loci) across the genome. RADseq libraries will be generated following the Gompert et al. (2014) ddRAD protocol. Library quality and quantity will be checked using a SyberGreen qPCR assay, a Qubit fluorometer, and a 4200 TapeStation. Libraries will be sequenced on an Illumina NextSeq 1000 high through-put sequencer at the US Fish and Wildlife Service Whitney Genetics Laboratory.

Data Analysis

Data analysis will occur in 2025. Sequence data will be analyzed following Gompert et al (2014). Barcode sequences attached to individual samples during library preparation are used to separate the pooled sequence data into separate sample files. Barcodes and low-quality sequences will be removed. All samples are then assembled to generate a “reference genome”. Samples will be individually compered to this reference genome to identify Single Nucleotide Polymorphisms (SNPs), or individual base pair differences between the reference and the sample. The SNPs will be filtered to remove low and high coverage SNPs. This removes very common SNPs that are less informative as well as very rare SNPs that may be sequencing error. Samples and their SNPs are then correlated to their sampling locations/type and Entropy will be used to cluster samples into “populations”.

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Appendix J | **Monthly Refugia Reports**

January 2024 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desirée Moore, Dominique Alvear, and Braden West

With contributions from

Dr. Katie Bockrath, Adam Daw, Jonathan Donahey, Richelle Jackson, Heidi Meador, and Shawn Moore

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Task 1 Refugia Operations

Species Collection

Adam Daw, Jonathan Donahey, Richelle Jackson, and Shawn Moore collected 66 Comal Springs fountain darters from Spring Island, Comal River on January 2 (Figure 1). 40 darters were taken to the Uvalde National Fish Hatchery (UNFH) and 26 were taken to the San Marcos Aquatic Resources Center (SMARC).

Dominique Alvear, Daw, Jackson, and S. Moore collected 22 Comal Springs fountain darters from the old channel area of the Comal River on January 29. The darters were retained for the UNFH.

Husbandry

Uvalde

All staff at the UNFH spent time updating various SOPs in conjunction with SMARC staff to ensure similar procedures are being conducted at both facilities.

Alvear and Daw traveled to the SMARC on January 4. Alvear conducted 64 fountain darter archived necropsies to look for a historical baseline of various parasites. Daw worked with SMARC husbandry staff to install the refugia building well water line, water quality probes, and diversion valve.

Alvear conducted quarterly inventories of the Comal Spring riffle beetle and the Comal Springs dryopid beetle boxes. The riffle beetle boxes contained larvae that were non target species, and those larvae were removed. Adults that were identified as non-target were also removed.

In a first for the EARP, two of the three remaining dryopid beetle larvae metamorphosed into adults! This is from the 15 F1 larvae that were originally found in December of 2023. The larvae were kept in boxes similar to riffle beetle boxes, modified to have additional substrate at the water/air interface. The larvae took about one year to metamorphose into adults, which is consistent with previous research by Randy Gibson (SMARC biologist).

Donahey completed construction of one controller box that will be used on the refugia hospital rack and began working on a second controller box to be placed in the invertebrate room.

Heidi Meador continued updated the plumbing on refugia tank 12. Meador repotted and incorporated 10 Texas wild rice plants into the refugia population.

Alvear and Daw cleaned Invertebrate Rack 1 and made some modifications to the system.

Daw started building a new quarantine rack in the quarantine building.

SMARC

Daw provided training to SMARC staff on wiring temperature, total gas pressure, and water pressure sensors to controller boxes. (Figure 2)

Daw, S. Moore, and Braden West finished the first phase of the supersaturation diversion project in the SMARC refugia (Figure 3). The system was tested with manual inputs on January 24. The system functioned as expected on January 31. West began designing a second system for the SMARC quarantine to function in tandem with the refugia system. These systems work in conjunction with each other to detect incoming supersaturated well water and divert it away from organisms.

West completed the CO_2 delivery system for the SMARC refugia, allowing individual gas delivery lines for each system.

S. Moore worked with SMARC maintenance specialist Juan Martinez to repair a pump, electrical plug, and receptacle in the greenhouse that were damaged in the January freeze.

S. Moore conducted Peck's cave amphipod inventories and incorporated individuals collected in December.

Jackson and S. Moore incorporated 10 Texas wild rice plants collected in December.

Jackson led efforts to organize and clear space in the SMARC quarantine in preparation to remove aging tanks from the space.

SMARC staff put newly revised SOPs to use. Staff identified deficiencies and worked together to add pertinent information.

Jackson designed a new collection cup for the Diversion Spring net. S. Moore assisted in construction of the new equipment.

Task 2 Research

Dryopid Life History

Dr. Matt Pintar (BIO-WEST) reconstructed the experimental setup to accommodate more individual housing chambers for mating, larval development, and paired choice experiments. Dr. Pintar paired four captive adults (two males, two females) and began monitoring for reproduction.

Dr. Pintar conducted three collection events for Comal Springs dryopid beetles in Comal Springs, but no dryopids were found.

San Marcos Salamander Mark Recapture

Several members of the SMARC staff, interns, and volunteers contributed to the collection, processing, and release of San Marcos salamanders in Spring Lake. Salamanders were collected from Spring Lake near the Hotel site January 3 (Table 2). Salamanders were collected from Spring Lake near the Diversion pipe January 23. The second week of sampling at the Hotel site and both weeks of sampling at the Eastern Spillway site were cancelled due to staff availability and dangerous water depths and flows immediately following a large rain event. All salamanders were released back to the area they were captured after they fully recovered. Across all sites, two salamander recaptures occurred in January (Table 2).

Reproductive Gene Expression in San Marcos Salamanders

Ruben Tover (University of Texas Austin) continued to work through samples for comparative gene expression analysis. Desiree Moore went to the UNFH to select a male and female F1 Texas blind salamander for tissue samples. The salamanders were brought back to the SMARC for holding until Tovar can process them.

Comal Springs Riffle Beetle Population Genetics

There are no updates to this project this month.

Tagging Aquatic Invertebrates

Jusitn Crow, Randy Gibson (SMARC biologists), and David Thomasson (Texas Fish and Wildlife Conservation Office) collected 200 *Heterelmis glabra* from lures set in the Devil's River in November to serve as a surrogate species for Comal Springs riffle beetle.

Dr. Shannon Brewer, Brian De La Torre (Auburn University), and D. Moore set up control and experimental housing for the Comal Springs riffle beetle tagging trials (Figure 4). Four experimental chambers (two with surrogate beetles, two with captive-reared Comal Springs riffle beetles) and three control chambers (two with surrogate beetles, one with captive-reared Comal Springs riffle beetles) were set up in the SMARC quarantine room. The experimental chambers were designed to passively scan tagged beetles. Thus far, seven beetles were scanned passively in the system.

Genetic Assessment of Peck's Cave Amphipod

There are no updates to this project this month.

Additional Accomplishments

Dr. Katie Bockrath met with partnered researchers and their grants management specialists to complete paperwork required to allocate 2024 research funds.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Alvear, Dr. Bockrath, Daw, D. Moore, and West worked on drafting the EARP 2023 annual report. The draft of the report was submitted to the EAA January 31.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss collections, husbandry, and ongoing research.

EARP staff attended the Texas Conservation Symposium. D. Moore and S. Moore presented the San Marcos salamander mark-recapture study and fountain darter archive presentations, respectively.

Summary of January Activities

- The EARP collected 66 Comal Springs fountain darters from Spring Island, 40 of which were retained for the UNFH, and 26 were retained for the SMARC
- The EARP collected 22 Comal Springs fountain darters from the old channel of the Comal River, all of which were retained for the UNFH
- SMARC staff collected 200 *Heterelmis glabra* for the invertebrate tagging research, all of which were retained for research at the SMARC
- The EARP 2023 annual report was submitted to the EAA

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for January 2024. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	--	--	0	0	28	11	61	289
Fountain darter: Comal	26	62	1	88	0	0	44	4	105	367
Comal Springs riffle beetle	NT	NT	--	--	32	0	0	15	32	1
Comal Springs dryopid beetle	NT	NT	--	--	--	0	0	1	0	7
Peck's cave amphipod	NT	NT	--	--	33	21	0	14	178	188
Edwards Aquifer diving beetle	NT	NT	--	--	--	--	--	--	--	--
Texas troglobitic water slater	NT	NT	--	--	--	--	--	--	--	--
Texas blind salamander	NT	NT	--	--	0	0	0	0	88	62
San Marcos salamander	NT	NT	--	--	0	0	3	8	145	156
Comal Springs salamander	NT	NT	--	--	0	0	1	0	57	83
Texas wild rice plants	NT	NT	--	--	10	10	10	0	178	198

Table 2. The number of tagged and recaptured San Marcos salamanders from each site each field day of the San Marcos salamander mark-recapture study. The number of untagged salamanders that were collected and released without tagging due to size restrictions or because tagging was completed is also reported.

Date	Site	# Tagged	# Recaptured	# Untagged	Total Capture
9-May-23	eastern spillway	82	0	5	87
10-May-23	diversion area	33	0	0	33
11-May-23	hotel area	53	0	8	61
30-May-23	eastern spillway	53	0	16	69
31-May-23	hotel area	22	0	0	22
12-Jun-23	eastern spillway	75	6	20	101
14-Jun-23	hotel area	74	6	25	105
20-Jun-23	diversion area	62	2	8	72
26-Jun-23	hotel area	0	9	21	30
27-Jun-23	eastern spillway	0	4	90	94
10-Jul-23	hotel area	0	3	19	22
12-Jul-23	diversion area	0	2	78	80
13-Jul-23	eastern spillway	0	4	53	57
8-Aug-23	eastern spillway	0	2	95	97
10-Aug-23	hotel area	0	3	54	57
22-Aug-23	hotel area	0	1	101	102
24-Aug-23	eastern spillway	0	0	108	108
6-Sep-23	diversion area	0	5	79	84
13-Sep-23	hotel area	0	3	23	26
14-Sep-23	eastern spillway	0	1	59	60
25-Sep-23	hotel area	0	0	51	51
27-Sep-23	eastern spillway	0	1	94	95
10-Oct-23	eastern spillway	0	3	145	148
11-Oct-23	diversion area	0	5	87	92
12-Oct-23	hotel area	0	1	43	44
23-Oct-23	hotel area	0	0	60	60
24-Oct-23	eastern spillway	0	1	104	105
8-Nov-23	diversion area	0	4	95	99
14-Nov-23	eastern spillway	0	2	90	92
16-Nov-23	hotel area	0	0	14	14
11-Dec-23	hotel area	0	0	8	8
12-Dec-23	eastern spillway	0	0	66	66
13-Dec-23	diversion area	0	5	84	89
3-Jan-24	hotel area	0	0	7	7

23-Jan-24	diversion area	0	2	55	57

Figure 1. Jonathan Donahey collecting data during fountain darter collection at the Comal River.

Figure 2. Daw provided demonstrations of sensor wiring to Shawn Moore.

Figure 3. The completed first phase of the supersaturation diversion project in the SMARC refugia. The system affords further security using an uninterruptable power supply (UPS). Supersaturated water is directed into trench drains.

Figure 4. Dr. Shannon Brewer and Brian De La Torre setting up the beetle tagging chambers.

February 2024 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dominique Alvear, Braden West and Dr. Katie Bockrath

With contributions from

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Task 1 Refugia Operations

Staffing

Adam Daw's last day at the Uvalde National Fish Hatchery (UNFH) was on February 16. Daw will be joining the National Centers for Coastal Science, NOAA at the Hollings Marine Laboratory in Charleston, South Carolina as an Environmental Scientist.

Species Collection

BIO-WEST beetle lures were collected and screened from February 28 to March 1. The beetles were split between the stations with the UNFH keeping 13 individuals and the San Marcos Aquatic Resources Center (SMARC) keeping 15 individuals.

Jackson and S. Moore checked the Diversion net twice weekly in February. Two San Marcos salamanders, one juvenile and one adult, were captured. Both individuals were released at their designated release site.

Jackson and S. Moore set traps for Texas blind salamander at Primer's Fissure and Johnson's Well in the Purgatory Creek Natural Area in San Marcos, Texas. Five Texas blind salamanders were encountered. Three individuals were retained for quarantine at the SMARC and two were released into their respective capture sites.

Husbandry

Uvalde

Dominique Alvear scheduled the spring fountain darter collection trips which are set to begin the first week of March. Preparation for the large number of fish began with quarantine racks being disinfected and prepared along with 26 aquaria to house the incoming fish.

Alvear continued with inventories of Peck's cave amphipod boxes. During the January amphipod collection, a brooding female was brought into the refugia and monitored bi-weekly to be able to detach the juveniles before they were eaten. Nine juveniles were attached to the female on February 23, and on February 26 the female was examined again. Five juveniles had been released and four remained attached. The four remaining were forcefully removed and placed in a small box. The other five were not found and assumed to have been eaten.

Jonathan Donahey constructed two controller boxes. One was taken to the SMARC, and the other was placed in the invertebrate room on rack 2.

Heidi Meador made progress on replumbing refugia tank 12 and began preparing for the annual task of rice repotting. Last spring, different types of pots were ordered to potentially find a better container for the Texas wild rice. The plants performed better in a type of air pruning pot. Therefore, staff ordered air pruning pots. Alvear and Meador put the pots together when they arrived.

In the absence of Daw, Alvear and Donahey continued working on updating the plumbing on quarantine systems (Figure 1).

SMARC

Dr. Katie Bockrath and Braden West traveled to the UNFH on February 16 to meet with staff. West took photographs of systems to replicate at the SMARC. West completed the replicated system on February 29.

Daw and West installed SMARC's first system controller box on the invertebrate rack system in the SMARC refugia.

West provided training and resources for identifying Comal Springs riffle beetle in the field to all EARP staff on February 28-March 1. West hosted the UNFH EARP in New Braunfels, where he trained staff on the processing of cotton lures and differentiation of Comal Springs riffle beetles from similar species in the field.

West completed construction on a pressurized upflow filter system in the SMARC greenhouse. This system combines a mechanical strainer with artificial media to filter suspended solids from influent water. The system also incorporates dual 120w UV filters to account for potential pathogenic transfer.

Task 2 Research

Dryopid Life History

Dr. Matt Pintar (BIO-WEST) continued to monitor the two pairs of drypoid beetles for evidence of reproduction. One pair produced an egg, which was transferred to a separate housing contain for observation The other pair did not produce an egg and unfortunately died in captivity. This pair had been held in captivity for three months. *Stenelmis*, the surrogate genus, has produced multiple eggs in captivity, suggesting the experimental captive holding practices are promising.

Dr. Pintar continued collection events for Comal Springs dryopid beetles in Comal Springs. Searches were conducted around Spring Island to minimize disturbances to the most productive Comal Springs riffle beetle sites where lures have been placed for biomonitoring. No dryopids were found.

San Marcos Salamander Mark Recapture

Several members of the SMARC staff, interns, and volunteers contributed to the collection, processing, and release of San Marcos salamanders in Spring Lake. Salamanders were collected from Spring Lake near the Hotel site February 7 and February 29 (Table 2). Monthly dive collections at Hotel were added to increase collections in deeper locations where snorkelers are unable to reach (Figure 2). Salamanders were collected from Spring Lake near the Diversion pipe February 14 (Table 2). Salamanders were collected from the Eastern Spillway site February 27 (Table 2, Figure 3). Many gravid females were observed at the Eastern Spillway site (Figure 4). The first week of sampling at the Eastern Spillway site was cancelled due to dangerous water depths and flows following a large rain event. All salamanders were released back to the area they were captured after they fully recovered. Across all sites, one salamander recapture occurred in February (Table 2).

Reproductive Gene Expression in San Marcos Salamanders

Ruben Tover (University of Texas Austin) continued to work through samples for comparative gene expression analysis and took CT scans of the salamanders collected for reproductive gene expression analysis. Ruben is ordering reagents and supplies for RNA extractions.

Comal Springs Riffle Beetle Population Genetics

Initial DNA extractions were too dilute to do enzyme cuts and have DNA remaining for secondary analyses, if needed. The DNA extractions were concentrated at the Abernathy Fish Technology Center in Abernathy, Washington. The DNA is ready for rehydration and enzyme shearing to generate a RADseq library to collect SNP data.

Tagging Aquatic Invertebrates

The four experimental chambers (two with surrogate beetles, two with captive-reared Comal Springs riffle beetles) and three control chambers (two with surrogate beetles, one with captive-reared Comal Springs riffle beetles) were checked and inventoried weekly. Temperature and flow were adjusted as needed. Beetles were observed moving through the scanning tube from one housing chamber to the other (Figure 5). Thus far, seven beetles were scanned passively in the system.

Genetic Assessment of Peck's Cave Amphipod

Peck's cave amphipod collected in November 2023 as bycatch during BIO-WEST Comal Springs riffle beetle lure collections were transferred to Dr. Chris Nice at Texas State University for DNA extraction and genetic analysis. Dr. Katie Bockrath met with Dr. Nice to discuss analysis approaches and expected product deliverables. Dr. Nice purchased reagents for DNA extractions and Next-Gen sequencing library preparation. Dr. Kate Bell reviewed previously used bioinformatic pipelines for sequence data analysis. Using previously collected sequence data on Peck's cave amphipod optimized the pipelines to ensure accurate and robust data analysis.

Additional Accomplishments

Dr. Katie Bockrath met with partnered researchers and their grants management specialists to complete paperwork required to allocate 2024 research funds.

Dr. Bockrath met with Dr. Scott Walker to discuss best approaches to filling the Husbandry lead position.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Dr. Bockrath addressed comments in the draft 2023 EARP Annual Report and added a description of the January 2023 supersaturation event as an appendix. Dr. Bockrath submitted the final report to the EAA on February 28, 2024.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss collections, husbandry, and ongoing research.

EARP staff attended the 2024 R2 FAC Science Symposium where Dominique Alvear presented background information on the upcoming fountain darter mortality project set to begin in March. Alvear was awarded the 2024 FAC Science Symposium's Best Presentation Runner-Up Award in the Early Career Category. D. Moore and S. Moore presented the San Marcos salamander mark-recapture study and fountain darter archive presentations, respectively.

Summary of February Activities

- The EARP collected 28 Comal Springs riffle beetles from BIO-WEST lures, 13 of which were retained for the UNFH, and 15 were retained for the SMARC
- The EARP collected five Texas blind salamanders from Primer's Fissure and Johnson's Well, three of which were retained for the SMARC

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for February 2024. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	--	--	0	0	25	1	36	288
Fountain darter: Comal	NT	NT	1	88	0	39	18	3	87	403
Comal Springs riffle beetle	15	13	16	34	0	0	NA	0	32	1
Comal Springs dryopid beetle	NT	NT	--	--	--	0	0	0	0	7
Peck's cave amphipod	5	NT	--	--	0	0	7	16	171	172
Edwards Aquifer diving beetle	NT	NT	--	--	--	--	--	--	--	--
Texas troglobitic water slater	NT	NT	--	--	--	--	--	--	--	--
Texas blind salamander	3	NT	2	5	0	0	0	1	88	61
San Marcos salamander	NT	NT	--	--	0	0	9	7	136	149
Comal Springs salamander	NT	NT	--	--	0	0	1	2	56	81
Texas wild rice plants	NT	NT	--	--	--	10	24	0	154	198

Table 2. The number of tagged and recaptured San Marcos salamanders from each site each field day of the San Marcos salamander mark-recapture study. The number of untagged salamanders that were collected and released without tagging due to size restrictions or because tagging was completed is also reported.

Date	Site	# Tagged	# Recaptured	# Untagged	Total Capture
9-May-23	eastern spillway	82	0	5	87
10-May-23	diversion area	33	0	0	33
11-May-23	hotel area	53	0	8	61
30-May-23	eastern spillway	53	0	16	69
31-May-23	hotel area	22	0	0	22
12-Jun-23	eastern spillway	75	6	20	101
14-Jun-23	hotel area	74	6	25	105
20-Jun-23	diversion area	62	2	8	72
26-Jun-23	hotel area	0	9	21	30
27-Jun-23	eastern spillway	0	4	90	94
10-Jul-23	hotel area	0	3	19	22
12-Jul-23	diversion area	0	2	78	80
13-Jul-23	eastern spillway	0	4	53	57
8-Aug-23	eastern spillway	0	2	95	97
10-Aug-23	hotel area	0	3	54	57
22-Aug-23	hotel area	0	1	101	102
24-Aug-23	eastern spillway	0	0	108	108
6-Sep-23	diversion area	0	5	79	84
13-Sep-23	hotel area	0	3	23	26
14-Sep-23	eastern spillway	0	1	59	60
25-Sep-23	hotel area	0	0	51	51
27-Sep-23	eastern spillway	0	1	94	95
10-Oct-23	eastern spillway	0	3	145	148
11-Oct-23	diversion area	0	5	87	92
12-Oct-23	hotel area	0	1	43	44
23-Oct-23	hotel area	0	0	60	60
24-Oct-23	eastern spillway	0	1	104	105
8-Nov-23	diversion area	0	4	95	99
14-Nov-23	eastern spillway	0	2	90	92
16-Nov-23	hotel area	0	0	14	14
11-Dec-23	hotel area	0	0	8	8
12-Dec-23	eastern spillway	0	0	66	66
13-Dec-23	diversion area	0	5	84	89
3-Jan-24	hotel area	0	0	7	7

23-Jan-24	diversion area	0	2	55	57
7-Feb-24	hotel area	0	0	75	75
14-Feb-24	diversion	0	1	76	77
27-Feb-24	eastern spillway	0	0	74	74
29-Feb-24	hotel area	0	0	8	8

Figure 1. Jonathan Donahey finished plumbing the return line on quarantine rack 8.

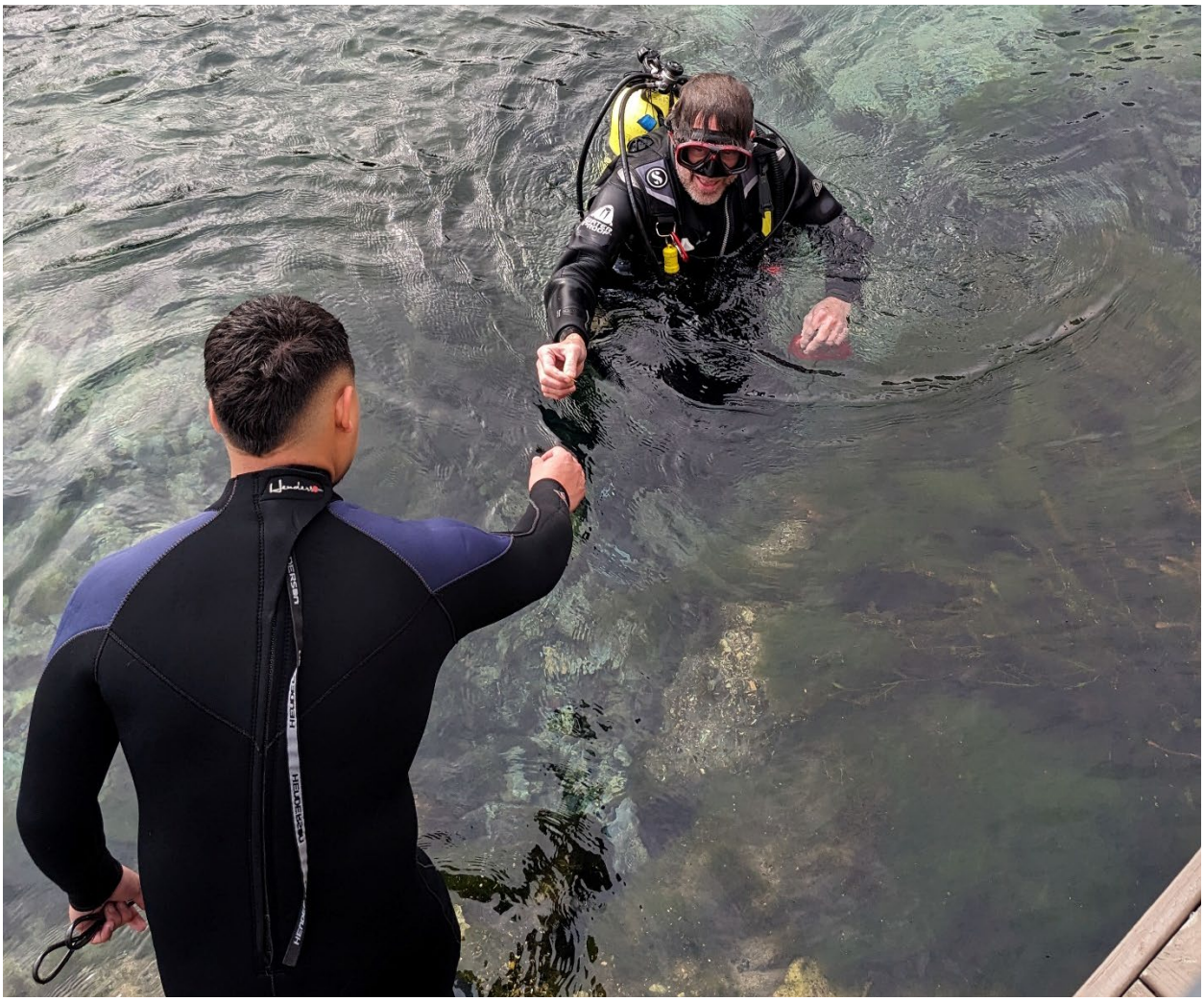

Figure 2. Isaiah Travino (Student Conservation Association intern) transfers San Marcos salamanders collected by Randy Gibson (USFWS Diver) to coolers on shore.

Figure 3. Shawn Moore bringing San Marcos salamanders to shore for scanning and measuring at the Eastern Spillway site.

Figure 4. Two anesthetized San Marcos salamanders collected at the Eastern Spillway site. Top: Gravid female on her back with visible eggs. Bottom: A non-gravid San Marcos salamander.

Figure 5. P-Chip tagged beetle on Velcro substrate moving through the scanning tube of the experimental scanning setup.

March 2024 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dominique Alvear, Braden West and Dr. Katie Bockrath

With contributions from

Desirée Moore, Jonathan Donahey, Richelle Jackson, Heidi Meador, and Shawn Moore

San Marcos Aquatic Resources Center

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Task 1 Refugia Operations

Species Collection

Jonathan Donahey, Richelle Jackson, Dominique Alvear and Braden West collaborated with Dr. Matt Pintar (BIO-WEST, Inc) to process cotton lures set by BIO-WEST for their annual biomonitoring. Edwards Aquifer Refugia Program (EARP) staff collected a total of nine Comal Springs riffle beetles. Three beetles were retained for refugia at the Uvalde National Fish Hatchery (UNFH), and six were retained for refugia at the San Marcos Aquatic Resources Center (SMARC).

March 4-7, Alvear, Donahey, Jackson, Heidi Meador, Shawn Moore, Scott Walker, and Nicolas Yvon had a busy week collecting San Marcos and Comal fountain darters for the spring collection of the mortality study. A total of 184 darters were collected from three sites in the San Marcos River and 253 darters were collected from three sites in the Comal River. All darters were transferred to the UNFH.

March 12, Alvear, Jackson, S. Moore, and Yvon, collected 55 Pecks cave amphipods from Spring Island. Of the 55 collected, 25 were taken back to the SMARC and 30 were taken to the UNFH.

March 14, Jackson, Meador, and S. Moore collected a total of 20 Texas wild rice plants from sections C and D in the San Marcos River. Of the 20 collected, 10 were taken back to the SMARC and 10 were taken to the UNFH.

Jackson and S. Moore checked the Diversion net twice weekly in March. Fifteen San Marcos salamanders and three Texas blind salamanders were captured in the net. All individuals were released at the designated release site.

Jackson, S. Moore, and West initiated an exploratory two-week sampling period beginning on March 25 in Texas State University's Artesian Well (Figure 1). No EAHCP-covered species were collected.

Husbandry

Uvalde

After the spring fountain darter collection, Alvear conducted 78 necropsies from the Comal darter cohort. Higher mortality was observed in the first 30 days of the quarantining period compared to the San Marcos darters, which had higher mortalities 40-60 days after collection.

Multiple parasites were found and preserved for potential sequencing or identification in the future (Figures 2,).

During quarterly Peck's cave amphipod inventories, Alvear observed a brooding female and determined the eggs were removable from the mother to increase chances of survival. Twelve juveniles were hand hatched, two of which appeared to be partially eaten by the adult female already, and placed in a smaller rearing box. The rearing box was inventoried seven days later, and ten juveniles were alive.

Meador began the annual task of Texas wild rice repotting. During the 2023 rice re-pot, Alvear placed 10 plants in new air pruning pots with an adjusted ratio of lava rock to pea gravel mixture. After a year in the new pots, the rice was removed and photographed (Figure 4). A substantial difference was noted in the development of the rice roots and overall health of the plant. It was determined that the new pots should be used in the future.

Donahey continued the construction of quarantine racks 9 & 10 along with the continued construction of controller boxes for future use in the refugia.

SMARC

Jackson and S. Moore visited the UNFH on March 13 for cross-training on husbandry techniques used at the UNFH.

Jackson and S. Moore completed inventories on all Peck's cave amphipod boxes in refugia at the SMARC.

Jackson and S. Moore continued repotting Texas wild rice plants and established a map of Texas wild rice plants in refugia tanks. Jackson and S. Moore incorporated new air pruning pots already in use at the UNFH. Repotted plants were moved to Refugia Tank 1, which was fitted with the newly completed pressurized upflow filter system. The new filter system demonstrated improvements in the amount of algal growth present in the tank.

West completed the SMARC's supersaturation diversion project (Figure 5). The system successfully detected and diverted supersaturated water from reaching animals held in refugia and quarantine.

West completed construction of the first controller box assembled at the SMARC. West installed the controller box on the RE-13 and RE-14 systems.

West installed Walchem controllers on three systems in the SMARC refugia. West connected flow meters, pH probes, and temperature probes to the Walchem controllers on each system.

Animal Health

S. Moore and West collected 11 fountain darters from the San Marcos River and 11 fountain darters from the Comal River as part of USFWS's annual Wild Fish Health Survey. West shipped the live fish to USFWS's Southwestern Fish Health Unit (SFHU) in Dexter, New Mexico. Immature *Centrocestus* cysts were observed on gill arches of 1 of 10 Comal River fish and 2 of 10 San Marcos River fish. *Monogenean* parasites were observed on five of ten Comal River fish and nine of ten San Marcos River fish.

Task 2 Research

Dryopid Life History

Dr. Matt Pintar (BIO-WEST) searched for dryopid beetles in the wild each week. Five beetles were found across two collection days in March.

Dr. Pintar continued to monitor the pairs of drypoid beetles for evidence of reproduction and completed additional replicates testing the response of dryopid beetles to the presence of a conspecific beetle (*Stenelmis* sp.). Additionally, Dr. Pintar performed some replicates examining the rate of gut clearing and consumption of different food types in dryopid adults.

Dr. Bockrath and D. Moore met with BIO-WEST to discuss potential shifts of this project toward field research given the scarcity of dryopid beetles for lab research.

San Marcos Salamander Mark Recapture

Several members of the SMARC staff, interns, and volunteers contributed to the collection, processing, and release of San Marcos salamanders in Spring Lake and the San Marcos River.

Salamanders were collected from the Eastern Spillway site March 12 and 26 (Table 2). Salamanders were collected from Spring Lake near the Hotel site by snorkelers March 25 (Table 2). Additionally, Hotel, Crator Bottom, Salt and Pepper 1 and 2, and Cabomba were sampled by divers March 14. Salamanders were collected from Spring Lake near the Diversion pipe March 13. All salamanders were released back to the area they were captured after they fully recovered. Across all sites, three salamander recaptures occurred in March (Table 2).

Reproductive Gene Expression in San Marcos Salamanders

Desiree Moore and Ruben Tover (University of Texas Austin) began RNA extractions of reproductive tissues dissected from male and gravid female salamanders at the SMARC.

Comal Springs Riffle Beetle Population Genetics

Dr. Bockrath determined the maximum volume to rehydrate Comal Springs riffle beetle DNA extractions in preparation for sequencing and ordered the requisite supplies.

Tagging Aquatic Invertebrates

The four experimental chambers (two with surrogate beetles, two with captive-reared Comal Springs riffle beetles) and three control chambers (two with surrogate beetles, one with captive-reared Comal Springs riffle beetles) were checked and inventoried weekly. Temperature and flow were adjusted as needed. Beetles were observed moving through the scanning tube from one housing chamber to the other. Due to an unexpected fungal growth in control chambers, the trial was ended early, and designs were adjusted for the next trials. Seven beetles were scanned passively in the system.

Genetic Assessment of Peck's Cave Amphipod

Dr. Chris Nice (Texas State University) received lab supplies for DNA extractions and Next-Gen sequencing library preparation. Dr. Kate Bell worked to set up the computer for the analyses by setting up the basic directory structure and installing the required software.

Genetic Assessment of Texas Blind Salamanders

Dr. Bockrath ordered supplies for DNA extraction and sequencing of Texas blind salamander tail clips. Erin Lowenberg (Student Conservation Association intern) archived Texas blind salamander mortalities to determine which specimens to include in the genetic assessment. Lowenberg and D. Moore tested DNA extraction on a subset of the mortalities held at the SMARC to confirm the extraction protocol.

Genetic Assessment of San Marcos Salamanders

Dr. Bockrath ordered supplies for DNA extraction and sequencing of San Marcos salamander tail clips. Lowenberg archived San Marcos salamander mortalities to determine which specimens to include in the genetic assessment.

SMARC staff tagged and tail clipped the San Marcos salamander F1s at the SMARC. Lowenberg and D. Moore tested DNA extraction on a subset of the mortalities held at the SMARC to confirm the extraction protocol.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

EARP staff submitted the 2025 Work Plan and Budget.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss collections, husbandry, and ongoing research.

Dr. Bockrath and D. Moore attended the EAHCP Science Committee meeting on March 7.

The Quarterly EARP/EAA meeting was held at the SMARC March 21. All EARP staff available attended the meeting.

Summary of March Activities

- The EARP collected a total of nine Comal Springs riffle beetles from BIO-WEST lures, three of which were retained for the UNFH, and six were retained for the SMARC
- The EARP collected 184 darters San Marcos salamanders the San Marcos River which were retained for the UNFH
- The EARP collected 253 darters from the Comal River, which were retained for the UNFH
- The EARP collected 55 Pecks cave amphipods from Spring Island, 25 of which were retained for the SMARC, and 30 were retained for the UNFH
- The EARP collected 20 Texas wild rice plants from sections C and D in the San Marcos River, 10 of which were retained for the SMARC, and 10 were retained for the UNFH

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for March 2024. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	11	184	0	11	0	0	5	1	31	287
Fountain darter: Comal	11	253	4	15	0	0	24	4	64	399
Comal Springs riffle beetle	6	15	4	25	0	0	0	0	32	1
Comal Springs dryopid beetle	NT	NT	--	--	--	0	0	0	0	7
Peck's cave amphipod	25	30	12	67	0	0	72	37	99	135
Edwards Aquifer diving beetle	NT	NT	--	--	--	--	--	--	--	--
Texas troglobitic water slater	NT	NT	--	--	--	--	--	--	--	--
Texas blind salamander	0	NT	3	3	0	0	0	0	88	61
San Marcos salamander	0	NT	15	15	0	0	1	7	135	142
Comal Springs salamander	NT	NT	--	--	0	0	2	2	46	79
Texas wild rice plants	10	10	--	--	--	0	9	0	145	198

Table 2. The number of tagged and recaptured San Marcos salamanders from each site each field day of the San Marcos salamander mark-recapture study. The number of untagged salamanders that were collected and released without tagging due to size restrictions or because tagging was completed is also reported.

Date	Site	# Tagged	# Recaptured	# Untagged	Total Capture
9-May-23	eastern spillway	82	0	5	87
10-May-23	diversion area	33	0	0	33
11-May-23	hotel area	53	0	8	61
30-May-23	eastern spillway	53	0	16	69
31-May-23	hotel area	22	0	0	22
12-Jun-23	eastern spillway	75	6	20	101
14-Jun-23	hotel area	74	6	25	105
20-Jun-23	diversion area	62	2	8	72
26-Jun-23	hotel area	0	9	21	30
27-Jun-23	eastern spillway	0	4	90	94
10-Jul-23	hotel area	0	3	19	22
12-Jul-23	diversion area	0	2	78	80
13-Jul-23	eastern spillway	0	4	53	57
8-Aug-23	eastern spillway	0	2	95	97
10-Aug-23	hotel area	0	3	54	57
22-Aug-23	hotel area	0	1	101	102
24-Aug-23	eastern spillway	0	0	108	108
6-Sep-23	diversion area	0	5	79	84
13-Sep-23	hotel area	0	3	23	26
14-Sep-23	eastern spillway	0	1	59	60
25-Sep-23	hotel area	0	0	51	51
27-Sep-23	eastern spillway	0	1	94	95
10-Oct-23	eastern spillway	0	3	145	148
11-Oct-23	diversion area	0	5	87	92
12-Oct-23	hotel area	0	1	43	44
23-Oct-23	hotel area	0	0	60	60
24-Oct-23	eastern spillway	0	1	104	105
8-Nov-23	diversion area	0	4	95	99
14-Nov-23	eastern spillway	0	2	90	92
16-Nov-23	hotel area	0	0	14	14
11-Dec-23	hotel area	0	0	8	8
12-Dec-23	eastern spillway	0	0	66	66
13-Dec-23	diversion area	0	5	84	89
3-Jan-24	hotel area	0	0	7	7

23-Jan-24	diversion area	0	2	55	57
7-Feb-24	hotel area	0	0	75	75
14-Feb-24	diversion	0	1	76	77
27-Feb-24	eastern spillway	0	0	74	74
29-Feb-24	hotel area	0	0	8	8
12-Mar-24	eastern spillway	0	0	39	39
13-Mar-24	diversion	0	2	51	53
14-Mar-24	hotel area	0	1	77	78
14-Mar-24	crater bottom	0	0	3	3
14-Mar-24	salt and pepper 1	0	0	1	1
14-Mar-24	salt and pepper 2	0	0	0	0
14-Mar-24	cabomba	0	0	0	0
25-Mar-24	hotel area	0	0	0	0
26-Mar-24	eastern spillway	0	0	19	19

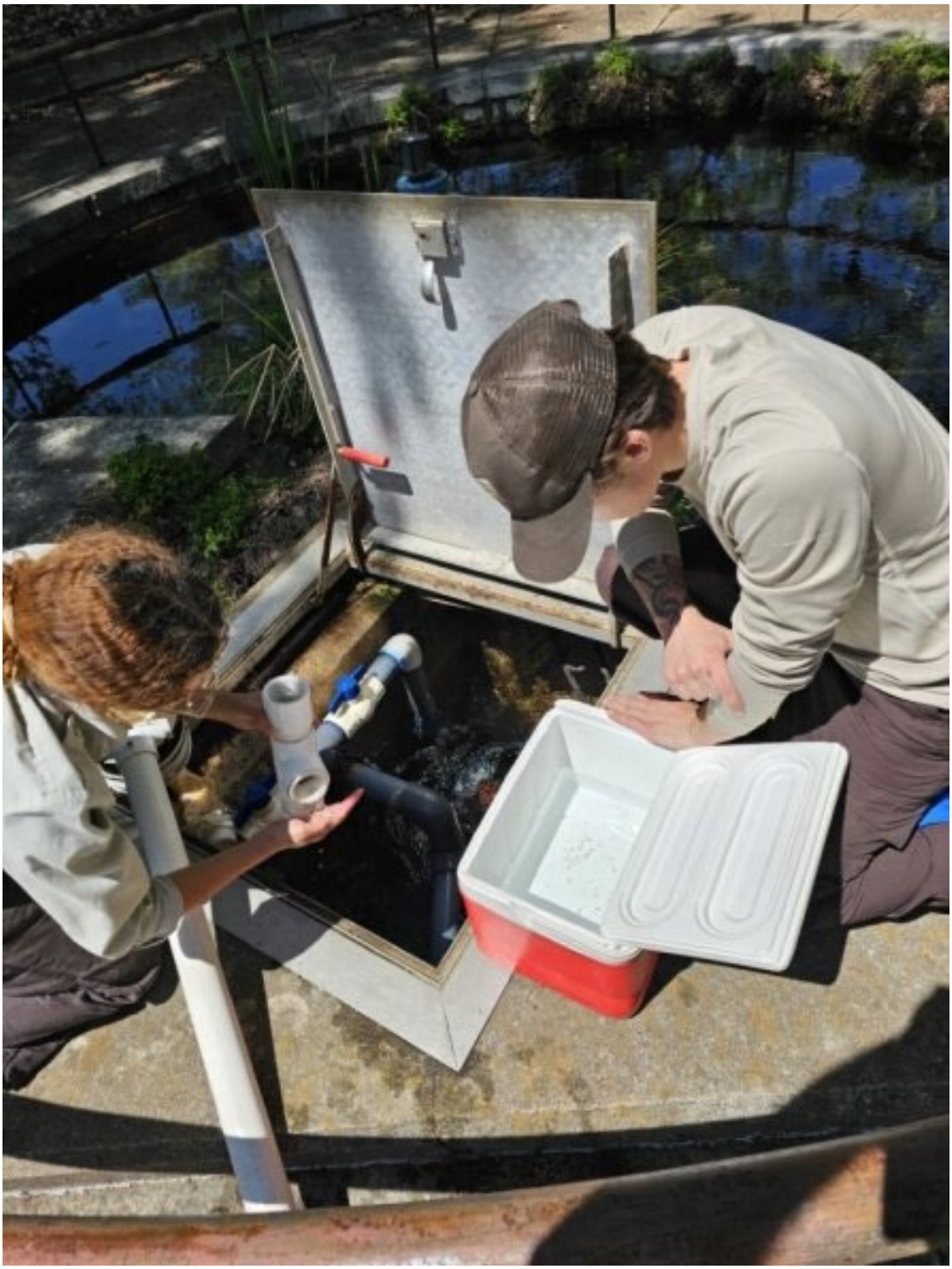

Figure 1. Richelle Jackson and Shawn Moore checking the net over the Artesian Well at Texas State University.

Figure 2. Parasite - from a Comal Springs fountain darter.

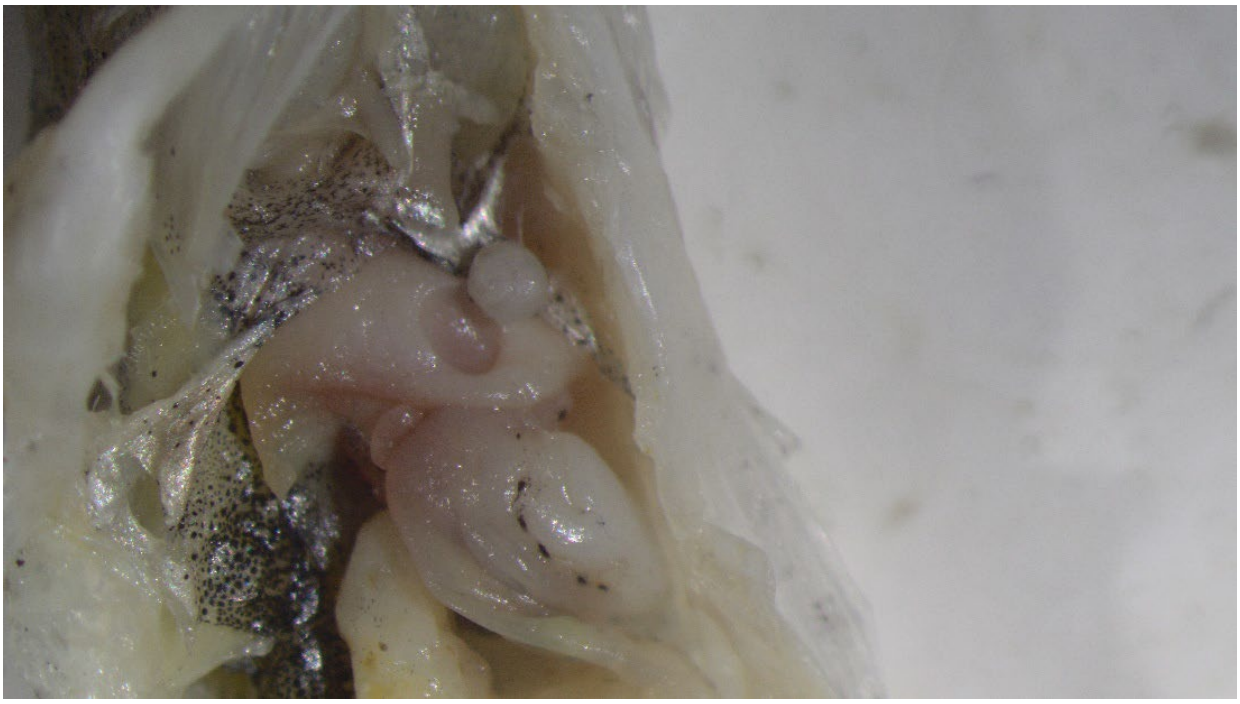

Figure 3. Parasite from a Comal Springs fountain darter.

Figure 4. The left panel shows roots from the older style Texas wild rice pots. The right panel shows a Texas wild rice plant in the air pruning pots with adjusted soil ratio.

Figure 5. The completed supersaturation diversion project in the quarantine room at the SMARC.

April 2024 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dominique Alvear, Braden West and Dr. Katie Bockrath

With contributions from

Desirée Moore, Jonathan Donahey, Richelle Jackson, Heidi Meador, and Shawn Moore

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Task 1 Refugia Operations

Species Collection

Jackson and S. Moore checked the Artesian well drift net every day from April 1st to April 5th. No EAHCP-covered animals were collected.

On April 13th Dominique Alvear, Braden West along with Dr. Matt Pintar (BIO-WEST) and Israel Prewitt (BIO-WEST) set cotton lures along Spring Runs 1-3.

On April 18th, Jackson and S. Moore coordinated the transfer of 291 Fountain darters collected by BIO-WEST in the San Marcos River. All organisms were retained for quarantine at the SMARC.

On April 25th, Jackson and S. Moore coordinated the transfer of 477 Fountain darters collected by BIO-WEST in the Comal River. All organisms were retained for quarantine at the SMARC.

On April 30th, Heidi Meador, Braden West, Shawn Moore and Richelle Jackson had assistance from divers Randy Gibson, Tommy Funk and Jacqualyn Halmbacher in the San Marcos River targeting section C for collection of Texas wild rice (Figure 1). Twenty-nine TWR plants were collected. Fifteen plants were retained for refugia at the UNFH and fourteen plants were retained for refugia at the SMARC.

Jackson and S. Moore checked the Diversion net on a twice weekly basis in April. Two Texas blind salamanders and eighteen San Marcos salamanders were collected. Both Texas blind salamanders were retained for refugia at the SMARC. All San Marcos salamanders were released.

Husbandry

Uvalde

Jonathan Donahey continued construction on quarantine rack systems.

Meador continued transfer and repotting of Texas wild rice into the new air pruning pots.

Alvear assisted in the annual Fish Health Inspection by taking buccal swabs of the salamanders requested for the inspection (Figure 2)

SMARC

Jackson and S. Moore incorporated quarantined Peck's cave amphipod and Comal Springs riffle

beetle into the Refugia population.

Jackson and S. Moore continued repotting of Texas wild rice into the new air pruning pots. They also transferred quarantined Texas wild rice to the Refugia population.

Jackson and S. Moore redesigned a drain for raceway tanks in the SMARC quarantine.

West designed and built an isolation area for Comal River Fountain darters in the SMARC quarantine.

Jackson, S. Moore, and West conducted five-day salt treatments on newly collected Fountain darters to reduce stress from collection.

Dr. Bockrath and Braden West completed the necessary purchasing documentation to purchase the remaining Walchem Controller units for both SMARC and UNFH. The controllers are now moving through the purchasing process.

Task 2 Research

Dryopid Life History

Dr. Matt Pintar (BIO-WEST) completed husbandry experiments assessing bio-film consumption on wood condition in different sources and at different rates. Dr. Pintar collated and organized dryopid beetle collection data from 1990 to 2022 and worked on designing alternative ways to detect, monitor and collect adult dryopid beetles.

San Marcos Salamander Mark Recapture

Several members of the SMARC staff, interns, and volunteers contributed to the collection, processing, and release of San Marcos salamanders in Spring Lake and the San Marcos River. Salamanders were collected from the Eastern Spillway site April 11 and 23 (Table 2). Salamanders were collected from Spring Lake near the Hotel site by snorkelers April 24 (Table 2). Hotel was sampled by divers April 9. Salamanders were collected from Spring Lake near the Diversion pipe April 10. All salamanders were released back to the area they were captured after

they fully recovered from sedation. Across all sites, 346 salamanders were collected, 2 of which were recaptures (Table 2).

Reproductive Gene Expression in San Marcos Salamanders

Ruben Tovar (University of Texas Austin) isolated RNA from embryos. Sheena Leelani and Dan Tatulescu (University of Texas Austin) presented posters and talks at the University of Texas Undergraduate Research Forum. The research presented is derived from the samples and CT scans generated for this study. Sheena Leelani presented on changes in eye volume through development for all three covered EAHCP salamander species. Dan A. Tatulesc presented of skull formation and its correlation to eye development between San Marcos salamanders and Texas blind salamanders.

Comal Springs Riffle Beetle Population Genetics

Bio-West and EARP staff set out lures across Spring Run 1, Spring Run 2, Spring Run 3, Spring Island, Western Shore and Upper Spring Run. Comal Springs riffle beetles will be retained for the Refugia unless the genetic assessment study needs individuals from areas of low representation. Lures will be retrieved in May.

Tagging Aquatic Invertebrates

Dr. Bockrath inventoried the surrogate beetles and determined that there were sufficient beetles, at the time of inventory, to conduct the second trial of the tagging study. The second trial requires 70 *Heterelmis glabra.* Eighty adults (5 with tags remaining from the first control trial) and many large larvae were present in the holding tube. Dr. Shannon Brewer (Auburn University) sent updated experimental tubes and control housings to the SMARC. Dr. Bockrath and Desiree Moore scheduled a meeting with Dr. Brewer and Brian De La Torre (Auburn University) to discuss changes to improve the second trial and potential dates for Dr. Brewer and De La Torre to visit the SMARC to set up the second trial.

Genetic Assessment of Peck's Cave Amphipod

Bio-West and EARP staff set out lures across Spring Run 1, Spring Run 2, Spring Run 3, Spring Island, Western Shore and Upper Spring Run. Peck's cave amphipods will be retained for the Refugia unless the genetic assessment study needs individuals from areas of low representation. Lures will be retrieved in May.

Genetic Assessment of Texas Blind Salamanders

No significant updates to report.

Genetic Assessment of San Marcos Salamanders

Erin Lowenberg and Dr. Bockrath finished extracting DNA from 450 San Marcos salamander tail clips collected during the mark and recapture study. DNA was quantified using a Qubit Fluorometer and DNA was concentrated to a minimum of 20ng/µl using a sodium acetate precipitation protocol (Figure 3). DNA isolations now meet the minimum DNA concentrations for ideal RAD-Seq library preparation and sequencing. Dr. Bockrath will be conducting library preparation in May.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

EARP staff submitted the 2025 Work Plan and Budget.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss collections, husbandry, and ongoing research.

Other Activities

The EARP was awarded the US Fish and Wildlife Service Southwest Region Team of the Year (Figure 4).

Summary of April Activities

- EARP regularly checked a net set on the Artesian Well, no organisms were collected.
- EARP regularly checked the Diversion net; 2 Texas blind salamanders were collected.
- EARP staff and BIO-WEST set lures at Spring Runs 1-3
- EARP staff collected Comal Springs fountain darters from BIO-WEST during their Spring biomonitoring effort.
- Progress was made on purchasing the remaining Walchem Controller units for both stations.
- Uvalde had their annual fish health inspection.
- Texas wild rice was repotted in the air pruning pots.
- The EARP team was awarded Team of the Year.
- Biofilm consumption experiments were concluded for the dryopid beetle and research switched to improved collection methods.
- EARP staff and Dr. Shannon brewer prepared for the second trial of the invertebrate tagging study.
- Ruben Tovar extracted RNA from salamander embryos.
- San Marcos Salamander DNA was concentrated in preparation for sequencing.
- EARP staff submitted the 2025 draft work plan and budget.

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for April 2024. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	291	NT	3	294	0	0	5	19	27	268
Fountain darter: Comal	477	NT	0	477	0	0	24	16	58	383
Comal Springs riffle beetle	NT	NT	--	--	12	0	0	0	44	1
Comal Springs dryopid beetle	NT	NT	--	--	--	0	0	0	0	7
Peck's cave amphipod	NT	NT	--	--	17	20	72	0	116	155
Edwards Aquifer diving beetle	NT	NT	--	--	--	--	--	--	--	--
Texas troglobitic water slater	NT	NT	--	--	--	--	--	--	--	--
Texas blind salamander	2	NT	2	4	0	0	0	0	99	61
San Marcos salamander	0	NT	18	18	0	0	1	5	133	137
Comal Springs salamander	NT	NT	--	--	0	0	2	1	47	141
Texas wild rice plants	14	15	--	29	10	10	0	0	155	204

Table 2. The number of tagged and recaptured San Marcos salamanders from each site each field day of the San Marcos salamander mark-recapture study. The number of untagged salamanders that were collected and released without tagging due to size restrictions or because tagging was completed is also reported.

Date	Site	# Tagged	# Recaptured	# Untagged	Total Capture
9-May-23	eastern spillway	82	0	5	87
10-May-23	diversion area	33	0	0	33
11-May-23	hotel area	53	0	8	61
30-May-23	eastern spillway	53	0	16	69
31-May-23	hotel area	22	0	0	22
12-Jun-23	eastern spillway	75	6	20	101
14-Jun-23	hotel area	74	6	25	105
20-Jun-23	diversion area	62	2	8	72
26-Jun-23	hotel area	0	9	21	30
27-Jun-23	eastern spillway	0	4	90	94
10-Jul-23	hotel area	0	3	19	22
12-Jul-23	diversion area	0	2	78	80
13-Jul-23	eastern spillway	0	4	53	57
8-Aug-23	eastern spillway	0	2	95	97
10-Aug-23	hotel area	0	3	54	57
22-Aug-23	hotel area	0	1	101	102
24-Aug-23	eastern spillway	0	0	108	108
6-Sep-23	diversion area	0	5	79	84
13-Sep-23	hotel area	0	3	23	26
14-Sep-23	eastern spillway	0	1	59	60
25-Sep-23	hotel area	0	0	51	51
27-Sep-23	eastern spillway	0	1	94	95
10-Oct-23	eastern spillway	0	3	145	148
11-Oct-23	diversion area	0	5	87	92
12-Oct-23	hotel area	0	1	43	44
23-Oct-23	hotel area	0	0	60	60
24-Oct-23	eastern spillway	0	1	104	105
8-Nov-23	diversion area	0	4	95	99
14-Nov-23	eastern spillway	0	2	90	92
16-Nov-23	hotel area	0	0	14	14
11-Dec-23	hotel area	0	0	8	8
12-Dec-23	eastern spillway	0	0	66	66
13-Dec-23	diversion area	0	5	84	89
3-Jan-24	hotel area	0	0	7	7

23-Jan-24	diversion area	0	2	55	57
7-Feb-24	hotel area	0	0	75	75
14-Feb-24	diversion	0	1	76	77
27-Feb-24	eastern spillway	0	0	74	74
29-Feb-24	hotel area	0	0	8	8
12-Mar-24	eastern spillway	0	0	39	39
13-Mar-24	diversion	0	2	51	53
14-Mar-24	hotel area	0	1	77	78
14-Mar-24	crater bottom	0	0	3	3
14-Mar-24	salt and pepper 1	0	0	1	1
14-Mar-24	salt and pepper 2	0	0	0	0
14-Mar-24	cabomba	0	0	0	0
25-Mar-24	hotel area	0	0	0	0
26-Mar-24	eastern spillway	0	0	19	19
9-Apr-24	hotel area	0	0	86	86
10-Apr-24	diversion area	0	1	100	101
11-Apr-24	eastern spillway	0	1	80	81
23-Apr-24	eastern spillway	0	0	42	42
24-Apr-24	hotel area	0	0	36	36

Figure 1. Divers assisting EARP staff in the collection of Texas wild rice from the San Marcos River. Top from left: Thomas Funk, Jacqualyn Halmbacher, Randy Gibson. Bottom from left: Shawn Moore, Richelle Jackson.

Figure 2. Alvear taking a buccal swab of a Texas Blind Salamander for the annual Fish Health Inspection at the Uvalde National Fish Hatchery

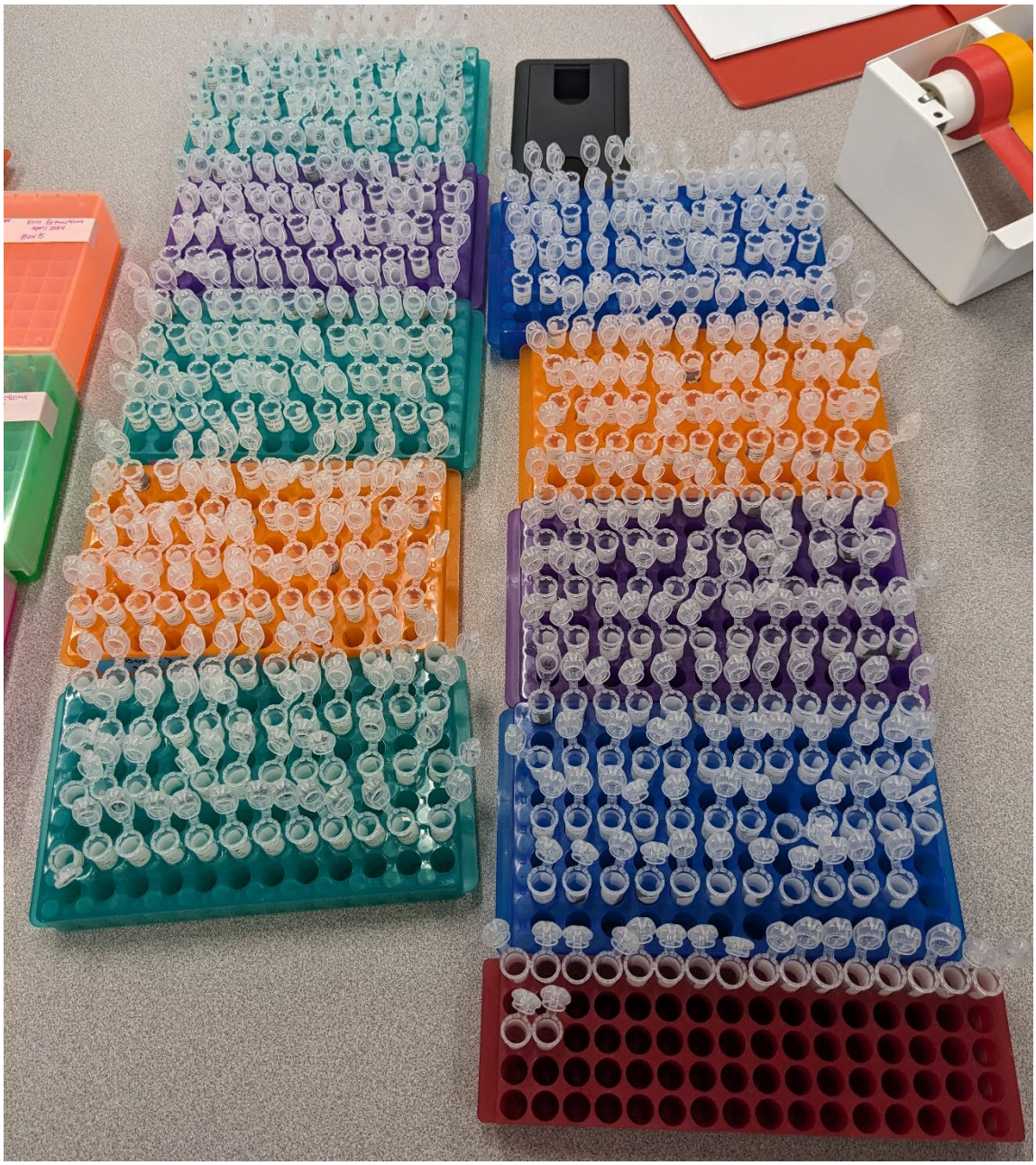

Figure 3. DNA extractions drying after being concentrated in preparation for sequencing. These DNA samples are from the 453 San Marcos salamanders tagged during the start of the Mark and Recapture Study.

Figure 4. Lisa Griego-Lyon, Desiree Moore, Braden West, and Jon Donahey accepting Team of the Year award.

May 2024 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dominique Alvear, Braden West and Dr. Katie Bockrath

With contributions from

Desirée Moore, Jonathan Donahey, Richelle Jackson, Heidi Meador, and Shawn Moore

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Task 1 Refugia Operations

Species Collection

On May 8th Dr.Scott Walker and Dominique Alvear from the Uvalde National Fish Hatchery (UNFH) assisted in the mark recapture study on the San Marcos salamander. Organisms from this study were kept for incorporation into the Refugia population; 42 salamanders were brought back to the UNFH and 54 were taken to the San Marcos Aquatic Resources Center.

On May 15th Alvear assisted Dr. Katie Bockrath, Shawn Moore and Dr. Matt Pintar (BIO-WEST) in the retrieval of lure set for the Comal Springs riffle beetle. Twelve beetles were brought back to the UNFH and nine were taken back to the SMARC.

On May 15th Richelle Jackson and Erin Lowenberg set traps for Texas blind salamanders in Primer's Fissure. Blowdown traps were checked on Mondays, Wednesdays, and Fridays until May 28th. A total of seven Texas blind salamanders were captured during the sample period, four were released and three were taken to the SMARC for refugia. Two of the seven captured animals were tagged recaptures. Both animals were tagged in the May 2023 sample period. One of the tagged animals was recaptured during every sample period since May 2023 and the other tagged animal had never been observed after tagging, with this being the only time it was recaptured.

On May 20th Alvear assisted in the mark-recapture collection of the San Marcos salamander in Spring Lake (Figure 1); 80 salamanders were brought back to the UNFH and 68 were taken to the SMARC.

On May 21st Jackson and S. Moore assisted in the final mark and recapture field collection for San Marcos salamander at the Eastern Spillway of the San Marcos River. Forty-four organisms were taken to the SMARC for refugia.

On May 30th Jackson, S. Moore, West, and Daniela Cortez (USFWS Student Trainee) collected Peck's cave amphipods from the Spring Island area of the Comal River in New Braunfels, TX (Figure 2). A total of 79 individuals were captured and three were released. Seventy-six individuals were taken to the SMARC for refugia.

Husbandry

Uvalde

Jonathon Donahey constructed and added airlines to the newly built quarantine racks.

Alvear repotted 67 Texas wild rice into air-pruning pots.

Meador conducted the semi-annual salamander inventory to ensure accurate numbers are being reported.

Alvear, Donahey and Meador cleaned the mezzanine and quarantine chiller room to help reduce dust and debris build up on the coils of the chillers in preparation for the extreme summer temperatures.

SMARC

Jackson, S. Moore, and West worked diligently in early May to ensure sufficient space was available in the SMARC quarantine to prepare for San Marcos salamander collections at the completion of the mark-recapture study on San Marcos salamanders.

Jackson and S. Moore continued caring for the three lots of Fountain darters collected during BIO-WEST biomonitoring surveys in April from the San Marcos and Comal Rivers. Repeated low-concentration salt treatments were used to reduce stress in the week following collection. Survival in San Marcos River Fountain darter lots (n=152, n=139) after 30 days were 94 and 92 percent, respectively. Survival in the Comal River Fountain darter lot (n=477) was 97 percent.

West completed the refit of the SMARC quarantine by constructing two physical barriers using clear water-resistant curtains. Biosecurity checkpoints consisting of disinfection footbaths were placed at both barriers. The addition of physical barriers further reduced the probability of disease transmission between systems (Figure 3).

Animal Health

Jackson and S. Moore collected skin swabs from San Marcos salamanders collected on May 8th from Diversion Springs.

Task 2 Research

Dryopid Life History

Dr. Matt Pintar (BIO-WEST) planned and constructed multiple designs of miniature drift nets and lures consisting of stakes of conditioned wood and cellulose sponges for testing in the field. Five drift nets and 34 wood/sponge lures were set around the Spring Island area and Western Shoreline.

San Marcos Salamander Mark and Recapture

Field collections for the mark and recapture effort concluded in May. Several members of the SMARC staff, interns, and volunteers contributed to the collection, processing, and release of San Marcos salamanders in Spring Lake and the San Marcos River. Salamanders were collected from the Eastern Spillway site May 21 (Table 2). Salamanders were collected from Spring Lake near the Hotel site by snorkelers May 06 (Table 2). Hotel was sampled by divers May 20. Salamanders were collected from Spring Lake near the Diversion pipe May 08. All salamanders were released back to the area they were captured after they fully recovered from sedation. Across all sites, 417 salamanders were collected. No recaptures were collected in May (Table 2).

Reproductive Gene Expression in San Marcos Salamanders

No significant updates to report.

Comal Springs Riffle Beetle Population Genetics

Lures previously set in Spring Run 1, Spring Run 2 and Spring Run 3 were retrieved and reset in May. All adults were retained for the Refugia population. One larva was collected from Spring Run 2 and five larvae were collected from Spring Run 3. All larvae were preserved in ethanol and retained for genetic analysis.

Tagging Aquatic Invertebrates

Dr. Bockrath set up the redesigned experimental tubes and control chambers on a flow-through system and ensured all housing received adequate flow (Figure 4). Housings were set up a month prior to adding beetles to allow for biofilms to grow on the Velcro substrate attached to the bottom of all housings. Dr. Bockrath met with Dr. Shannon Brewer (Auburn University) and her graduate student Brian De La Torre to coordinate a week for them to visit SMARC and start the next tagging trial. Dr. Bockrath and Dr. Brewer discussed modifications to the tagging process and housing designs that should reduce stress during tagging and improve flow while in the housings. Dr. Bockrath inventoried the *Heterelmis glabra* tube to ensure there were sufficient beetles to conduct the second trial. Dr. Bockrath applied the methods developed during the first trial to practice applying p-Chip tags to preserved Comal Springs riffle beetles (Figure 5).

Genetic Assessment of Peck's Cave Amphipod

Six Peck's cave amphipods were collected from Spring Run 2 and Spring run 3 during the Comal Springs riffle beetle lure check. The individuals will be transferred to Dr. Chris Nice (Texas State University) in June for analysis. Dr. Nice has purchase reagents for DNA extraction and sample preparation for sequencing. Dr. Kate Bell (Texas State University) is optimizing sequencing methods and data analysis scripts.

Genetic Assessment of Texas Blind Salamanders

Methods are being validated in the San Marcos salamander genetic assessment effort and wild caught and captive breed individuals are being identified for sequencing from preserved refugia mortalities and tail clips.

Genetic Assessment of San Marcos Salamanders

Dr. Katie Bockrath worked with Dr. Chris Nice to prepare the San Marcos salamander samples for sequencing. All DNA samples were sheared using two restriction enzymes that produced consistent cut sites across samples so that the samples are directly comparable. Samples were

individually barcoded and amplified for downstream bioinformatic analysis. Dr. Bockrath identified a USFWS lab that can size select the samples to a specific size range suitable for sequencing. Dr. Bockrath will send the samples off for size selection and sequencing in June.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss collections, husbandry, and ongoing research.

USFWS Southwest Regional Director, Amy Lueders, visited the station where we were able to highlight the EARP and the EAHCP (Figure 6).

Summary of May Activities

- 288 San Marcos salamanders were collected from Spring Lake (Diversion, Hotel and Eastern Spillway). 166 were brought to SMARC and 122 were brought to UNFH.
- 17 Comal Springs riffle beetles were collected from Spring Run 2 and Spring Run 3.
- Texas blind salamander traps were set in Primer's Fissure. Seven Texas blind salamanders were observed; three of which were collected.
- 76 Peck's cave amphipods were collected from the Comal River and brough to SMARC.
- Quarantine racks were constructed at UNFH.
- Texas wild rice was repotted into the air pots at both SMARC and UNFH.
- SMARC prepared quarantine space for the San Marcos salamander collections.
- Biosecurity curtains were put in place in quarantine at SMARC for additional separation between the Comal Springs fountain darters permanently held in quarantine from any new collections going through quarantine.
- Repeated salt treatments for San Marcos and Comal Springs fountain darters resulted in very high overall survival; 85% and 95%, respectively.

- The collections for San Marcos salamander mark-recapture study concluded. All salamanders collected in May were retained for the Refugia.
- Dr. Shannon Brewer and Brian De La Torre revised the experimental tubes and control boxes and sent them to SMARC. Tubes and boxes were set up on a flow-through system to build up biofilm prior to starting the next trial.
- Library preparation is complete for the genetic assessment of San Marcos salamanders. Samples should be sent off for sequencing in June/July.
- Collections for the genetic assessment of Comal Springs riffle beetle and Peck's cave amphipod have concluded.

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for May 2024. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	--	--	0	162	0	34	27	410
Fountain darter: Comal	NT	NT	--	--	0	116	14	1641	44	492
Comal Springs riffle beetle	9	12	--	--	0	15	0	0	44	28
Comal Springs dryopid beetle	NT	NT	--	--	0	0	0	0	0	7
Peck's cave amphipod	86	NT	3	89	0	0	0	0	116	155
Edwards Aquifer diving beetle	NT	NT	--	--	0	--	--	--	--	--
Texas troglobitic water slater	NT	NT	--	--	0	--	--	--	--	--
Texas blind salamander	6	NT	7	13	0	0	1	1	97	60
San Marcos salamander	165	118	8	209	0	0	8	8	127	129
Comal Springs salamander	2	NT	0	2	8	0	1	5	53	75
Texas wild rice	NT	NT	--	0	0	0	0	4	155	200

Table 2. The number of tagged and recaptured San Marcos salamanders from each site each field day of the San Marcos salamander mark-recapture study. The number of untagged salamanders that were collected and released without tagging due to size restrictions or because tagging was completed is also reported.

Date	Site	# Tagged	# Recaptured	# Untagged	Total Capture
9-May-23	eastern spillway	82	0	5	87
10-May-23	diversion area	33	0	0	33
11-May-23	hotel area	53	0	8	61
30-May-23	eastern spillway	53	0	16	69
31-May-23	hotel area	22	0	0	22
12-Jun-23	eastern spillway	75	6	20	101
14-Jun-23	hotel area	74	6	25	105
20-Jun-23	diversion area	62	2	8	72
26-Jun-23	hotel area	0	9	21	30
27-Jun-23	eastern spillway	0	4	90	94
10-Jul-23	hotel area	0	3	19	22
12-Jul-23	diversion area	0	2	78	80
13-Jul-23	eastern spillway	0	4	53	57
8-Aug-23	eastern spillway	0	2	95	97
10-Aug-23	hotel area	0	3	54	57
22-Aug-23	hotel area	0	1	101	102
24-Aug-23	eastern spillway	0	0	108	108
6-Sep-23	diversion area	0	5	79	84
13-Sep-23	hotel area	0	3	23	26
14-Sep-23	eastern spillway	0	1	59	60
25-Sep-23	hotel area	0	0	51	51
27-Sep-23	eastern spillway	0	1	94	95
10-Oct-23	eastern spillway	0	3	145	148
11-Oct-23	diversion area	0	5	87	92
12-Oct-23	hotel area	0	1	43	44
23-Oct-23	hotel area	0	0	60	60
24-Oct-23	eastern spillway	0	1	104	105
8-Nov-23	diversion area	0	4	95	99
14-Nov-23	eastern spillway	0	2	90	92
16-Nov-23	hotel area	0	0	14	14
11-Dec-23	hotel area	0	0	8	8
12-Dec-23	eastern spillway	0	0	66	66
13-Dec-23	diversion area	0	5	84	89
3-Jan-24	hotel area	0	0	7	7

23-Jan-24	diversion area	0	2	55	57
7-Feb-24	hotel area	0	0	75	75
14-Feb-24	diversion	0	1	76	77
27-Feb-24	eastern spillway	0	0	74	74
29-Feb-24	hotel area	0	0	8	8
12-Mar-24	eastern spillway	0	0	39	39
13-Mar-24	diversion	0	2	51	53
14-Mar-24	hotel area	0	1	77	78
14-Mar-24	crater bottom	0	0	3	3
14-Mar-24	salt and pepper 1	0	0	1	1
14-Mar-24	salt and pepper 2	0	0	0	0
14-Mar-24	cabomba	0	0	0	0
25-Mar-24	hotel area	0	0	0	0
26-Mar-24	eastern spillway	0	0	19	19
9-Apr-24	hotel area	0	0	86	86
10-Apr-24	diversion area	0	1	100	101
11-Apr-24	eastern spillway	0	1	80	81
23-Apr-24	eastern spillway	0	0	42	42
24-Apr-24	hotel area	0	0	36	36
06-May-24	hotel area	0	0	42	42
08-May-24	diversion area	0	0	103	103
20-May-24	hotel area	0	0	195	195
21-May-24	eastern spillway	0	0	77	77

Figure 1. Dominique Alvear putting aiding snorkelers and divers with releasing San Marcos salamanders after scanning for p-Chip tags in a mark and recapture study.

Figure 2 Richelle Jackson and Daniela Cortez (USFWS Student Trainee) collected Peck's cave amphipods from the Spring Island area of the Comal River, New Braunfels TX.

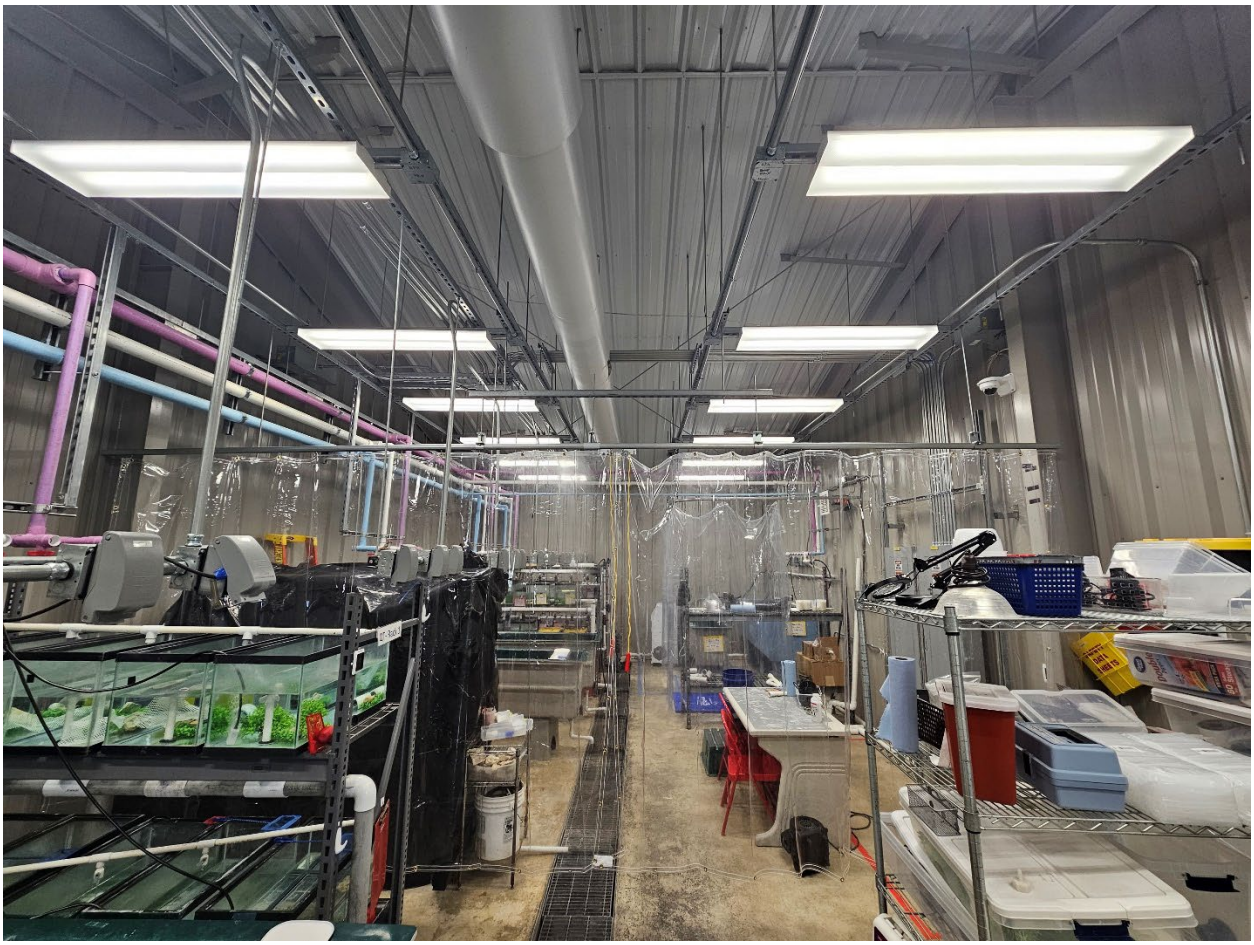

Figure 3 The SMARC quarantine space was subdivided using clear water-resistant curtains to increase biosecurity between groups of organisms.

Figure 4. Improved experimental tubes and control chambers for CSRB tagging.

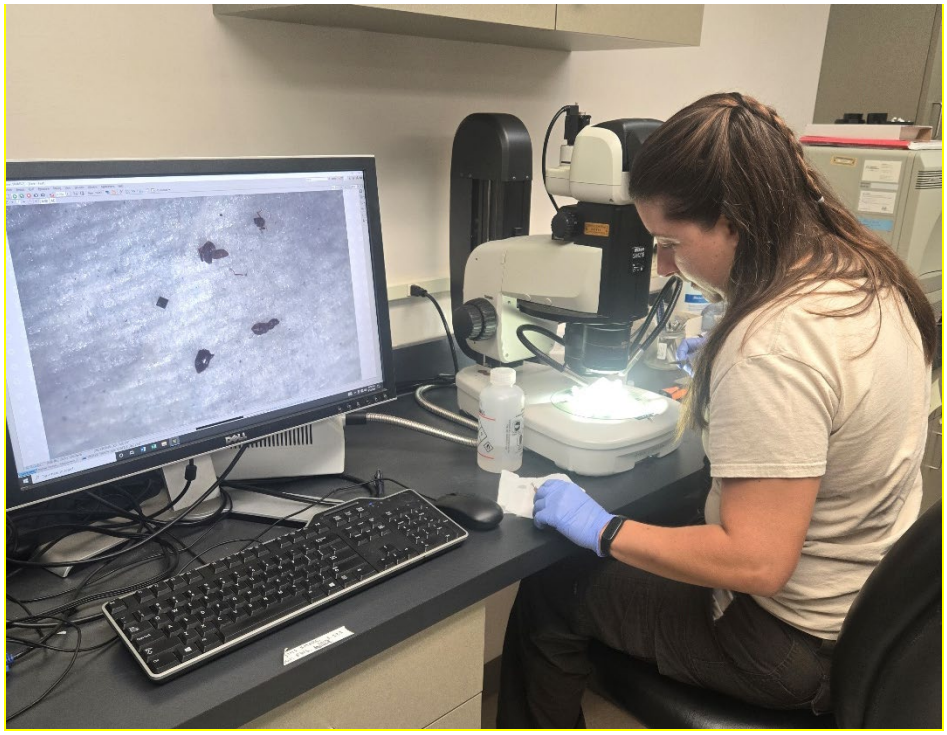

Figure 5. Dr. Kate Bockrath practicing applying p-Chip tags to preserved Comal Springs riffle beetles using methods developed in the first trial of the invertebrate tagging project.

Figure 6. Desiree Moore (Right) showing Regional Director, Amy Lueders (Left) Texas blind salamanders while discussing the EARP and Texas blind salamander biology.

June 2024 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dominique Alvear, Braden West and Dr. Katie Bockrath

With contributions from

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Task 1 Refugia Operations

Species Collection

On June 5, Dominique Alvear met Braden West and Dr. Matt Pintar (BIO-WEST) at the Comal Springs system to retrieve experimental lures consisting of stakes of conditioned wood and cellulose sponges. Alvear and West collected 12 Peck's cave amphipod (PCA), 35 Comal Springs riffle beetle (CSRB), and 5 Comal Springs dryopid beetle (CSDB). All animals were retained and taken back to the SMARC for refugia.

On June 18, Dr. Katie Bockrath, Richelle Jackson, Shawn Moore, and Braden West collected 70 PCA and 26 Comal Springs salamanders from the Spring Island area of New Braunfels, TX. All animals were retained and taken back to the SMARC for refugia.

On June 27, EARP staff Jackson, Heidi Meador, Moore, and Braden West were assisted by divers Justin Crow (SMARC), Thomas Funk, and Jacqualyn Halmbacher (both Inks Dam National Fish Hatchery) to collect Texas Wild rice plants from the San Marcos River. Ten plants were taken back to the Uvalde National Fish Hatchery and 30 to the San Marcos Aquatic Research Center.

Jackson and Moore continued to check the Diversion net twice weekly throughout the month of June. The net captured 15 San Marcos salamanders (SMS) and 6 larval Texas blind salamanders (TBS). Four of the six TBS were found dead on capture, and the remaining two were retained and taken back to the SMARC for refugia.

Husbandry

Uvalde

Alvear began preparing the Quarantine building for the summer collection portion of the fountain darter parasite and mortality study in addition to extra holding tanks in case of salvage being triggered.

Meador finished the annual task of repotting all the Texas Wild Rice!

Jonathan Donahey continued replumbing and constructing new controller boxes for use in the Refugia. Donahey also added a CO_2 line on an invertebrate rack to help with stabilizing the pH and help prevent calcium build up.

Alvear and Meador worked on replacing a pump on a Refugia tank. (Figure 1)

Alvear, Donahey and Meador did the annual "Clean the Mezzanine" on top of the Refugia, chillers were cleared of cobwebs and excess dirt to help the chillers from overheating during the

Summer.

Braden West visited the UNFH on June 26 to drop off new controller boxes that had been delivered to the SMARC and to touch base with the Uvlade crew on various aspects in the Refugia to ensure consistency across stations.

SMARC

Jackson and Moore continued tagging all Texas blind salamanders held in refugia, finishing an additional tank on June 6.

Jackson and Moore completed salamander inventories while simultaneously shuffling animals between tanks for deep cleaning.

West coordinated field work between USFWS and BIO-WEST to further boost refugia numbers at each facility.

West replaced critical Total Gas Pressure (TGP) sensors on the SMARC supersaturation diversion.

Jackson and Moore finished incorporating San Marcos River Fountain darters and Comal River Fountain darters after a 60-day observational quarantine. Both lots of fish exhibited 90-plus percent survival rates.

West installed new non-slip flooring mats in the SMARC greenhouse. West also worked with Juan Martinez (Facilities Maintenance Specialist, SMARC) to replace aging electrical hardware in the SMARC greenhouse.

The remaining 15 Walchem Intuition 9 water treatment controllers were delivered to the SMARC on June 17 (Figure 2).

Animal Health

Jackson and Moore finished collecting skin swabs from San Marcos salamanders held in the SMARC quarantine. Skin swabs were transferred to Erin Lowenberg (SCA) for DNA extraction and qPCR analysis.

The Southwestern Fish Health Unit (USFWS, Southwestern Native Aquatic Resources and Recovery Center) conducted their annual fish hatchery inspection at the SMARC on June 25. Fish Health Unit staff sacrificed 30 San Marcos River Fountain darters and 40 Comal River Fountain darters for parasite enumeration.

Task 2 Research

Dryopid Life History

Dr. Matt Pintar (BIO-WEST) deployed 20 small drift nets between Spring Island, the Western Shoreline, and Spring Run 3. In total, 33 sites were sampled and for over 500 trap-days. No dryopids or other endangered beetles were caught in nets, but 12 *Stygobromus* were found.

Dr. Pintar conducted collections from wood disks set at the Spring Island and Western Shoreline areas. Water levels dropped after initial placement and some disks were above the water line at the time of retrieval. At Spring Island, 13 wood disks produced 9 dryopids, 99 *Heterelmis*, and 11 *Stygobromus*. At the Western Shoreline a single disc remained below the water had 13 *Heterelmis* and one *Stygobromus*. All Western Shoreline and Spring Island disks were reset at deeper locations. New wood lures were constructed and set at Spring Island and Western Shoreline, with more to be set in the spring runs.

Dr. Pintar examined locations in the Upper Spring Run area where dryopids were previously found, but exact collection locations were either not flowing or did not seem suitable.

Six wood stakes in the Spring Island backwater area produced 1 dryopid, 179 *Heterelmis*, and 9 *Stygobromus*. Stakes were reset in the same locations.

San Marcos Salamander Mark and Recapture

Desiree Moore conducted a one-way ANOVA and post-hoc pairwise t-tests to determine differences in salamander size among sites. The results showed that salamanders at the Eastern Spillway site were larger than salamanders at the Diversion ($P = < 0.001$) and Hotel ($P = < 0.001$) sites. The salamanders collected at the Diversion site were larger than those collected at the Hotel site ($P < 0.001$).

Additionally, D. Moore conducted a chi-squared test to determine salamander sex ratio differences among sites. Sex ratios were not significantly different among sites ($X^2 = 1.050$, $P = 0.592$). Additionally, sex ratios did not significantly differ from an equal sex ratio ($X^2 = 1.4376$, $P = 0.6967$).

Reproductive Gene Expression in San Marcos Salamanders

Ruben Tovar (University of Texas Austin) extracted RNA from gonad tissues from adult male and female San Marcos salamanders, San Marcos salamander embryos and adult male and female Texas blind salamanders. RNA was quantified using a Quibit fluorometer and RNA is in quantities sufficient for sequencing.

Comal Springs Riffle Beetle Population Genetics

Dr. Katie Bockrath extracted DNA from all adult and larval beetles retained from recent field collections. Dr. Bockrath prepared all 253 beetles for sequencing by competing double restriction enzyme cuts to generate consistent sequencing sites, individually barcoded all genetic samples for unique identification in downstream bioinformatic analysis, and PCR amplified genetic products to ensure adequate genetic target material is present for sequencing. The samples will be sent off for size selection and sequencing in July/August.

Tagging Aquatic Invertebrates

Dr. Shannon Brewer and Brian De La Torre (Auburn University) visited the San Marcos Aquatic Resources Center to set up a second tagging trial using the upgraded experimental tubes and control chambers (Figure 3). *Heterelmis glabra* were tagged with p-chip transponders and placed in one of four experimental tubes and one control chamber. Tubes were checked weekly to ensure water temperature and flow were within optimal range. The directional flow through the tubes was switched each week to encourage movement across the scanning laser located between the two halves of the experimental tubes. The control beetles were inventoried weekly for survival. Temperature and flow were monitored. Movement data from the experimental tubes was downloaded weekly and sent to Dr. Brewer and De La Torre. Beetles continue to move and are successfully scanned by the p-chip scanning laser.

De La Torre and Dr. Bockrath collected H*yalella* from the Comal Springs system to test an alternative to p-chips. Using the same methods to glue p-chips to CSRB, De La Torre glued QR codes printed on write in the rain paper to *hyalella* to see if they would be a viable option for PCA tagging and an alternative to p-chips. The tagged *hyalella* did not appear to be impacted by the QR code and continued to move around their enclosure as if they were not tagged.

Genetic Assessment of Peck's Cave Amphipod

No significant updates to report.

Genetic Assessment of Texas Blind Salamanders

Erin Lowenberg (Student Conservation Association) pulled Texas blind mortalities and tail clips from the tissue archive and extracted DNA from these tissues. West and Alvear gathered the tail clips and mortalities at Uvalde National Fish Hatchery and brought them back to San Marcos Aquatic Resources center for DNA extraction.

Genetic Assessment of San Marcos Salamanders

Dr. Bockrath sent off the sequencing library (total DNA) for size selection to Dr. Nathan Whelan's (USFWS) lab at Auburn University. The DNA size range of the sequencing library was selected to be between 300-450 base pairs for targeted high-quality sequencing. Size selection was very successful and quality control checks confirmed the library was selected to the target range. Dr. Bockrath received the size selected sequencing library from Auburn University and will send it off for sequencing in July/August.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss collections, husbandry, and ongoing research.

SMARC staff hosted Dr. Chad Furl and Kristy Smith (Edwards Aquifer Authority) for the Quarter 2 meeting in 2024.

Summary of June Activities

- Remaining water quality controllers delivered and controller boxes continue to be constructed and plumbed into the Refugia spaces.
- Fountain darter survival after a 60-day quarantine period increased to 90+%.
- EARP staff collected CSRB and PCA from BIO-WEST experimental lures.
- Texas wild rice was repotted at UNFH and non-slip flooring was installed in the greenhouse at SMARC.
- SMARC had their annual fish health inspection.
- Sequencing libraries were completed for the San Marcos salamander and Comal Springs riffle beetle genetic assessments.
- Experimental lures (BIO-WEST) continue to attract CSRB, PCA and dryopid beetles.
- The second invertebrate tagging trial is under way and QR codes were glued to *Hyalella* sp. to test as an alternative tagging for PCA and CSRB.
- EARP staff met with EAA for Quarter 2 meeting at the SMARC.

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for June 2024. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	--	--	171	0	17	34	198	376
Fountain darter: Comal	NT	NT	--	--	409	0	12	5	453	487
Comal Springs riffle beetle	44	12	--	56	0	0	0	0	53	16
Comal Springs dryopid beetle	5	NT	--	--	0	0	0	0	0	7
Peck's cave amphipod	82	NT	7	89	0	0	0	0	126	155
Edwards Aquifer diving beetle	NT	NT	--	--	0	--	--	--	--	--
Texas troglobitic water slater	NT	NT	--	--	0	--	--	--	--	--
Texas blind salamander	2	NT	4	6	0	0	0	0	99	60
San Marcos salamander	0	NT	27	27	0	84	11	11	116	202
Comal Springs salamander	26	NT	1	27	0	0	5	0	48	75
Texas wild rice	30	10	--	40	12	11	0	0	167	211

Figure 1. Dominique Alvear (left) and Heidi Meador (right) wiring and preparing a new pump to be replaced on a Refugia tank.

Figure 2 Garrison Engstrom (Student Conservation Association intern) assisted EARP staff by unloading the shipment of Walchem controllers.

Figure 3. Dr. Shannon Brewer and Brian De La Torre set up the invertebrate tagging trial. Experimental tubes with p-chip scanners are placed on a flow through system. P-chip scanners are placed above the scanning chamber of each experimental tube. Data is automatically collected as tagged beetles move across the scanning chamber.

July 2024 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dominique Alvear, Braden West and Dr. Katie Bockrath

With contributions from

Jonathan Donahey, Richelle Jackson and Shawn Moore

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Staffing Updates

The Edwards Aquifer Refugia Program said goodbye to three of our team members in July. Desiree Moore took a Biologist position with the Devil's Hole pupfish program at US Fish and Wildlife Service Ash Meadows National Refuge in Amargosa Valley, Nevada. Heidi Meador accepted a Biological Science Technician position with the Southwestern Native Aquatic Resources and Recovery Center in Dexter, New Mexico. Although part of "Base" staff at the UNFH, Nicholas Yvon assisted the EARP when an extra set of hands was needed during collections or daily husbandry duties when staff was short. Yvon accepted a position with the Maine Department of Inland Fisheries & Wildlife working with Brook Trout. We wish them all the best of luck in their new ventures and thank them for their hard work and dedication to the US Fish and Wildlife Service and the Edwards Aquifer Refugia Program.

Task 1 Refugia Operations

Species Collection

Jackson and Moore continued to routinely monitor the drift net over Diversion Spring in Spring Lake, San Marcos, TX. Two Texas blind salamanders were retained for the Refugia and all San Marcos salamanders were too young for collection and were released.

On July 2 and July 8, Dominique Alvear, Shawn Moore and Braden West assisted Dr. Matt Pintar (BIO-WEST) in retrieving experimental lures from the Comal Springs System. 107 Comal Springs riffle beetles and 13 Comal Springs Dryopid beetles were retained for incorporation into Refugia.

On July 8, Alvear, Nicholas Yvon, Heidi Meador and Jonathan Donahey worked in teams to begin the summer fountain darter collection. Alvear and Yvon sampled at Spring Island where 101 Comal Springs fountain darters were collected. Donahey and Meador sampled the Old Channel where 101 Comal Springs fountain darters were collected. On July 9, Alvear, Donahey and Yvon sampled at Llanda Lake where 99 darters were collected. All darters collected on July 8 and 9 were taken to the Uvalde National Fish Hatchery for the Refugia standing stock population. The darters were monitored for mortality and necropsies performed on mortalities for parasite inspection and enumeration.

On July 24, Dr. Katie Bockrath, Richelle Jackson, Shawn Moore and Alvear collected Comal Springs salamander and Peck's Cave amphipods at Spring Island. Twenty Comal Springs salamanders were captured via dip net along with 85 Peck's Cave amphipods. All salamanders and amphipods were taken to the Uvalde National Fish Hatchery for incorporation into Refugia.

Husbandry

SMARC

Shawn Moore closely monitored San Marcos Fountain darters moved from the quarantine into the Refugia population. There were a small number of mortalities following transfer to the refugia. Moore worked with Richelle Jackson to apply a 3% salt treatment and increased live feed habitat cover to mitigate stress. The darters stabilized within three weeks.

West worked with Juan Martinez (Facilities Specialist, SMARC) to troubleshoot ongoing electrical issues in the SMARC greenhouse. Martinez guided West in replacing a GFCI circuit breaker. West also replaced a variable speed pump serving tank 3.

West worked closely with USFWS Information Resources and Technology Resources (IRTM) to implement wireless internet connectivity to the Whalchem Controllers at SMARC. West installed both the router and network switch in the SMARC EARP building and started running the cable from the server closet to the Refugia.

West began training Jackson and Moore in the construction of accessory boxes for the Whalchem controllers in the Refugia. Jackson and Moore began construction of a box while following along with West on the construction of a second box (Figure 1). West used this time to proof the Walchem controller and accessory box construction and programming SOP.

Uvalde

Jon Donahey and Dominique Alvear were kept busy with plenty of chiller maintenance and replacements, as well as tank construction in the Refugia and in the Quarantine buildings.

Alvear monitored the summer fountain darter collection for the Refugia and performed necropsies to assess parasite presence and load as mortalities occurred.

Donahey administered a salt treatment bath for a fountain darter tank in the Refugia that had a spike in mortality after the fish were moved from Quarantine to the Refugia.

Animal Health

Jackson and Moore collected skin swabs from Comal Springs salamanders collected in June. Samples were provided to Erin Lowenberg for analysis and Bd testing.

Jackson, Moore, and West collected and transported skin swabs from multiple lots of salamanders held in the refugia for the Southwestern Fish Health Unit's annual fish hatchery inspection.

Task 2 Research

Dryopid Life History

All remaining drift nets deployed in the Comal Springs system were removed in early July. No Comal Springs dryopid beetles (CSDB) were found in any of the deployed drift nets.

BIO-WEST and EARP staff retrieved 5 wood stakes and 36 wood disks from the Spring Island and Western Shoreline area. Comal Springs riffle beetles (CSRB), Peck's cave amphipods (PCA) and CSDB were collected from each location and lure type. CSRB and CSDB were retained for incorporation into the Refugia population. Wood disks and stakes were reset at Spring Island and Western Shore. The type of lure, number of lures, and number of organisms collected from each lure type and location are detailed below.

Location	Lure Type	# Lures	# CSRB Adults	# CSRB Larvae	# CSDB	# PCA
Western Shore	Wood Disks	16	46	2	1	1
Spring Island	Wood disks	20	153	35	12	10
Spring Island	Wood Stakes	5	10	8	0	1

Matt Pintar (BIO-WEST) set wood disks, stakes and cotton lures across locations in the Comal Springs system.

Five disks were set at Spring Run 1, four disks were set at Spring run 2, and twelve disks were set at Spring Run 3. Three wood stakes were set in silty backwaters round Spring Island to identify potential dryopid sampling location. A lack of flow prevented wood stakes from being set in the Upper Spring Run area.

To compare the efficacy of the disks and stakes to the historically used cotton lures, six cotton lures were set at Spring run 3, five were set at Western Shore and 10 cotton lures were set at Spring Island.

San Marcos Salamander Mark and Recapture

Desiree Moore began writing the report for the mark and recapture research project. One-way ANOVA and post-hoc pairwise t-tests were used to determine differences in salamander size among sites. A chi-squared test was used to determine differences in sex ratio among sites. Final recapture rates using p-Chips was determined for each site. Salamander length was different

among all sites with Eastern spillway having the largest salamanders and hotel having the smallest. Sex ratios did not significantly differ from equal and were not significantly different among sites. Total recapture rate across sites was 14%. Recapture rate at Diversion was 21%, Hotel was 15% and Eastern Spillway was 10%.

Reproductive Gene Expression in San Marcos Salamanders

Ruben Tovar (University of Texas, Austin) continued to isolate RNA from tissues collected from male and female San Marcos salamanders.

Comal Springs Riffle Beetle Population Genetics

Dr. Katie Bockrath ran quality control checks on the completed sequencing library to confirm the protocol was successful. Dr. Bockrath coordinated sending the sample other USFWS labs for size selection and sequencing.

Tagging Aquatic Invertebrates

Dr. Bockrath and Randy Gibson (SMARC) collected 200 *Heterelmis glabra* from Finnigan Springs to serve as surrogate species for the Comal Spring riffle beetle during the tagging trials. Dr. Bockrath coordinated dates with Dr. Shannon Brewer and David De La Torre for visiting the SMARC and setting up the next round of tagging trials. The second tagging trial is ongoing. Flows and temperatures for the control and experimental chambers were checked weekly. Experimental tubes were flipped weekly to encourage movement. Movement data was sent to Dr. Brewer and De La Torre weekly.

Genetic Assessment of Peck's Cave Amphipod

No significant updates to report.

Genetic Assessment of Texas Blind Salamanders

No significant updates to report.

Genetic Assessment of San Marcos Salamanders

Dr. Bockrath received the size selected sequencing library from the USFWS genetics lab at Auburn University, who completed the size selection protocol. Dr. Bockrath quality control checked the size selected sequencing library to confirm it was size selected to the target range and to quantify the final library prior to sending the library off to the USFWS Midwest Fisheries Center Whitney Genetics Lab (WGL) for sequencing. The library is as expected and was sent to WGL for sequencing.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss collections, husbandry, and ongoing research.

EARP staff met with John Bogess (Edwards Aquifer Authority) to participate in an interview covering the EARP staff's USFWS Region 2 Team of the Year Award (Figure 2).

EARP staff at SMARC provided a tour of the Refugia to Edwards Aquifer Authority staff and interns on July 10 and July 29.

Summary of July Activities

- The EARP lost three team members in July: Desiree Moore, Heidi Meador and Nicholas Yvon.
- The drift net over Diversion Spring was routinely monitored. Two Texas blind salamanders were retained for the Refugia.
- 301 fountain darters were collected form the Comal Springs system for the summer refugia collection. Collections occurred at Spring Island, Old Channel and Landa Lake. The fish were monitored, and mortalities were necropsied for parasite and general inspection.
- EARP staff collected riffle beetles and dryopid beetles from experimental lures set out by BIO-WEST. 107 riffle beetles and 13 dryopid beetles were retained for the Refugia.
- EARP staff collected 20 Comal Springs salamanders and 85 Peck's Cave amphipods from the Spring Island area for the Refugia.
- EARP staff repaired electrical issues in the SMARC greenhouse, replaced chillers in the Uvalde Refugia, and build tank systems in the Uvalde Quarantine.
- The report for the San Marcos salamander mark and recapture project has been drafted. Additional analyses are need.
- Ruben Tovar continues to isolate RNA from San Marcos salamander reproductive tissues.
- 200 *Heterelmis glabra* were collected from Finnigan Springs for the invertebrate tagging project. The ongoing trial was monitored, and data sent to Dr. Brewer weekly.
- The San Marcos salamander RadSeq library was sent off for sequencing for the population genetic assessment project.
- The Comal Spring riffle beetle RadSeq library (population genetic assessment project) was quality checked prior to sending off for size selection and sequencing.
- EARP staff met with John Bogess and participated in an interview for the EAHCP Stewardship Newsletter.
- EARP staff provided tours to EAA staff and interns.

Tables and Figures

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for July 2024. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	--	--	0	0	48	22	150	354
Fountain darter: Comal	0	301	0	301	0	0	9	5	442	482
Comal Springs riffle beetle	67	40	0	107	0	0	9	0	44	16
Comal Springs dryopid beetle	1	12	0	--	5	0	0	0	7	7
Peck's cave amphipod	0	85	7	92	102	0	7	0	221	155
Edwards Aquifer diving beetle	NT	NT	--	--	--	--	--	--	--	--
Texas troglobitic water slater	NT	NT	--	--	--	--	--	--	--	--
Texas blind salamander	2	0	4	2	2	0	1	0	100	60
San Marcos salamander	0	0	29	29	159	0	3	4	272	198
Comal Springs salamander	0	20	0	20	25	0	0	1	73	74
Texas wild rice	NT	NT	--	--	0	6	0	0	167	217

Figure 1. Richelle Jackson (left), Shawn Moore (middle), and Braden West (right) assembling accessory boxes for the SMARC refugia. Photo credit: Dr. Katie Bockrath, USFWS.

Figure 2. The Edwards Aquifer Refugia Program team at the Uvalde National Fish Hatchery Edwards Aquifer Refugia. Back row from left to right is Robert Quinones, Nicholas Yvon, Dr. David Britton, Dr. Scott Walker and Braden West. Bottom row from left to right is Heidi Dunn, Jon Donahey, Dr. Katie Bockrath, Richelle Jackson, Shawn Moore and Dominique Alvear. Photo credit: John Bogess, Edwards Aquifer Authority.

August 2024 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dominique Alvear, Braden West and Dr. Katie Bockrath

With contributions from

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Staffing Updates

Jonathan Donahey said farewell to the Uvalde National Fish Hatchery (UNFH) on August 30th. Donahey accepted a technician position at the London State Fish Hatchery in London, Ohio. We will greatly miss Jonathan and appreciate the time he had at the UNFH.

Task 1 Refugia Operations

Species Collection

The Summer San Marcos fountain darter collections took place August 12 thru the 14. Dominique Alvear and Donahey collected fountain darters from three locations in the San Marcos River and brought them back to the UNFH. One hundred darters were collected from below Spring Lake Dam, ten of those darters were sent to fish health for testing. Seventy-nine darters were collected from the Rio Vista Dam, ten of those darters were sent to fish health for testing. Forty-nine darters were collected from Spring Lake.

Richelle Jackson and Shawn Moore set Texas blind salamander traps in Primer's Fissure and Johnson's Well on August 21. Jackson and Moore checked the traps on Mondays, Wednesdays, and Fridays for two weeks until the end of August. The traps were removed on September 3. Three Texas blind salamanders were captured between both wells. One was retained for refugia and the remaining two were returned to their respective capture locations.

Jackson, Moore, and Garrison Engstrom (Student Conservation Association) checked the Diversion Springs net twice weekly in August (Figure 1). A total of 27 San Marcos salamanders were captured. One San Marcos salamander was retained for refugia, 26 were released. One Texas blind salamander was captured in the net and released.

Husbandry

SMARC

Braden West completed the installation and troubleshooting of the SMARC EARP network switch for the automatic notification of water quality to EARP staff.

West continued training Jackson and Moore on the construction of controller accessory boxes. Staff worked weekly to move through the construction protocol.

West brought all invertebrate inventories up to date.

A Comal Springs fountain darter mortality event occurred in August, where 246 Comal Springs

fountain darters died (55% loss). The cause of the mortality event is a suspected fungal infection that evaded detection until mortalities occurred. Upon discovery, fish were immediately removed from their tanks and placed in new tanks for a formalin and salt treatments in attempts to prevent further mortalities. SMARC staff plan to send some of the mortalities to the USFWS Fish Health Unit for inspection and guidance on future prevention and treatment.

Uvalde

In preparation for Donahey's departure, time was spent finishing tank construction in various systems in the Refugia and Quarantine systems. Alvear assisted in the replacing multiple chillers throughout the month.

Task 2 Research

Dryopid Life History

Dr. Matt Pintar (BIO-WEST) continued testing and refining techniques to detect and collect drypoid beetles and other invertebrates. Both wood disks and wood stakes have continued to be very productive in the Spring Island area with 20 dryopid beetle adults and >300 Comal Springs riffle beetle adults and larvae collected in August. Wood have not always remained in springs and are generally more difficult to manage than the wood disks, thus wood stake testing will continue into September. Dr. Pintar finished the second monthly survey of wood disks from the Western Shoreline and the first survey of wood disks set in Spring Runs 1, 2, and 3. The numbers of invertebrates found on wood disks in those areas during August were much lower (31 Comal Spring riffle beetle adults and larvae), but flows were also low, and the number of available sites was limited. Four wood disks were set in the Upper Spring Run area; available sites were very limited there due to lack of flow. Lastly, Dr. Pintar completed an initial paired comparison of wood disks versus cotton lure efficacy within 16 springs that showed no statistical difference in Comal Springs riffle beetle numbers between methods, but all dryopids were found on wood disks. This comparison is being repeated in September.

San Marcos Salamander Mark and Recapture

Marina Draeger was selected to fill the Student Conservation Association internship. Draeger processed most of the photos taken of the San Marcos salamanders initially collected and tagged for the mark and recapture study. Draeger clipped photos to show only the heads from snout to just behind the gills. Draeger downloaded Wild.ID and was able to upload photos of salamanders from the mark and recapture events. Wild.ID is a program used to assess recapture rates for trail cam images and images of animals with unique markings. Photos have been used to mark and recapture Barton Springs salamanders, but using photos to for mark and recapture assessment in

San Marcos salamanders is untested and it is not known if San Marcos salamanders maintain unique markings on their head. A test was run using a duplicate salamander photo to confirm that the software can identify a salamander based on the markings on their head. Marina is processing photos from a collection date that had a known p-chip recapture and this dataset will be tested in Wild.ID to test if the program can identify a recapture with a known dataset.

Reproductive Gene Expression in San Marcos Salamanders

No significant updates.

Comal Springs Riffle Beetle Population Genetics

Dr. Katie Bockrath sent the samples off for size selection to the Auburn University, USFWS Genetics Lab. Austin Hannah (USFWS) size selected the genetic sample to 340-400 bps and sent the size selected sample to Zeb Woiak at the USFWS Midwest Fisheries Center, Whitney Genetics Lab for sequencing. Dr. Bockrath submitted sequencing datasheets to Woiak and sequencing is scheduled to occur mid-September.

Tagging Aquatic Invertebrates

Dr. Bockrath collected 200 *Heterelmis glabra* from the Devils River and brough them back to the SMARC. *H. glabra* has served as the surrogate species for *H. comalensis* for this tagging study. The second tagging trial is ongoing. Dr. Bockrath checked flows and temperatures for the control and experimental chambers weekly. Experimental tubes were flipped weekly to encourage movement. Movement data was sent to Dr. Brewer and De La Torre (Auburn University).

Genetic Assessment of Peck's Cave Amphipod

No significant updates to report.

Genetic Assessment of Texas Blind Salamanders

Erin Lowenberg (Student Conservation Association) continued to extract DNA from preserved mortalities. Lowenberg worked with Moore and Jackson to take tail clips of Fx Texas blind salamanders and extracted DNA from those samples.

Genetic Assessment of San Marcos Salamanders

Dr. Bockrath sent sequencing datasheets to Zeb Woiak (USFWS) at the Midwest Fisheries Center, Whiney Genetics Lab. Sequencing is scheduled for mid-September.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

All EARP staff meet weekly to discuss husbandry updates, collections (previous and upcoming), and research updates.

Dr. Bockrath met with Dr. Tim Bonner (Meadows Center) to discuss fountain darter collections at Spring Lake.

Summary of August Activities

- Jonathan Donahey left the EARP team.
- 231 San Marcos fountain darters were collected from three locations in the San Marcos River.
- Texas blind salamanders were collected from Primer's Fissure and Johnson's Well
- The Diversion net was checked twice weekly and both San Marcos and Texas blind salamanders were collected.
- When comparing cotton lures and the wood disks, there is not difference in the number of CSRB collected between the lure types, but dryopid beetles were only found on wood disks.
- Photo editing of tagged and untagged San Marcos salamanders continues and Wild.ID photo recognition testing is underway.
- Sequencing for the CSRB, PCA and SMS genetic assessments are in progress.
- DNA extractions for TBS genetic assessment continues.
- 200 *Heterelmis glabra* were returned to the SMARC for additional invertebrate tagging tirals.

Tables and Figures

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for August 2024. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census	Total Program Census
Fountain darter: San Marcos	NT	231	--	231	0	0	36	14	114	340	454
Fountain darter: Comal	NT	NT	0	301	0	0	246	0	196	482	678
Comal Springs riffle beetle	248	NT	246	494	24	20	0	0	68	36	104
Comal Springs dryopid beetle	19	NT	1	20	3	23	0	0	3	30	33
Peck's cave amphipod	6	NT	--	6	0	52	33	0	188	207	395
Edwards Aquifer diving beetle	NT	NT	--	--	--	--	--	--	--	--	--
Texas troglobitic water slater	NT	NT	--	--	--	--	--	--	--	--	--
Texas blind salamander	1	NT	3	4	0	0	0	0	100	60	160
San Marcos salamander	1	NT	26	27	0	0	21	7	251	191	442
Comal Springs salamander	NT	NT	0	20	0	0	1	1	72	81	153
Texas wild rice	NT	NT	--	--	30	0	3	10	194	207	401

Figure 1. Garrison Engstrom (SCA) assisted EARP staff in checking the Diversion Spring net in August.

September 2024 Monthly Activity Report:

Edwards Aquifer Refugia Program

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Staffing Updates

Interviews were held to hire a SCA Intern to be stationed at the Uvalde National Fish Hatchery (UNFH). A selection was made, and the candidate will start mid-November.

Candidates were selected for interviews to fill the Vice-Adam Husbandry and Collections Lead position stationed at Uvalde. There were 11 qualified applicants and interviews were extended to four candidates.

Task 1 Refugia Operations

Species Collection

In collaboration with Dr. Matt Pintar (BIO-WEST, Inc.), Braden West collected 233 Comal Springs riffle beetles (CSRB), 13 Comal Springs dryopid beetles (CSDB), and 16 Peck's cave amphipods (PCA) from September 9-11. Of the 233 CSRB, West retained 137 adult beetles. Of the 13 CSDB, West retained all but one, a field mortality. Of the 16 PCA, West retained 13. All retained organisms were transported to the San Marcos Aquatic Resources Center (SMARC) for quarantine and incorporation into the Refugia.

On September 18, Alvear and Moore traveled from the UNFH to meet West and Jackson, and Erin Lowenberg (Student Conservation Association) at Spring Island, New Braunfels, TX. Sixty-eight PCA and eleven Comal Springs salamanders (CSS) were collected. All PCA were returned to the UNFH and all CSS were returned to the SMARC for quarantine and incorporation into Refugia at their respective facilities.

Jackson and Moore continued to routinely monitor the Diversion Springs drift net through September. A total of 17 juvenile San Marcos salamanders were collected throughout the month. All salamanders were released at their designated release site in Spring Lake.

Husbandry

SMARC

On September 25 the SMARC EARP received a shipment of live blackworms. Staff reintroduced blackworms as a food source for Fountain darters, following extended supply chain issues with the manufacturer. Moore washed the worms thoroughly and introduced the worms to the new culture tank (Figure 1). Moore placed half PVC pieces in the culture tank to attract and remove flatworms (bycatch from the supplier) from the system, curtailing competition between blackworms and flatworms. On September 30 Moore and Jackson added more pea gravel substrate to the system and fed the worms spirulina sinking tablets.

West began pilot trials on the enrichment of artemia nauplii at the end of September in order to supplement fatty acid intake of juvenile fish to boost long-term survival and development. West used established protocols to culture the artemia nauplii for 24 hours, then created a 1% solution of artemia nauplii and concentrated enrichment liquid. After rinsing excess enrichment from the artemia, West fed the solution to one tank of captive-produced Fountain Darters. No deleterious effects were observed.

On September 11, Moore p-chipped and tail clipped 9 Texas Blind salamanders for the Texas Blind salamander genetic assessment and moved them to a clean tank. Moore continued monitoring the salamanders closely the following week.

Uvalde

On September 9, Dominique Alvear incorporated 145 Comal Spring fountain darters from the Spring seasonal darter collection.

Shawn Moore traveled and stayed at the Uvalde National Fish Hatchery to assist Alvear the week of September 16. Alvear and Moore moved Texas Wild Rice from tank 11 to tank 14 as the algae had become hard to manage. Moore then power washed tank 11 to remove the remaining debris.

Task 2 Research

Dryopid Life History

Dr. Matt Pintar (BIO-WEST) continued monthly monitoring and testing of methods used to detect and collect *Stygoparnus*. Dr. Pintar completed the final month of testing wood stakes in the Spring Island backwaters. The 8 stakes were removed. At some sites stakes were replaced with variations of the wood disk method.

In September, wood stakes produced:

- 45 *Heterelmis* larvae,
- 4 *Heterelmis* adults, and
- 1 *Stygobromus.*

Wood stakes have proven more difficult to manage (sometimes they float away) and produce mostly *Heterelmis* larvae. Wood discs were checked and reset in the same locations with some slight modifications to continue testing designs. In total, 66 *Stygoparnus* have been found since early June.

Additionally, 20 cotton lures were set in springs adjacent to wood discs to repeat a comparison of lure effectiveness (initial study was July to August).

Wood Disk Collections in September					
Location	# Disks	# CSRB Adults	# CSRB Larvae	# CSDB	# PCA
Spring Island	22	200	180	8	19
Spring Run 1	3	0	0	0	0
Spring Run 2	5	30	17	0	0
Spring Run 3	9	2	13	0	1
Upper Spring Run	3	0	0	0	0
Western Shore	15	35	4	2	0
Total	57	267	214	10	20

San Marcos Salamander Mark and Recapture

Physical markings on the heads of aquatic salamanders have been used as "tags" to identify individual salamanders during mark and recapture studies. The EARP took pictures of all tagged and collected San Marcos salamanders during the p-chip mark and recapture study to compare efficacy of photos and p-chips as tagging methods. The p-chip recapture data has been analyzed and we are in the process of analyzing the photos, which require cropping and organizing. Marina Draeger (Student Conservation Association) continued to crop photos for analysis using Wild-ID.

Reproductive Gene Expression in San Marcos Salamanders

Ruben Tovar (University of Texas, Austin) submitted all samples for RNA sequencing and is currently awaiting data from the genomics facility.

Comal Springs Riffle Beetle Population Genetics

Samples were sequenced at USFWS Midwest Fisheries Center, Whitney Genetics Lab. Approximately 900 million reads were sequenced across two sequencing runs. Sequence quality was very high with over 95% of the sequence reads being of the highest sequencing quality (Q score >30).

Tagging Aquatic Invertebrates

The second tagging trial is ongoing. Dr. Bockrath checked flows and temperatures for the control and experimental chambers weekly. Experimental tubes were flipped weekly to encourage movement. Movement data was sent to Dr. Brewer and De La Torre (Auburn University) for analysis.

Hurricane Helene further delayed the start of the third trial. Dr. Brewer and Del La Torre are scheduled to visit SMARC to start the third trial in early October.

Genetic Assessment of Peck's Cave Amphipod

All PCA have been transferred from USFWS to Dr. Chris Nice (Texas State University). Dr. Nice is preparing the sample for sequencing. Genetic Assessment of Texas Blind Salamanders

Genetic Assessment of Texas Blind Salamanders

Erin Lowenberg continued to extract DNA from preserved mortalities. Lowenberg worked with Moore and Jackson to take tail clips of Fx Texas blind salamanders and extracted DNA from those samples.

Genetic Assessment of San Marcos Salamanders

Lowenberg extracted DNA from preserved San Marcos salamander mortalities.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Dr. Bockrath worked on 2024 research reports.

Task 6 Meetings and Presentations

All EARP staff meet weekly to discuss husbandry updates, collections (previous and upcoming), and research updates.

EARP staff had a quarterly meeting with Kristy Smith and Dr. Chad Furl to discuss 2024 research progress and 2025 research plans.

Summary of September Activities

Hiring:

- Student Conservation Association husbandry intern selected for Uvalde. Intern will start in mid-November.
- 3 candidates were interviewed to fill the Husbandry and Collections lead position at Uvalde.

Collections:

- 137 adult Comal Springs riffle beetles
- 13 Comal Springs dryopid beetles
- 81 Peck's cave amphipod
- 11 Comal Springs salamanders

Husbandry:

- The EARP was able to get black worms again and started a black worm culture tank.
- Artemia was enriched with lipids to improve nutritional value of food items offered to fountain darters.
- 9 wild stock Texas blind salamanders were p-chipped and tail clipped. Tail clips will be used for a genetic assessment in 2025.
- 145 Comal spring fountain darters were incorporated into the refugia at Uvalde
- Moore spent a week at Uvalde helping out while they are short staffed.

Research:

- Photos continue to be processed for the San Marcos salamander mark and recapture study.
- Dr. Matt Pintar continued to collect drypoid beetles using conditioned wood disks. Wood stakes remain less effective as wood disks.
- DNA sequence data was returned and at a very high quality for the Comal Spring riffle beetle genetic assessment. Dr. Bockrath is working on processing the data.
- DNA sequencing is ongoing for:
    - San Marcos salamander genetic assessment
    - Peck's cave amphipod genetic assessment
    - Reproductive gene expression assessment
- Tails clips are being collected for the Texas blind salamander genetic assessment.
- Hurricane Helene further delayed the start of the third invertebrate tagging trail. Dr. Brewer plans to visit SMARC at the beginning of October.

Tables and Figures

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for September 2024. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census	Total Incorporated Program Census
Fountain darter: San Marcos	NT	NT	-	0	0	0	8	11	106	329	435
Fountain darter: Comal	NT	NT	-	0	0	145	41	7	175	620	795
Comal Springs riffle beetle	137	NT	96	233	0	0	NA	NA	68	36	104
Comal Springs dryopid beetle	13	NT	0	13	0	0	NA	NA	3	30	33
Peck's cave amphipod	13	68	3	84	0	0	14	NA	174	207	381
Edwards Aquifer diving beetle	NT	NT	-	0	0	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	-	0	0	0	0	0	0	0	0
Texas blind salamander	NT	NT	-	0	3	0	0	1	103	59	162
San Marcos salamander	17	NT	17	17	0	0	10	8	246	183	429
Comal Springs salamander	11	NT	0	11	0	0	1	2	71	79	150
Texas wild rice	NT	NT	-	0	0	0	4	15	190	192	382

Figure 1. Shawn Moore and Erin Lowenberg assisted in washing blackworms to remove waste and bycatch. Justin Crow (Herpetologist, SMARC) and Marina Draeger (SCA) supervised.

October 2024 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear and Dr. Katie Bockrath

With contributions from

Richelle Jackson and Shawn Moore

San Marcos Aquatic Resources Center
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Task 1 Refugia Operations

Species Collection

Edwards Aquifer Refugia Program (EARP) staff continued to monitor the drift net over Diversion Springs in Spring Lake. Eleven San Marcos salamanders were captured in the net during October. Of the eleven captured individuals, ten were released at a pre-designated release point. One individual was retained and returned to the SMARC for incorporation into the refugia population.

On October 31, the Diversion Springs drift net was found to be slumped against the bottom of Spring Lake. Staff observed minimal flow and no observable sediment disturbance. Following a discussion with Texas State University/Meadows Center staff, the EARP made the decision to remove the collection cup from the drift net. Removal of the collection cup will allow staff to make necessary repairs to the net.

During the weeks of October 7 and October 14, EARP staff from both the SMARC and UNFH traveled to six different sites in the San Marcos and Comal Rivers as part of seasonal Fountain darter collections. In total, 311 San Marcos River Fountain darters and 130 Comal River Fountain darters were retained for quarantine and incorporation at the SMARC. Please see the below table for a site-by-site breakdown:

Table 1. Collection location and numbers for San Marcos and Comal Springs fountain darters. "Collected" is the number of individuals captured. "Retained" is the number of individuals brought back to the Refugia. Totals for each river system are in bold.

Site	Collected	Retained
San Marcos River – Spring Lake	98	98
San Marcos River – Eastern Spillway	124	104
San Marcos River – William & Eleanor Crook Park	110	109
Comal River – Spring Island	119	104
Comal River – Old Channel	11	11
Comal River – Landa Lake	15	15
San Marcos River - Total	**332**	**311**
Comal River - Total	**145**	**130**

Jackson, Moore, and West conducted a field collection of San Marcos salamander from the eastern spillway of the San Marcos River on October 2 (Figure 1). EARP staff were joined by Kristina Tolman (Edwards Aquifer Authority) as well as Megan Bean (USFWS Austin Ecological Services Field Office, HCP Coordinator). Staff captured 44 salamanders and retained 42. Two individuals were caught and released. The remaining salamanders were returned to the SMARC for incorporation into the refugia population.

In collaboration with Dr. Matt Pintar and Israel Prewitt (both BIO-WEST, Inc.), West collected 230 Comal Springs riffle beetles (CSRB), 9 Peck's cave amphipods (PCA), and 16 Comal Springs dryopid beetles (CSDB). Of the 230 CSRB, West retained 211. All PCA, CSRB, and CSDB were also retained for quarantine and incorporation at the SMARC.

Figure 1. Shawn Moore (Biological Science Technician) snorkeling to collect San Marcos salamanders from the eastern spillway of the San Marcos River.

Table 2. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for October 2024. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC Collections	UNFH Collections	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	311	NT	0	0	12	31	97	298
Fountain darter: Comal	130	NT	0	0	14	28	148	592
Comal Springs riffle beetle	211	NT	19	0	NA	NA	87	36
Comal Springs dryopid beetle	16	NT	10	0	NA	NA	13	30
Peck's cave amphipod	9	NT	0	0	21	NA	153	207
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	0	NT	0	0	0	0	103	59
San Marcos salamander	43	NT	0	0	9	2	236	181
Comal Springs salamander	NT	NT	0	0	0	0	71	79
Texas wild rice	NT	NT	0	0	14	42	176	150

Husbandry

SMARC

Jackson and Moore, along with Marina Draeger (Student Conservation Association intern), conducted the annual inventory of all Texas wild rice plants held in the refugia program at the SMARC. Staff removed mortalities, replaced weak pumps, and constructed a new map outlining the locations of plants in each tank.

Jackson, Moore, and West constructed additional racks, flow bars, and air lines in the SMARC quarantine facility on October 4th to accommodate the additional Fountain darters maintained at the SMARC.

West repeated additional artemia enrichment trials assessing the longevity of previously enriched artemia in hatcheries. West found that artemia experienced increased longevity and survival when maintained at 24° Celsius after hatching, a significant departure from the optimum hatching temperature of 30° Celsius.

Dr. Katie Bockrath, Jackson, Moore, and West each attended the Edwards Aquifer Authority Annual appreciation event in San Marcos on October 24th.

Uvalde

Dr. Scott Walker and Dominique Alvear attended the Edwards Aquifer Authority Annual appreciation event in San Marcos on October 24th.

Walker and Alvear repotted a Texas Wild Rice tank after noticing unusually high mortality and a decline in overall health.

Task 2 Research

Dryopid Life History

BIO-WEST continued monthly monitoring and testing of methods used to detect and collect drypoid beetles. Wood disks were checked and reset, except in sites used for Comal Springs riffle beetle biomonitoring. Low flow conditions and declining water levels left 6 discs dry when checked in early October (two each in Spring Run 1, Spring Run 2, and the Upper Spring Run). Total numbers of invertebrates found in October were:

Table 3. Collection location and numbers of invertebrates captured on wood disks in the Comal Spring system.

Location	Number of Disks	Drypoid Beetles	Comal Springs riffle beetle Adults	Comal Springs riffle beetle Larvae	Peck's cave amphipods
Spring Run 1	1	0	0	0	0
Spring Run 2	4	0	23	6	0
Spring Run 3	8	0	8	0	1
Upper Spring Run	1	0	0	0	0
Spring Island	25	16	117	212	4
Western Shore	16	0	21	16	0

A second round of the paired comparison between cotton lures and wood discs was completed. This paired study has indicated no differences in efficacy between the two methods for Comal Springs riffle beetles, but 100% of drypoid beetles have been found on wood discs.

San Marcos Salamander Mark and Recapture

Dr. Katie Bockrath estimated population size* of San Marcos salamanders using two metrics, the Peterson-Lincoln Index and the Schnable Index. The Peterson-Lincoln Index takes all collections and recaptures into account in a single estimate while the Schnable Index accounts for individual collection events. The assumptions for both indices were violated, compromising the validity of the population estimate, and the more robust program, MARK, will be used to more accurately estimate population size. The assumptions for both indices are:

- The population is closed meaning births, deaths, and movements are negligible during the study period.
- Equal capture probability where all individuals in the population have the same probability of capture during a given sampling occasion.
- Tags are not lost or overlooked between sampling occasions.
- Tagged individuals from the first sampling occasion are completely mixed into the population as a whole, not just the specific sampling site.
- Sampling time is negligible in relation to intervals between samples.
- Release is made immediately after the sample.

The table below shows the population estimate at three regularly surveyed locations and a total estimate when considering all three locations.

Table 4. Population estimations of San Marcos salamanders at three locations using the Peterson-Lincoln and Schnable Indices.

	Peterson-Lincoln Index	Schnable Index
Location	Population Size Estimate	Population Size Estimate
Hotel	6,384.93	5,682.30
Diversion	2,974.48	2,712.45
Eastern Spillway	12,457.20	11,372.52
All Locations Together	19,886.32	18,392.20

* Population sizes are preliminary estimates only and are not to be taken as actionable values.

Marina Draeger (SCA) continued to crop photos taken for all tagged and collected salamanders for mark and recapture assessment using unique marking on the salamander heads.

Reproductive Gene Expression in San Marcos Salamanders

Ruben Tovar (University of Texas, Austin) received data for all tissue types, except gonad tissue. The gonad tissue was sent off for sequencing and data is expected in early November. Thus far, gene expression profiles significantly differ between life stage, species and dwelling (surface or subterranean). In the PCA plots below, 13% of gene expression variation appears to be due to dwelling or sightedness. In the top figure, there is division between subterranean and surface species indicated by the lack of random mixing of the red and blue dots. Additionally, 23% of

gene expression variation appears to be due to life stage. In the second PCA plot, early developmental life stages are clustered to the left (blue) while later life stages cluster to the right (purple and red). Further investigation and incorporation of gonad gene expression data is needed to identify what genes or genetic pathways are driving the differentiation between species and samples.

Comal Springs Riffle Beetle Population Genetics

Dr. Bockrath worked with Dr. Chris Nice (Texas State University) to analyze the genetic data received from the USFWS Midwest Fisheries Center, Whitney Genetics Lab.

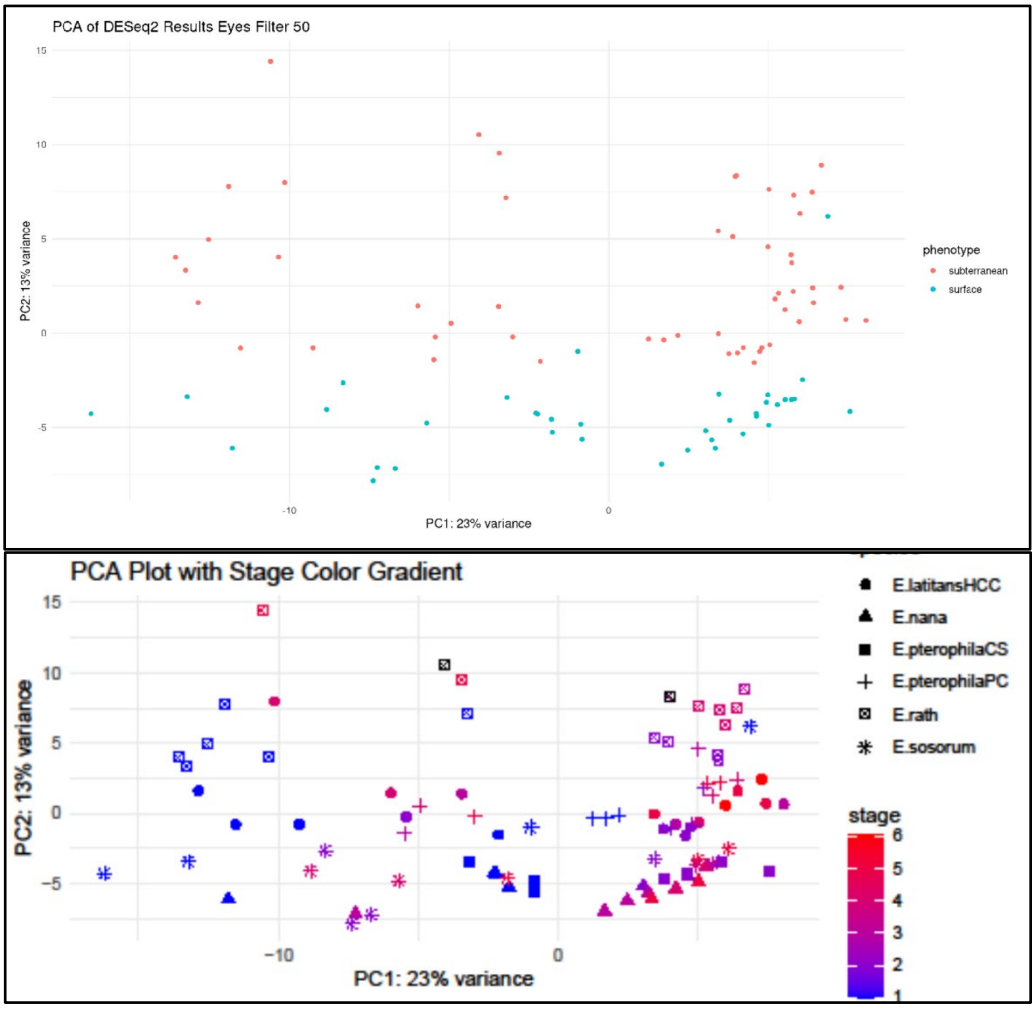

Figure 2. PCA plot of RNA gene expression data associated with subterranean or surface species of aquatic salamanders. The Y-axis explains 13% of variance associated with the sightedness or blindness (suface vs subterranean) species (Top). The x-axis explains 23% of variance in the data and appears to be associated with life stage (Bottom).

Table 5. Number of samples collected from adult and larvae Comal Springs riffle beetles. The mean, median and total number of raw sequences passing quality control standards are reported for each sample type.

Sample Type	Number of Samples	Mean Number of Sequences Per Sample	Median Number of Sequences Per Sample
Adult Beetle	128	1594481	1229313
Larval Beetle	125	1662213	1415818
Total	253	418522178	

Tagging Aquatic Invertebrates

Brian De La Torre and Dr. Shannon Brewer visited the SMARC at the end of October to set up the third p-chip tagging trial and to test QR codes for tagging Comal Springs riffle beetles.

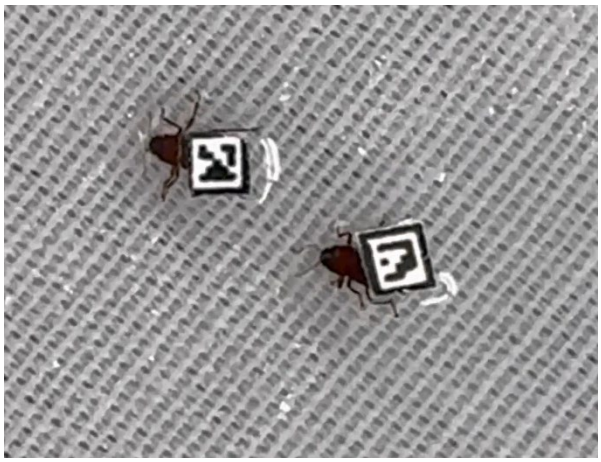

Figure 3. Two **Heterelmis glabra** *riffle beetles with 1.5x1.5mm QR codes glued to their carapaces.*

Genetic Assessment of Peck's Cave Amphipod

Dr. Chris Nice (Texas State University) processed the collected individuals for sequencing.

Genetic Assessment of Texas Blind Salamanders

Erin Lowenberg (SCA) and Shawn Moore continued to take tail clips from captive held Texas blind salamanders for eventual population genetic analyses.

Genetic Assessment of San Marcos Salamanders

The tail clips collected from all tagged San Marcos salamanders during the Mark and Recapture study were sent off for sequencing at the USFWS Midwest Fisheries Center, Whitney Genetics Lab.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Dr. Bockrath worked on 2024 research reports.

Dr. Bockrath reviewed performance reports for partnered researcher in GrantSolutions.

Task 6 Meetings and Presentations

All EARP staff meet weekly to discuss husbandry updates, collections (previous and upcoming), and research updates.

Dr. Bockrath met with Randy Gibson (SMARC), Dr. David Hoffman (Texas State University) and Enzo Silvagni (Texas State University) to discuss potential 2025 research around determining ideal, maximum and minimum thermal tolerances for EAHCP covered species.

Dr. Bockrath met with Dr. Shannon Brewer and Brian De La Torre (Auburn University) to discuss suitability of using of p-chips and/or QR codes for mark and recapture studies on riffle beetles.

November 2024 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear and Dr. Katie Bockrath

With contributions from

Richelle Jackson and Shawn Moore

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Task 1 Refugia Operations

Staffing Updates

On November 18, Noel Valenzuela-Charro began his internship through the Student Conservation Association. Noel is from San Antonio, Texas and is interested in Aquatic Biology. He will be stationed at the Uvalde National Fish Hatchery assisting the EAR program until May 18, 2025. We are excited to have him in the program.

Species Collection

Braden West assisted Dr. Matt Pintar and Israel Prewitt (BIO-WEST) with checking wood disks for beetles in the Comal Springs system. West processed the disks and assisted in recording data. West collected all adult Comal Springs riffle beetles (CSRB) and Comal Springs dryopid beetles (CSDB) found on the disks. A total of 94 adult CSRB and 23 adult CSDB were caught and transported to the SMARC for incorporation into the refugia population.

On Monday November 25, Jackson and Moore set traps for Texas blind salamanders in Johnson's well and Primer's fissure in the Purgatory Creek Natural Area, San Marcos, TX. Staff checked traps on Monday, Wednesday, and Friday. Three Texas blind salamanders were caught in November and transported to the SMARC for incorporation into the refugia population.

Husbandry

SMARC

Jackson, Moore, and West continued monitoring San Marcos and Comal River Fountain darters collected in October. Fish were maintained in the SMARC quarantine for a 60-day period to allow for additional observation. Staff treated the fish with anti-parasitic treatment on November 1 and November 15. A total of 340 fish (262 San Marcos and 78 Comal) remained in quarantine through the end of November.

West incorporated quarantined invertebrates from previous collection events. 359 Comal Springs riffle beetles, 9 Comal Springs dryopid beetles, and 18 Peck's cave amphipods were incorporated into the Refugia population.

West and Moore tested larger polycarbonate habitat boxes for use in the refugia currently used to house salamanders in quarantine. They purchased and tested new 12-quart containers. The new containers used the same overall height as the incumbent 8-quart containers, not requiring staff to alter culture rack systems. Unfortunately, the new containers' sidewalls were slightly more angled than previous designs, leading to leaky bulkheads. West and Moore further altered the

drain design to alleviate this issue. The bulkheads need to be precisely positioned to prevent leaking, but the new habitat boxes allow for more boxes to be on a rack.

Jackson worked on archiving mortalities, addressing incomplete archival entries and filling in data where necessary.

Moore acid washed refugia tanks RE-05 and RE-06 and started the acid washing process on an additional tank, RE-07.

Uvalde

Dominique Alvear and Noel finished repotting Texas Wild Rice (TWR) after an unusually high number of mortalities. TWR was transferred to a new tank and is doing better. Once the old rice tank was empty Noel began power washing to remove excess algae and prepare it for a mild acid wash to remove the calcium build up (Figure 1). Alvear and Noel conducted the semi-annual salamander inventories (Figure 2). All Texas Blind salamanders, Comal Springs salamanders and San Marcos salamanders were accounted for, and Refugia numbers were updated.

Figure 1. Noel power washing algae buildup from Texas Wild rice tank.

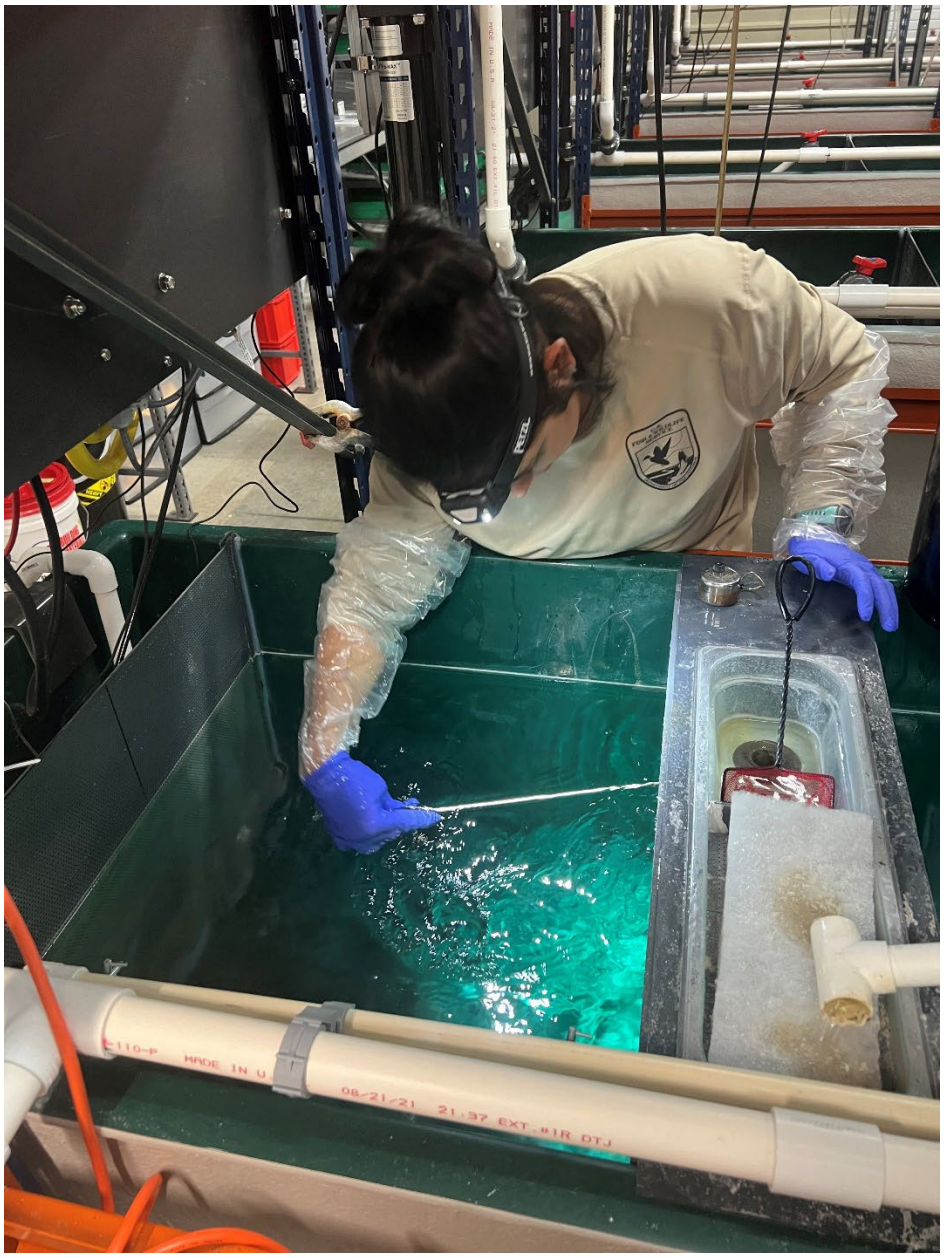

Figure 2. Dominique catching San Marcos salamanders for semi-annual inventory

During routine morning temperature checks Noel alerted Alvear that a tank was out of optimal temperature range, animals were immediately transferred into a chilled tank. After further investigation with Val Cantu (Base Biologist), the chiller cooling the tank was working as expected but circuit in the controller box had burned out, causing the controller box to not report higher temperatures to the chiller and the chiller not adjusting the temperature in the tank. Alvear and Cantu were able to replace the circuit and get the tank back to optimal temperature range (Figure 3). The circuit is likely a faulty part and was quickly and easily replaced with

replacement parts. No other circuits have failed on any other controllers at UNFH or SMARC.

The “Summer Collection” of the San Marcos fountain darters were incorporated into the Refugia population on November 20 after an extended quarantine period due to higher mortality rate. A total of 84 darters were added.

Figure 3. Dominique Alvear replacing a burnt circuit in the system controller box.

Fish Health

Moore swabbed all remaining San Marcos salamanders held in the SMARC quarantine. Moore tendered the samples to Erin Lowenberg (SCA) for disease testing prior to incorporation.

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for November 2024. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC Collections	UNFH Collections	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	0	84	3	0	54	391
Fountain darter: Comal	NT	NT	0	0	16	45	126	547
Comal Springs riffle beetle	94	NT	359	0	NA	NA	446	36
Comal Springs dryopid beetle	23	NT	9	0	NA	NA	22	30
Peck's cave amphipod	NT	NT	18	0	NA	NA	171	207
Edwards Aquifer diving beetle	NT	NT	0	0	0	NA	0	0
Texas troglobitic water slater	NT	NT	0	0	0	NA	0	0
Texas blind salamander	3	NT	0	0	1	0	102	59
San Marcos salamander	NT	NT	0	0	6	32	227	149
Comal Springs salamander	NT	NT	0	0	0	6	82	73
Texas wild rice	NT	NT	0	0	0	22	176	128

Task 2 Research

Dryopid Life History

BIO-WEST submitted their draft report discussing the captive housing and propagation efforts. The report also discussed the findings associated with modified luring methods. The draft report has been submitted to the EAA for review.

San Marcos Salamander Mark and Recapture

Marina Dreager (SCA) finished cropping the photos for all p-chipped tagged salamanders, creating the reference data base. Marina cropped photos of salamanders taken during a spring 2023 sampling event that resulted in a p-chip recapture. Marina is processing these photos through Wild.ID to see if the photo of the recaptured p-chip tagged salamander matches to the reference photo library, thus providing preliminary evidence that photos of the markings on San Marcos salamander heads can be used for future mark and recapture studies.

The draft report covering the p-Chip recapture work has been submitted to the Edwards Aquifer Authority for review.

Reproductive Gene Expression in San Marcos Salamanders

The draft report has been submitted to the Edwards Aquifer Authority for review.

Comal Springs Riffle Beetle Population Genetics

Dr. Bockrath began analyzing over 500 million sequences representing a total of 253 individuals, first focusing on assessing population structure and genetic diversity. Initial assessments indicate that genetic structure located at Spring Island and Western Shore is distinct from Spring Run 2 and Spring Run 3. Additionally, genetic lineages represented in Spring Runs 2 and 3 are absent from Spring Island and Western Shore, and vice versa (Figure 4). The representation of a unique genetic lineage and relative uniformity in Spring Runs 2 and 3 indicates unique subpopulations relative to the main river channel and the potential of a reduction in genetic diversity due to reduction in population size (bottleneck) from a decrease in habitat availability. These results suggest that spring flows in the Spring Runs must be maintained at a sufficient minimum flow rate to prevent the Spring Runs from drying to prevent further reductions in population size, the loss of unique genetic (and thus adaptive) diversity, and potential local species extirpation from habitat loss. Additional genetic analyses, which are forthcoming, are required to expand upon these preliminary findings. The draft report has been submitted to the Edwards Aquifer Authority for review.

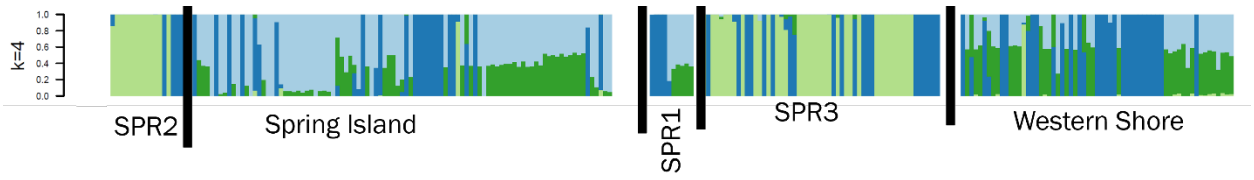

Figure 4. Population Genetic Structure bar plot. Vertical bars represent an individual included in the study. Individuals are grouped by sampling location (population). The different colors represent genetic lineages. Each individual (vertical bar plot) is assigned to a

genetic lineage (color). Those with a single color were 100% assigned to that genetic lineage. Those with multiple colors were assigned to multiple lineages, indicative of retained ancestral genetic diversity.

Tagging Aquatic Invertebrates

Dr. Bockrath checked control chambers and experimental tubes weekly. Flow and temperature remained within target ranges. The number of living beetles in control chambers was recorded and the experimental tubes were flipped. Movement data was sent to Brian De La Torre for analysis. Beetles are retaining the QR code tags with mixed success. Some beetles lost their codes, some retaining their tags and moving round unimpeded (Figure 5), and some have died after tagging. The draft report has been submitted to the Edwards Aquifer Authority for review.

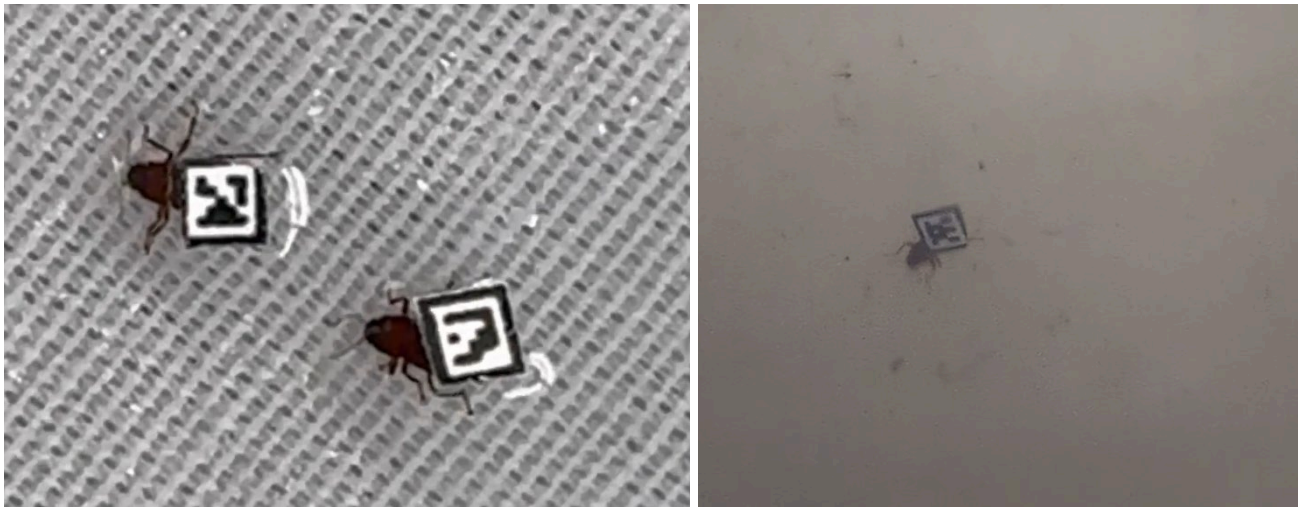

Figure 5. Comal Springs riffle beetles tagged with 1.5mm x 1.5mm QR codes. The picture on the right shows beetles right after tagging with QR codes. The picture on the left shows a beetle in a flow through tube with an intact QR code.

Genetic Assessment of Peck's Cave Amphipod

Dr. Chris Nice (Texas State University) is analyzing the genetic data and conducting population genetic assessments. The draft report has been submitted to the Edwards Aquifer Authority for review.

Genetic Assessment of Texas Blind Salamanders

Erin Lowenberg (SCA), Shawn Moore, Richelle Jackson, Garrison Engstrom (SCA), Marin DeBolt (SCA), and Braden West continued to take tail clips from captive held Texas blind salamanders. Erin Lowenberg continued to extract DNA from these tail clips. The draft interim report has been submitted to the Edwards Aquifer Authority for review.

Genetic Assessment of San Marcos Salamanders

The draft interim report has been submitted to the Edwards Aquifer Authority for review.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

Dr. Katie Bockrath submitted draft reports for research conducted in 2024. Dr. Bockrath also submitted proposals for 2025 research and an updated 2025 work plan.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss collections (previous and upcoming), husbandry needs and updates and research progress and updates.

Dr. Bockrath met with Dr. Chad Furl, Ed Oborny, Dr. Matt Pintar, and Kyle Sullivan to review the CSRB occupancy and population genetics preliminary results.

Dr. Bockrath met with Dr. Chad Furl for the EARP quarterly meeting to discuss 2024 research, 2025 research plans and the 2025 work plan.

December 2024 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear and Dr. Katie Bockrath

With contributions from

Richelle Jackson and Shawn Moore

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Task 1 Refugia Operations

Staffing Updates

The Uvalde National Fish Hatchery welcomed two new members onto the Edwards Aquifer Refugia Program: Matthew "Tanner" Donelon and Kallan Padget.

Matthew "Tanner" Donelon began working on December 2, Matthew filled the role of Biological Science Technician. Matthew graduated from Texas State University with a M.S. in Wildlife Ecology and is excited to begin his career in conservation.

Kallan Padget began on December 16 as the EARP Lead Biologist. Padget moved from New Mexico where he was an Assistant Hatchery Manger for 7 years and raised cutthroat trout, rainbow trout and Kokanee Salmon.

Species Collection

On December 2, 4, and 6, Richelle Jackson, Shawn Moore, and West checked Texas blind salamander (TBS) traps in Johnson's Well and Primer's Fissure, Purgatory Creek Natural Area, San Marcos, TX. Four TBS were captured on these days. Three were released and one was retained for refugia at the SMARC.

On December 5 and 6, Braden West met Dr. Matthew Pintar (BIO-WEST, Inc.) at Landa Park and Spring Island, New Braunfels, TX. West assisted Dr. Pintar on the final round of wood disc checks. West collected a total of 51 adult Comal Springs riffle beetle (CSRB), 8 Comal Springs dryopid beetle (CSDB), and 7 Peck's cave amphipods (PCA). All animals were retained for refugia at the SMARC.

On December 11 Dominique Alvear from the UNFH met Moore, Jackson and West at the eastern spillway of the San Marcos River for a Texas wild rice collection. (Figure 3) Thirty-five wild rice plants were collected and distributed among the refuges.

On December 18 Alvear, Moore, Jackson and West met at Spring Island to collect Peck's cave amphipods. Ninety-eight amphipods were collected and split between the UNFH and SMARC.

Husbandry

SMARC

On December 13 Moore and Jackson separated live, healthy blackworms from the culture tank to restart the culture. All gravel substrate was removed, disinfected and replaced in a new tank.

Jackson, Moore, and West each conducted end of year inventories on all species.

Moore and Jackson incorporated Fountain darter lots 1024A, 1024B, 1024C into the refugia.

Jackson made routine updates to the Fountain darter transport protocol to prepare for the January collection events.

Uvalde

Noel Valenzuela-Charro, Donelon and Alvear transferred and combined the remaining plants in tank 13 to be able to remove excess algae and calcium build up in the tank.

Alvear trained Donelon and Padget on how to conduct fountain darter inventories and all Refugia populations were counted as part of the semi-annual inventory.

Alvear also conducted Pecks Cave amphipod inventories.

Fish Health

Moore swabbed all remaining San Marcos salamanders held in the SMARC quarantine. Moore tendered the samples to Erin Lowenberg (SCA) for disease testing prior to incorporation.

On December 4 Jackson and Moore necropsied and archived fountain darter mortalities from the Fall collection. No parasites were observed.

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for December 2024. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC Collections	UNFH Collections	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	253	0	19	58	288	333
Fountain darter: Comal	NT	NT	85	0	18	108	193	439
Comal Springs riffle beetle	51	NT	103	0	5	NA	544	36
Comal Springs dryopid beetle	8	NT	23	0	NA	NA	45	30
Peck's cave amphipod	52	48	0	0	61	62	110	145
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	0	1	1	101	58
San Marcos salamander	NT	NT	0	0	4	9	224	140
Comal Springs salamander	NT	NT	0	0	1	0	81	73
Texas wild rice	20	15	0	0	0	2	176	126

Task 2 Research

Dryopid Life History

The draft report has been submitted to the EAA for review.

San Marcos Salamander Mark and Recapture

The draft report has been submitted to the EAA for review.

Reproductive Gene Expression in San Marcos Salamanders

The draft report has been submitted to the Edwards Aquifer Authority for review.

Comal Springs Riffle Beetle Population Genetics

The draft report has been submitted to the Edwards Aquifer Authority for review.

Tagging Aquatic Invertebrates

The draft report has been submitted to the Edwards Aquifer Authority for review.

Genetic Assessment of Peck's Cave Amphipod

Dr. Chris Nice (Texas State University) completed analyzing the genetic data and conducting population genetic assessments.

There is no genetic structure for Peck's cave amphipod across the Comal Springs System when compared to other species/populations. When the data is analyzed for just the PCA group (excluding non PCA species) PCA still break out as one group for the Comal Springs system, but we see more genetic diversity represented and it is evenly represented across Spring Runs. There

is no change in population or genetic structure in Peck's cave amphipod between time points, suggesting PCA populations do not seem to be significantly impacted by droughts and the collection locations (Spring Run 1-3, Spring Island, and Western Shore) are all well connected. Additional population genetic assessments (Tajima'S D) show values very close to 0, suggesting PCA is not under a lot of genetic selection pressure and has not undergone a lot of population size changes. PCA are distinct from other *Stygobromus* species, which means their population is indeed mainly in the Comal Springs system, with some other populations in nearby springs fed by the Edwards Aquifer. The draft report has been submitted to the Edwards Aquifer Authority for review.

Genetic Assessment of Texas Blind Salamanders

The draft report has been submitted to the EAA for review.

Genetic Assessment of San Marcos Salamanders

The draft interim report has been submitted to the Edwards Aquifer Authority for review.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

Dr. Katie Bockrath submitted draft reports for research conducted in 2024. Dr. Bockrath also submitted proposals for 2025 research and an updated 2025 work plan.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss Husbandry needs, Collection plans, and Research updates.

Dr. Bockrath presented the CSRB genetic assessment study at the EAHCP CSRB working group meeting and the EAHCP End of Year Joint Committee Meeting.

Figure 3. Dominique Alvear posing with a Texas wild rice tiller in the San Marcos River.

Appendix K | **USFWS Southwestern Native Aquatic Resources and Recovery Center Fish Health Unit reports**

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/1089 November 25, 2024

Memorandum
To: Katie Bockrath PhD, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 25-04).

On October 24, 2024, Southwestern Fish Health Unit (SFHU) staff received 30 fountain darters (*Etheostoma fonticola*) collected from the San Marcos River (GNIS ID: 1375972), Texas. One fish was received dead on arrival and not included for testing. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for health testing as part of an ongoing parasite enumeration study. San Marcos ARC staff recorded collection of fountain darters at the "Eastern Spillway" site – a spillway from Spring Lake into the San Marcos River – at latitude 29.8901° and longitude -97.9337° in Hays County. The river water temperature was 24°C. Originally collected from the river on October 7, 2024, these fish were held at San Marcos ARC until shipping. No treatments were administered to these fish prior to shipment.

All fish were humanely euthanized using buffered MS-222. Assays and examinations for the sampled fish included virology and parasitology. Viral screening of 29 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. Gill necropsies and microscopic observations for parasites were completed on 10 fish. Screening for *Centrocestus formosanus* involved inspecting the left gill set of each fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2020 edition) and standard SFHU protocols.

Results:
Immature *Centrocestus formosanus* were observed in 4 of 10 fish and mature *Centrocestus formosanus* were observed in 1 of 10 fish examined. Monogenetic trematodes were also observed on the gills from 4 of 10 fish and *Ichthyobodo sp*. were observed in 1 of 10 fish. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is also included in a separate file with my email of this report. No viruses were detected by cell culture.

If you have any further inquiries regarding test methodology or outcomes, or require further assistance from the SFHU, feel free to reach out to the staff at the Southwestern Fish Health Unit. Please reference case history number 25-04 for all follow-up correspondence.

cc:
Huseyin Kucuktas, PhD, Southwestern Fish Health Unit
Scott Walker, PhD, Uvalde National Fish Hatchery
Jennifer Howeth, PhD, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 25-03

Date examined: 10-24-24 Date Collected: 10/16/2024

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)	175	422	372	205	268	212	162	220	119	144
Total Length (mm)	27	35	36	28	31	29	27	30	25	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, 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
Immature (left gills only)	L	1, 0, 0,0	3, 7, 4, 2	7, 4, 2, 1	0, 0, 0, 0	0, 1, 0, 0	0, 0, 1, 0	0, 0, 0, 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, 3, 0	0, 1, 0, 0	0, 0, 0, 1	0, 0, 0, 0	0, 0, 0, 1	0, 1, 0, 0	0, 0, 0, 0
Myxobolus sp.	L	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0
Other	L	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0

Examiner signature _____

Revised on 9/20/2017

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

In Reply Refer To:
FWS/R2/FR-SFHU/1088 November 25, 2024

Memorandum
To: Katie Bockrath PhD, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the Comal River, Texas (Case Number 25-03).

On October 24, 2024, Southwestern Fish Health Unit (SFHU) staff received 45 fountain darters (*Etheostoma fonticola*) collected from the Comal River (GNIS ID: 1372140), Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for health testing as part of an ongoing parasite enumeration study. San Marcos ARC staff recorded collection of fountain darters at the "Spring Island" sample site, at latitude 29.7175° and longitude -98.1317° in Comal County. The river water temperature was 24°C. Originally collected from the river on October 16, 2024, these fish were held at San Marcos ARC until shipping. No treatments were administered to these fish prior to shipment.

All fish were humanely euthanized using buffered MS-222. Assays and examinations for the sampled fish included virology and parasitology. Viral screening of 45 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. Gill necropsies and microscopic observations for parasites were completed 10 fish. Screening for *Centrocestus formosanus* involved inspecting the left gill set of each fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2020 edition) and standard SFHU protocols.

Results:
Immature *Centrocestus formosanus* were observed on 5 of 10 fish examined. Monogenetic trematodes were also observed on the gills from 5 of 10 fish examined. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is also included in a separate file with my email of this report. No viruses were detected by cell culture.

If you have any further inquiries regarding test methodology or outcomes, or require further assistance from the SFHU, feel free to reach out to the staff at the Southwestern Fish Health Unit. Please reference case history number 25-03 for all follow-up correspondence.

cc:
Huseyin Kucuktas, PhD, Southwestern Fish Health Unit

Scott Walker, PhD, Uvalde National Fish Hatchery
Jennifer Howeth, PhD, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 25-04

Date examined: 10-24-24 Date Collected: 10/07/2024

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)	313	419	281	528	322	355	117	157	320	99
Total Length (mm)	32	36	34	39	33	36	24	28	33	24

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

Monogenea	L	0, 0, 0, 0	0, 0, 0, 0	1, 0, 1, 0	0, 0, 0, 0	0, 0, 0, 0	0, 1, 0, 0	2, 1, 2, 1	0, 0, 0, 0	0, 0, 0, 0	0, 2, 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, 1, 1, 0

Ichthyobodo

Examiner signature

Revised on 9/20/2017