IMPLEMENTATION OF THE EDWARDS AQUIFER REFUGIA PROGRAM UNDER THE EDWARDS AQUIFER HABITAT CONSERVATION PLAN

ANNUAL REPORT 2025

CONTRACT NO. 16-822-HCP

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The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

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EXECUTIVE SUMMARY

BACKGROUND

On January 1, 2017, a contract (Contract # 16-822-HCP) between the Edwards Aquifer Authority (EAA) and the U.S. Fish and Wildlife Service (USFWS) was initiated for the operation and maintenance of a series of refugia for ten species endemic to the Edwards Aquifer. These refugia were covered by the Edwards Aquifer Habitat Conservation Plan (EAHCP) Section 5.1.1. The contract spans a performance period beginning January 1, 2017, and continues until March 31, 2028. This is the ninth annual report of the contract covering the calendar year of 2025. The ninth year of the contract focused on maintaining the existing standing stocks and conducting research while facing a significant drought and staff changes.

The major objectives of the USFWS Refugia Program are to 1) develop and provide a fully functioning refugia for the Covered Species; 2) conduct research to expand knowledge of the Covered Species with a focus on Refugia needs; 3) develop and refine animal rearing

methods and captive propagation techniques for the Covered Species; 4) reintroduce species, in the event of a loss of species populations in their native environment, and monitor recovery; and 5) attend meetings and provide oral presentations to EAHCP Science Committee, Implementing Committee, and EAA Board of Directors as requested by the EAHCP Program Manager.

COLLECTIONS

Collection events occurred in every month of 2025. Collection numbers by month and species are shown in Table 1. Edwards Aquifer diving beetles (*Haideoporus texanus*), San Marcos gambusia (*Gambusia georgei*), and Texas troglobitic water slaters (*Lirceolus smithii*) were not collected in 2025; all other covered species were collected in 2025.

Figure 1. Shawn Moore (USFWS) collecting San Marcos Salamanders at the Eastern Spillway site in the San Marcos River, San Marcos, Texas.

Table 1. Counts of individuals captured in 2025 by species and month. Collection counts are provided for the San Marcos Aquatic Resources Center (before the slash) and Uvalde National Fish Hatchery (after the slash). "-" indicates there were no collections for either station. CSRB = Comal Springs riffle beetles, CSDB = Comal Springs dryopid beetles, PCA = Peck's cave amphipods, CSFD = Comal Springs fountain darters, SMFD = San Marcos fountain darters, TXBS = Texas blind salamanders, CSS = Comal Springs salamanders, SMS = San Marcos salamanders, and TWR = Texas wild-rice. The number captured may not reflect the number retained for refugia or research purposes, as some individuals may have been released.

	CSRB	CSDB	PCA	CSFD	SMFD	TXBS	CSS	SMS	TWR
JAN	-	-	-	106/0	183/91	-	-	-	-
FEB	31/9	-	162/50	-	-	5/0	-	-	-
MAR	6/15	-	-	-	-	1/0	-	2/0	-
APR	0/84	0/48	0/38	677/0	129/219	-	-	25/55	30/60
MAY	0/83	0/45	-	2295/0	-	5/0	-	7/0	0/40
JUN	0/12	0/18	50/220	-	-	-	0/1	52/0	-
JUL	-	-	-	-	-	5/0	-	-	0/50
AUG	-	-	-	-	-	3/0	-	-	-
SEP	41/0	-	197/0	-	-	-	-	2/89	-
OCT	0/56	0/6	217/140	-	331/0	-	-	10/64	-
NOV	0/68	-	-	-	124/0	5/0	-	-	57/80
DEC	-	-	-	-	213/0	-	-	-	-

RESEARCH

The EARP planned to carry out 5 research projects in 2025, several with external partners. These research projects focused on species covered by the Edwards Aquifer Habitat Conservation Plan, including two invertebrates (Comal Springs riffle beetle and Peck's cave amphipod) and the San Marcos and Texas blind salamanders. Research areas included genetic

assessments of wild populations, refining equipment to determine thermal tolerances of covered species, and mark and recapture of wild populations. All research was conducted to improve successful completion of their life cycles, promote reliable reproduction, and establish baselines for species reintroductions.

USFWS staff expanded on a mark-recapture study using p-Chip tagged wild San Marcos salamanders that examined recapture rate, movement, demographics and estimated population size. During this effort, photos were captured of all collected salamanders. Unique markings on each of the salamander's heads were used to identify recaptured salamanders. This effort expands on the p-chip mark and recapture study by drastically increasing the sample size, thus statistical power, to determine if salamanders are moving between sampling locations and better estimate the population size. Callender year 2025 efforts were focused on editing photos to a standard orientation and running a subset of the photos through Wild.ID to confirm San Marcos salamanders could be identified by markings on their heads. Using the p-chip recapture data from the previous study, tagged salamanders were consistently identified by the marking on their heads. Using said photos, additional recapture data was collected from untagged salamanders that were identified as recaptures via their unique markings. This effort will conclude in 2026. The interim report for this study is included in Appendix B.

A study developing tagging methodology for invertebrates, led by Dr. Shannon Brewer of the U.S. Geological Survey, Alabama Cooperative Fish and Wildlife Research Unit, was concluded this year. A literature review was conducted to identify potentially suitable tags for testing using the surrogate species *Heterelmis glabra*. Two tags were identified and tests, p-Chips and QR codes. A tagging protocol was developed for Comal Springs riffle beetle using superglue to affix both a p-Chip tag and a QR code to the elytra. Survival and retention of tagged beetles was assessed. Additionally, a passive method for conducting inventories in the Refugia was tested using custom flowthrough tubes with a scanning window where the tagged beetles were automatically scanned as they pass through the window. Final analysis was concluded in 2025. A final report for this study is included in Appendix C.

In 2025, Enzo Silvagni and Dr. David Huffman (Texas State University) worked to modify and refine a Thermal Biology Assessment Devise (TBAD) that will allow USFWS to test thermal maximum and minimum tolerances as well as preferred thermal ranges for Edwards Aquifer Habitat Conservation Plan covered species. The prototype was developed and built by Dr. Huffman and his former student, Matthew (Tanner) Donolan. Enzo refined the device to change temperatures more rapidly, reduce the amount of thermal variance at the extreme ends of the thermal range and it is now mobile. The instrument will be housed at the San Marcos Aquatic Resources Center where thermal tolerance testing will occur in 2026. The report on the build out of the TBAD and its performance are found in Appendix D.

USFWS staff experimented with two different strategies for excluding Peck's cave amphipod (PCA) neonates from adults in hopes of preventing cannibalism during the captive breeding process. Gravid female PCA were either held in passive exclusion chambers while their neonates developed or the developed neonates were physically removed from the gravid female. In the passive exclusion tubes, developed neonates had the opportunity to physically separate from the female by moving through a mess screen, large enough for them but too small for the adult, into a different chamber. Adult and neonate PCA were observed every 10 days. One neonate was successfully removed from a gravid female. At the conclusion of this study, this neonate remains active and growing. Unfortunately, the gravid female died two weeks post removal. Regular checks of the gravid females in the passive exclusion tubes allowed us to monitor how many neonates were present and how long females remained gravid before releasing their brood or slowly eating them. At the conclusion of this study, no females released fully developed neonates while in the passive exclusion tubes. While in the tubes, the brood size of each female reduced over time without eggs/neonates being released suggesting the female continued to cannibalize from her brood. This study will continue as an ongoing husbandry project. The report is located in Appendix E.

USFWS staff, Dr. Kate Bell and Dr. Chris Nice (Texas State University) planned to execute a population genetic study of San Marcos Salamanders using 453 tail clips collected during the p-Chip mark and recapture study in 2023, wild caught captive held salamanders, and captive

bred salamanders in the Refugia. Unfortunately, this research was not completed in 2025. This project has been moved to 2026. There will not be a report for the 2025 Annual Report.

BUDGET

The Aquifer Refugia Program did not exceed the allocated budget defined in the 2025 Refugia Work Plan previously approved by the EAA Board of Directors. The Refugia Program spent approximately $1,087,158 in 2025. Research activities accounted for $292,622, and approximately $762,845 was spent on collections, husbandry, and propagation. Approximately $31,712 was spent on reporting, meetings, and presentations. Most unspent funds in Tasks 1 and 2 will move to a Task 1 and 2 Reserve Funds, respectively, to hold until need requires the program to request those funds in a Work Plan and Budget.

INTRODUCTION

BACKGROUND

The activities reported herein are in support of the Federal Fish and Wildlife Incidental Take Permit (ITP) for the EAA (TE-6366A-1, Section K) and fulfillment of Contract #16-822-HCP between the Edwards Aquifer Authority (EAA) and the U.S. Fish and Wildlife Service (USFWS) as outlined within the 2025 Edwards Aquifer Refugia Work Plan. The overarching goal of the Edwards Aquifer Refugia Program conducted by the USFWS is to assist the EAA in compliance with its ITP and to meet its obligation within EAHCP section 5.1.1. The refugia contract covers ten different species including seven endangered species, one threatened species, one species no longer petitioned for listing, and two species currently proposed for listing (see **Error! Reference source not found.** for a list of Covered Species).

The Edwards Aquifer Refugia Program's purpose is to house and to protect adequate populations of the Covered Species for re-introduction into the Comal or San Marcos systems in the event a population is lost following a catastrophic event such as a long-term drought or major flood. In addition, the Refugia Program conducts research activities to expand knowledge of the species' habitat requirements, biology, life histories, and effective reintroduction techniques. Captive assurance populations of these species are maintained in refugia in San Marcos, Texas and Uvalde, Texas. See the appropriate sections of this report for further details on each of the species collected and maintained and the section on research activities.

The EAA-USFWS contract awarded the Region 2 Fish and Aquatic Conservation Program (FAC) with $18,876,267 over a period of performance spanning January 1, 2017 until March 31, 2028. The monetary support of the Refugia augments the existing financial and physical resources of two USFWS facilities and provides resources to house and protect adequate populations of the Covered Species. Support is also provided for research activities aimed at enhancing the maintenance, propagation, and genetic management of the Covered Species held in refugia (**Error! Reference source not found.**), as well as for salvage and restocking as necessary. The monetary support is allocated into six tasks: 1) Refugia Operations, 2) Research,

3) Species Husbandry and Propagation, 4) Species Reintroduction, 5) Reporting, and 6) Meetings and Presentations.

Table 2. Eleven species identified in the Edwards Aquifer Habitat Conservation Plan and listed for coverage under the Incidental Take Permit within the federal Endangered Species Act (ESA)

Common Name	**Scientific Name**	**ESA Status**
Fountain darter	*Etheostoma fonticola*	Endangered
Comal Springs riffle beetle	*Heterelmis comalensis*	Endangered
San Marcos gambusia	*Gambusia georgei*	Extinct*
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*	Petition Rescinded**
Comal Springs salamander	*Eurycea pterophila*	Petition Rescinded†
Texas troglobitic water slater	*Lirceolus smithii*	Petition Rescinded‡

* The San Marcos gambusia was proposed for removal from the ESA due to extinction on September 29, 2021 (Federal Register Document Number 2021-21219; U.S. Fish and Wildlife Service 2021).

** The Edwards Aquifer diving beetle petition for listing under the ESA was rescinded on 6/17/2025 (Federal Register docket number FWS–R2–ES–2024–0105, published document: 2025-10777 (90 FR 25559)).

†The Comal Springs salamander was petitioned for listing under the ESA as "*Eurycea* sp. 8" but has subsequently been identified as a common species, *Eurycea pterophila*, and is no longer petitioned for listing under the ESA.

‡The Texas troglobitic water slater was removed from petition consideration November 29, 2023 (Federal Register 88 FR 83368 2023-25586)

OBJECTIVES

1. **Further develop and provide fully functioning refugia for the EAHCP Covered Species.**
   USFWS will work toward fully functioning refugia operations for all the Covered Species. Fully functioning refugia populations are those that can be predictably collected, maintained, and bred with statistical confidence. The primary refugia will be located at the San Marcos Aquatic Resources Center (SMARC), with a secondary refugia population located at the Uvalde National Fish Hatchery (UNFH).
2. **Conduct research as necessary to expand knowledge of the EAHCP Covered Species.**
   USFWS and/or subcontractors will conduct research as necessary to expand knowledge of the Covered Species for the Aquifer Refugia Program. Research will follow the Edwards Aquifer Refugia Research Goals and Plan and be developed with consultation with the Edwards Aquifer Chief Science Officer. Research will include, but may not be limited to, species' physiology, husbandry requirements, propagation techniques, health and disease issues, life histories, genetics, and effective reintroduction techniques.
3. **Develop and refine animal care/husbandry methods and captive propagation techniques for the EAHCP Covered Species.**
   USFWS will maintain Standing Stock populations and continue to refine care techniques to increase survivorship, efficiencies, and organismal welfare. Staff will develop propagation techniques in case reintroduction of species into the wild becomes necessary.
4. **Reintroduce species populations, in the event of a loss of EAHCP Covered Species in their native environment and monitor recovery.**
   The reintroduction strategy will continually evolve as more information is learned about the species.
5. **Attend meetings and provide oral presentations to Science Committee, Implementing Committee, and EAA Board of Directors as requested by the EAHCP Program Manager.**
   The Edwards Aquifer Refugia Program staff will keep partners appraised of refugia activities.

PERSONNEL

The USFWS managed the Edwards Aquifer Refugia Program with dedicated staff at two geographically separated facilities: the SMARC and UNFH (Table 3). Both facilities are administratively managed under the direction of a single Center Director, Dr. Scott Walker (Acting) with the assistance of the Deputy Center Director, Dr. Katie Bockrath (Acting). Kallan Padget, based at the UNFH, led the Refugia Husbandry and Collections team for both facilities in 2025. Dr. Katie Bockrath also serves as the Refugia Research Lead, and the point of contact for the Edwards Aquifer Refugia Program by coordinating all research activities, project plans, reporting and budgets in 2025. The Edwards Aquifer Refugia Program underwent staff changes in 2025. The program welcomed Matthew (Tanner) Donelon as a Biological Science Technician at UNFH this year and hosted Noel Valenzuela-Charro for a one-year internship. Marisol Farias also began a one-year internship at SMARC working on PCA captive propagation. Shawn Moore and Richelle Jackson departed the program in May of 2025. Biological Scientists Braden West and Dominique Alvear remain with the program, bringing consistent continuity to each facility through staff and intern changes.

Table 3. USFWS Refugia Program Staff

San Marcos Aquatic Resources Center	
Dr. Scott Walker (acting)/Vacant	Center Director
Dr. Katie Bockrath (acting)/Vacant	Deputy Center Director
Dr. Katie Bockrath	Refugia Research Team Lead
Vacant	Refugia Biologist
Braden West	Refugia Biologist
Vacant	Biological Science Technician
Vacant	Biological Science Technician
Uvalde National Fish Hatchery	
Scott Walker	Uvalde National Fish Hatchery Project Leader
Kallan Padget	Refugia Husbandry and Collections Team Lead
Dominique Alvear	Refugia Biologist
Matthew (Tanner)Donelon	Biological Science Technician
Vacant	Biological Science Technician

Day-to-day operations were managed by the Lead Biologists providing supervision, mentorship, and training to the Refugia Biologists, Biological Science Technician and Interns (see Table 3 for staffing chart). The Lead Biologists managed and coordinated species collections, husbandry, propagation, research, and field activities related to species covered under the contract. They also arranged purchases, oversaw facility maintenance repairs, developed and implemented budgets, and organized all activities that were related to the contract. The Lead Biologists provided proper and efficient use of facilities and staff resources to ensure that contractual obligations are met in a timely manner. In coordination with the Center Director and Deputy Center Director, they prepared all written materials required for reporting. They communicated regularly with the EAA, USFWS personnel, researchers, and other partners.

Dr. Katie Bockrath, Refugia Research Lead, coordinated research efforts across stations. Dr. Bockrath, with help from Refugia staff, prepared the annual report, annual work plans, and monthly reports. Dr. Bockrath developed research activities and reports, developed and managed the Refugia Program budget, and established and oversaw outside research agreements.

Kallan Padget, Refugia Husbandry and Collections Lead, coordinated the husbandry and collections across stations. Padget, with help from supporting staff, prepared the annual report, annual work plans, and monthly reports. Kallan Padget managed refugia purchase needs oversaw development and implementation of husbandry standard operating procedures, designed and oversaw construction of refugia system improvements and coordinated collection activities.

Dominique Alvear and Braden West, Refugia Biologists, worked with Padget to manage the husbandry and collections across stations. They contributed to the annual report and monthly reports, developed and implemented husbandry standard operating procedures, designed and constructed refugia holding systems. The biologists performed quality control for daily and collection data records, ensured biosecurity adherence, and assisted with research activities.

Biological Science Technician Matthew (Tanner) Donelon and Intern Noel Valenzuela-Charro (Student Conservation Association), carried out collections and daily husbandry duties at the UNFH. They constructed, maintained, and monitored holding systems for refugia species. The technicians performed daily data recording, animal husbandry, promoted biosecurity, and assisted with research activities. Additionally, they managed logs and databases, authored and edited standard operating procedures (SOPs), and contributed to monthly reports.

Marisol Farias (Student Conservation Association intern) worked with Dr. Katie Bockrath and Braden West to execute data collection, inventory procedures, and system construction for the Peck's cave amphipod (PCA) captive propagation research project. Farias maintained partially recirculating systems used for invertebrate culture in the EARP. Farias performed daily data recording, promoted biosecurity, and assisted with animal husbandry activities.

BUILDING CONSTRUCTION

Staff made improvements to the EARP building at SMARC. Due to reduced staffing after May of 2025, Braden West focused on husbandry and collections activities for the bulk of the year. West and Padget were able to make some notable progress with replumbing tank systems in the refugia. The new system mirrors the systems that have been built at Uvalde and feature CO2 injection, Low Head Oxygenators with biofiltration, and UV sterilizers. These new systems significantly improve animal health through the use of UV sterilizers, biofiltration and added oxygenation. The desired result is increasing animal longevity which will decrease the demand to collect more animals from the wild. These systems reduce the risk of gasification events by minimizing well water supplied to culture tanks while encouraging degassing of water through use of Low Head Oxygenation.

Staff at the SMARC conducted repairs of the Edwards Aquifer Refugia building in 2025. In March, technicians noticed several points in the building where condensation was dripping onto the refuge floor. Closer inspection revealed water pooling above insulation batts in the crown of the building. Technicians Shawn Moore and Richelle Jackson moved animals into the quarantine building and out of the threat of danger, while West worked with Juan Martinez (Facilities Operations Specialist, SMARC) to determine the extent of water intrusion. Staff found condensation had soaked insulation batts spanning the entire crown section of the refuge building, prompting replacement of the insulation and increased moisture mitigation measures. Staff purchased a roll of insulation material, repaired damaged vent fans, and installed a high capacity de-humidifer to mitigate condensation in the Refugia. Juan Martinez, Braden West, Mike Montagne, Dr. Katie Bockrath, and Dr. Scott Walker worked to deconstruct the building's superstructure to access the affected sections of ceiling insulation (Figure 2). Repairs lasted several weeks, beginning in mid-March and were completed in early May.

Figure 2 Juan Martinez using a compact scissor lift machine to repair waterlogged insulation in the SMARC refugia building.

Kallan Padget traveled between the UNFH and the SMARC to ensure homogeneity in system design. Focus was put into standardizing the plumbing layout of systems between UNFH and SMARC. Installation of bypass valves, pressure sensors and a splitter bar for water delivery to Low Head Oxygenators are some of the key features of this system. All of the systems utilize the same controller boxes and Walchem systems that were installed in 2023. The concept of recirculating aquaculture systems with automated water quality monitoring remains the end goal.

Uvalde National Fish Hatchery EARP staff completed plumbing design on seven recirculating aquaculture systems in the refugia this year. The focus of these efforts was to update plumbing, install low head oxygenators, install UV filtration, and update drainage systems for better exchange rates during cleaning. Three chillers were replaced this year at Uvalde. Two Raypaks and one JBJ chiller were installed to replace Aqualogic chillers that went out of service. The JBJ chillers are smaller and more efficient than chillers used in the past and have worked very well since installation. As staff have updated plumbing on these systems, they have noticed a substantial improvement in monthly survival and sustainment of refugia populations.

Staff also made adjustments to the Texas wild-rice culture tanks this year. Padget installed flow bars at the front of every tank to create a laminar flow pattern. This is intended to better simulate the conditions that plants experience in a river environment. The pump disposition and number was changed to include pumps in the middle and back of the tanks in addition with the pumps present at the front of each tank. The purpose of this design is to encourage the water to continue moving through the tank in a laminar formation. This design also creates a back current that allows the water to move in the middle and back parts of the tanks in a circular formation.

In May of this year there was a gasification event at UNFH. This resulted in a mortality event in the quarantine building with a full depopulation of Comal Springs fountain darters and most of the Comal Salamanders. Due to the gasification event, UNFH staff redesigned the incoming water line in the quarantine building and installed a water collection head box to function as a multipurpose tool for controlling water quality and distribution. Staff started this project by installing an automated turnoff valve into the quarantine building that is designed similarly to the other three turn off valves at SMARC and UNFH. A Walchem controller will trigger the valve to divert water to a bleed off valve if a gasification event occurs. This will reopen once the high dissolved gas has been relieved. In addition to the turn off valve, a collection head box has been installed. At the front of the headbox is a J-pipe oxygen infuser. The purpose of this design is to entrain oxygen into the incoming well water. This is necessary because even under conditions where water is not gasified in a lethal amount, the gas ratios of oxygen to nitrogen may remain out of balance. Installation of the J-pipe helps mitigate the

problems associated with maintaining animals with gasified ground water. Water enters the head box and is pumped to a chiller and re-introduced to the head box. Utilizing a chiller to cool the well water minimizes the work that the tank chillers need to perform and will reduce operational costs to cool water. The sump also uses several bypass and bleed off valves that can be turned on to reduce gas pressure if needed. With several different scenarios kept in mind this head box design paired with the automated turn off valve adds several layers of protection against future gasification events.

COVERED SPECIES ANALYSIS

Collections of the Covered Species continued to work toward standing stock targets as outlined in the Contract and the 2025 EA Refugia Work Plan (Tables 4 and 5). For many species, the acclimation to captive systems can be achieved relatively quickly; this is particularly true for Texas wild-rice, San Marcos fountain darters, and San Marcos salamanders.

After consultation with the EAA staff, our other partners, and experts in the field, we decided to reduce the number of invertebrate collection events and numbers of CSRB held in refugia to minimize any negative effects that collection events might have on wild populations in the Comal Springs system due to drought conditions.

The Covered Species knowledge matrix (Table 6) was updated to reflect the current standing for all Covered Species across five distinct areas that make up a complete refugia: Collections, Husbandry, Propagation, Genetics, and Reintroduction. Texas wild-rice and the fountain darter have the highest knowledge score of all covered species. Texas wild-rice is in complete refugia.

Figure 3. Texas blind salamander in EA Refugia.

Table 4. Number of organisms incorporated in the SMARC Refugia Standing Stock in 2025, the end of year census, and overall survival rate.

Species		SMARC Incorporated into Refugia	SMARC End of Year Census	SMARC Survival Rate
Fountain darter - San Marcos *Etheostoma fonticola*		217	264	56%
Fountain darter – Comal Springs *Etheostoma fonticola*		1832	694	71%
Comal Springs riffle beetle *Heterelmis comalensis*		10	98	21%
Comal Springs dryopid beetle *Stygoparnus comalensis*		0	4	10%
Peck's cave amphipod *Stygobromus pecki*		379	243	51%
Edwards Aquifer diving beetle *Haideoporus texanus*		0	0	-
Texas troglobitic water slater *Lirceolus smithii*		0	0	-
Texas blind salamander *Eurycea rathbuni*		14	97	82%
San Marcos salamander *Eurycea nana*		111	242	67%
Comal Springs salamander *Eurycea* ***pterophila***		0	74	82%
Texas wild-rice *Zizania texana*		45	160	68%

Notes: Incorporated refers to organisms that have passed their 30-day quarantine period where they have been evaluated for health and suitability for inclusion into refugia populations; also, they have been cleared by USFWS Fish Health Unit where applicable. End of year census number is of those incorporated. Survival rate = (end of year census/ (start of year inventory + # incorporated)))*100. Survival rate does not include any individuals in quarantine or any mortality during quarantine period or those sacrificed for research or Fish Health diagnostics. Further details of these numbers can be found in the supporting sections of each species.

Table 5. Number of organisms incorporated in the UNFH Refugia Standing Stock in 2025, the end of year census, and overall survival rate.

Species		UNFH Incorporated into Refugia	UNFH End of Year Census	UNFH Survival Rate
Fountain darter - San Marcos *Etheostoma fonticola*		75	148	40%
Fountain darter – Comal Springs *Etheostoma fonticola*		498	549	62%
Comal Springs riffle beetle *Heterelmis comalensis*		153	92	48%
Comal Springs dryopid beetle *Stygoparnus comalensis*		77	25	32%
Peck's cave amphipod *Stygobromus pecki*		350	353	68%
Edwards Aquifer diving beetle *Haideoporus texanus*		0	0	--
Texas troglobitic water slater *Lirceolus smithii*		0	0	--
Texas blind salamander *Eurycea rathbuni*		0	55	96%
San Marcos salamander *Eurycea nana*		191	249	77%
Comal Springs salamander *Eurycea* ***pterophila***		1	1	2%
Texas wild-rice *Zizania texana*		205	205	61%

Notes: Incorporated refers to organisms that have passed their 30-day quarantine period where they have been evaluated for health and suitability for inclusion into refugia populations; also, they have been cleared by USFWS Fish Health Unit where applicable. End of year census number is of those incorporated. Survival rate = (end of year census / (start of year inventory + # incorporated)) * 100. Survival rate does not include any individuals in quarantine or mortality during quarantine period or those sacrificed for research or Fish Health diagnostics. Further details of these numbers can be found in the supporting sections of each species.

Table 6. Updated table shows the level of knowledge known for each covered species. Knowledge score is a gradient from 0 to 5, where 0 is complete lack of knowledge and 5 indicates documented procedures for that species exist. 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	4	3	4
Texas wild-rice	5	5	5	5	5
Texas blind salamander	4	5	4	3	1
San Marcos salamander	5	5	3	3	1
Comal Springs salamander	5	4	3	3	1
Comal Springs riffle beetle	5	5	4	4	3
Comal Springs dryopid beetle	4	3	2	3	2
Texas troglobitic water slater	1	0	0	1	1
Peck's cave amphipod	5	4	2	4	2
Edwards Aquifer diving beetle	1	0	0	0	1

Our Standing Stock goal for fountain darters is 1,000 fish per river (San Marcos and Comal) divided between the two facilities. Standing stock goals for San Marcos fountain darters are below target numbers in 2025. This is due to some changes in the way sampling and incorporation are now being performed at both facilities. Staff anticipate that target numbers will be achieved early in 2026. In the summer, due to a drought, the Comal River spring flow conditions reached critically low levels. These critically low river flows triggered the first salvage event for the Refugia Program (See Reintroduction Section for details). A salvage operation was implemented and over two thousand Comal Springs fountain darters were collected in the span of a week. 400 fish were collected during routine collection efforts and an additional 1600 fish were collected during the Salvage event. This collection effort was followed in the fall by the program's first reintroduction event for fountain darters. Numbers incorporated, end of the year census, and survival rates can be found in Table 7.

Table 7. Fountain darter Refugia population figures

		Beginning of Year Census	Incorporated 2025	Reintroduced	Transferred to UNFH	End of Year Census	In Quarantine End of Year	Target Goal 2025 Work Plan	Percent Survival [2]
San Marcos	SMARC	255	217	0	0	264	166	500	56
	UNFH	294	75	0	0	148	289	500	40
Comal Springs	SMARC	164	1832	500	525	694	0	500	71
	UNFH	391	498	0	0	549	0	500	62

[1]The number of fountain darters incorporated into the refugia is counted after a minimum 30-day quarantine period or when fish are cleared by Fish Health. During this period, fish are evaluated for health and suitability for inclusion into the refugia.

[2] Survival rate = (end of year census / (start of year inventory + # incorporated)))*100. Survival rate does not include any mortality during quarantine period or those sacrificed for research or Fish Health diagnostics. Fish removed from the refugia as part of the facilities yearly animal health inspection are not included in the moralities and calculated Percent Survival.

COLLECTIONS

In 2025, the collection of San Marcos fountain darters was focused at the beginning and end of the year. During the warmer months of the year, staff opted not to collect fountain

darters due to warmer temperatures and lower river flows. These conditions can result in mortality rates over 50 percent and the program shifted to collecting the majority of fish during the winter months because higher survival is observed during this time. Refugia Staff were challenged with collecting a majority of its Comal Springs fountain darters during a heat wave at the end of May that coincided with the salvage event. A bulk of Comal Springs fountain darters collected and incorporated this year were from the salvage event. Refugia staff observed high mortality rates in this lot of fountain darters that were likely fueled by the unfavorable collection conditions. Refugia staff conducted collections for San Marcos and Comal Springs fountain darters in the months of January, April, May, October, November and December. A total of 1,290 San Marcos fountain darters and 3,078 Comal Springs fountain darters were collected. Of the 1,291 San Marcos fountain darters, 1,289 were retained, with 581 transferred to the SMARC and 710 to the UNFH for incorporation into refugia. Roughly 280 of these fountain darters were lost at Uvalde in the gasification event in May during their quarantine period. All 3,078 Comal Springs fountain darters were retained at the SMARC. 525 were later transferred to Uvalde after construction of the collection head box and automated valve. 500 Comal Springs fountain darters were later reintroduced to the Comal River at the sight of collection. The end of year census numbers for the Comal Springs and San Marcos fountain darters are shown in the Table 7, above.

Refugia staff also collaborated with BIO-WEST to collect fountain darters during biomonitoring efforts in April and October. Refugia staff received 679 San Marcos fountain darters and 677 Comal Springs fountain darters. These collection numbers are included in the collections described above and fish were transferred to the SMARC for incorporation into the refugia.

Subsets of fountain darters collected in January, March, August, and October were sent directly to the USFWS Southwestern Fish Health Unit (SFHU) in Dexter, New Mexico for parasite enumeration and viral analysis, in compliance with the program's biosecurity plan. Additionally, in compliance with USFWS fish health inspections conducted at all hatcheries, 40 San Marcos fountain darters and 60 Comal Springs fountain darters fish were euthanized for inspection.

QUARANTINE PROCEDURES

Refugia staff transported all fountain darters from both systems to the SMARC for an initial holding period. Staff observed improved survival rates when dip-net-collected fish transportation to UNFH is delayed by 2 weeks. A standard fountain darter intake and quarantine procedure was used at both facilities. During this time, fish were administered a 3 g/L salt treatment daily for 3 days and fed a diet of Artemia while they adjusted to their new captive environment. After 2 weeks, subsets of the collections were transported to Uvalde and the quarantine period was reset. The quarantine period for fountain darters at both facilities was extended until staff observed no mortalities for five calendar days, often reaching 50-60 days. The quarantine areas are separate, biologically secure areas away from the refugia systems, preventing the spread of disease and aquatic nuisance species. To minimize stress, temperature acclimation progressed at a rate of one degree Celsius per hour. Darters were then transferred to clean flow-through quarantine tanks. Fish sent to the USFWS SFHU for routine parasitology and health screening were not treated for parasites and were SFHU as soon as possible.

HUSBANDRY

All culture systems were monitored multiple times daily for proper water flow and temperature, reproduction (eggs), and mortalities. Deceased fish were immediately removed from the systems. If warranted, deaths were necropsied for parasites and preserved in vials containing 95% non-denatured ethanol. If parasites were noted during the necropsy or there was an increase in mortality in a tank, either a 1-hour static bath of 1-3ppt salt was administered, according to the Southwestern Fish Health Unit recommendations.

Fountain darters at both facilities were housed in large, insulated fiberglass systems with either flow-through chilled well water (SMARC) or partial recirculation through heater-chiller units (UNFH) to maintain water temperature at 21 ℃ (ranging between 19–23 ℃). Water quality parameters including dissolved oxygen, pH, and total gas pressure were checked weekly.

Staff focused on debris management and water quality maintenance through husbandry. Staff siphoned, scrubbed and performed water exchanges on tanks daily to ensure fresh, clean water is in constant supply to the tanks and that bacteria and parasite loads are as low as possible. These practices along with UV sterilizers and biofiltration help staff continue to improve husbandry effectiveness. Each tank system had dedicated equipment (nets, cleaning supplies) to prevent the potential spread of pathogens from system to system. If equipment was shared, it was cleaned and disinfected between systems. Fish were fed daily, varying between live amphipods, live black worms, live *Artemia*, live *Daphnia* sp., frozen mysid shrimp, and refrigerated Copepods.

SURVIVAL RATES

Historically at both the SMARC and UNFH, survivorship of newly collected fountain darters from the Comal River was poor in comparison to fountain darters collected from the San Marcos River, even when these were collected during the same time period and held in similar conditions. This has been an ongoing pattern for Comal Springs fountain darters since collections were restarted in 2017 after Comal Springs fountain darters were found to test positive for Largemouth bass virus (LMBV). Given the history of low intake survival rates, the EARP suspended collections of Comal Springs fountain darters for the refugia stock in the fall of 2019. Starting in 2022 and continuing into this year, Comal Springs fountain darters were collected again in larger numbers because of low spring flow. Survival rates of Comal Springs fountain darters were highly variable during their 30-day quarantine period. Between 2022 and 2024, individual lots of fish exhibited survival rates ranging from as low as 0% to as high as 85%. Once out of the quarantine period, survival is on par with San Marcos fountain darters. Necropsies of darter mortalities have revealed internal parasites in some individuals, which may be causing some of the mortalities. The reason for the large variance in early survival rates is unknown.

Biologists saw continued poor survival for Comal Springs fountain darters in 2025. The reason for this is likely due to the unfavorable conditions from which the darters were collected during the salvage paired with higher-than-normal densities that the fish experienced once

transported to captivity. Another factor that contributed to the low survival was the gasification event that occurred at Uvalde in the spring. Not including this gasification event, the survival rate for Uvalde this year was 89 percent. Survival of San Marcos fountain darters was low and reduced mortality coincided with improved feeding and cleaning practices. Survival percentages in the refugia at the SMARC was 56% for the San Marcos River population and 71% for the Comal Springs population. This year staff noticed mortalities occurred during high stress moments. This included post collection mortality and mortality following any moves or census events. There may also be a relationship between mortality and cleaning. Once staff identified husbandry weaknesses and improved daily maintenance to be more thorough and precise, mortality began to decline. At UNFH, the survival rate was 40% for the incorporated San Marcos population and 62% for the Comal River population.

MAINTENANCE OF SYSTEMS

Refugia systems are now deep cleaned bi-annually with muriatic acid to remove calcium carbonate deposits that form within the tank, plumbing, chiller, and pump casing that can affect functionality. When systems were empty, they were bleached with 20ppm free chlorine for 24 hours followed by neutralization with sodium thiosulfate (UNFH) or the tank surface sprayed with 1% Virkon (SMARC). Water lines, hoses, valves, and restrictors were frequently checked for wear and clogs and were cleared, rebuilt, or replaced as needed.

CAPTIVE PROPAGATION

This year staff did not focus on establishing a captive broodstock. Once staff have restored refugia populations to meet the standing stock and have achieved a baseline for survival year to year they will work on captive broodstock and recruitment of F1 generations. Staff will opportunistically collect and hatch eggs as needed.

COMAL SPRINGS RIFFLE BEETLE (*HETERELMIS COMALENSIS*), ENDANGERED

Comal Spring riffle beetle (CSRB) collection by EARP staff for standing and refugia stocks occurred from February – June and September – November from around Spring Island. Staff are now working in connection with BIO-WEST Inc. to collect riffle beetles as part of a population study, from which some individuals were transferred to refugia staff. Standing stock numbers were reduced to 75 individuals per station until better knowledge of population numbers and meaningful standing stock numbers are derived (Table 8).

Table 8 Comal Springs riffle beetle refugia population figures

	Beginning of Year Census	Incorporated 2025	End of Year Census	In Quarantine End of Year	*Target Goal 2025 Work Plan	Percent Survival
SMARC	456	10	98	0	75	21
UNFH	36	153	92	38	75	48

Survival rate = (end of year census / (start of year inventory + # incorporated)))*100. Survival rate does not include any individuals in quarantine or mortality during quarantine period.

COLLECTIONS

Refugia staff collected CSRB in concert with BIO-WEST research and biomonitoring in April, May, June, October, November, and December. A total of 405 CSRB were collected from lures at Spring Island. 344 CSRB were retained by the EARP in 2025, of which 41 were transferred to the SMARC and 303 to the UNFH for incorporation.

QUARANTINE

Incoming CSRB were quarantined at the SMARC and the UNFH. CSRB were acclimated to quarantine water conditions at a rate not exceeding one degree Celsius every half-hour. During the quarantine period, staff monitored for potential aquatic nuisance species that may have come in with the collection, the general health of the organisms, or any large die-offs that might indicate a disease. If none of these events occurred, CSRB joined the Refugia population in a container labeled by collection date at the end of the 30-day quarantine period.

HUSBANDRY

All systems were evaluated daily for water temperature, adequate flow, and clear drain screens to maintain drainage and water level. CSRB refugia systems were not siphoned because adults, larvae, or eggs could easily be discarded along with debris. Comal Springs riffle beetles feed predominantly on biofilm; therefore there has been no traditional feeding schedule. Alternatively, leaves, wood, and cotton cloth containing biofilm were used in each system, providing food. Inventories were conducted every two to three months and new biofilm material was added as needed.

Culture boxes used to house CSRB were square black plastic containers with a manifold that delivers water through a spray bar onto the side of the container that flows down into the water. Containers contained leaves, conditioned wood, biofilm cloth, and mesh for structure and habitat. The systems were cleaned during inventory at which time staff checked water lines, hoses, and valves for functionality and cleaned or replaced them as needed. Air space and emergent structure was provided in box containers housing larvae.

SURVIVAL RATES

Because CSRB have an average life span of approximately one year and adults of unknown age are collected from the field, high annual mortality rates are expected due to senescence. Historically, about half of CSRB collected perish by six months in captivity. The small size of CSRB makes it difficult to assess mortality on a day-to-day basis. Therefore, mortalities are calculated as inventories are conducted, where the number of dead or missing CSRB equates to the number of mortalities for that time-period. The 2025 survival rates for CSRB in refugia at the SMARC were 21% and 48% at the UNFH. These metrics highlighted how much the refugia population can correct in a single year and aligns with staff expectations on life span.

CAPTIVE PROPAGATION

To encourage production of offspring, male and female wild stock were housed together. During inventories, larvae were placed into a separate container from wild stock adults. Staff observed higher reproduction and metamorphosis of CSRB relative to previous years suggesting recent improvements to culture systems and husbandry methods are beneficial. Offspring production was minimal and the beetle population in the refuge declined due to natural senescence. There were 112 F(1) offspring produced at SMARC this year with 26 Fx remaining at the end of the year.

COMAL SPRINGS DRYOPID BEETLE (*STYGOPARNUS COMALENSIS*), ENDANGERED

Given the low numbers of Comal Springs dryopid beetles (CSDB) historically collected in the field, yearly population goals were set at 20 individuals at each site in the Work Plan for this species. Numbers incorporated, end of the year census, and survival rates can be found in Table 9.

Table 9. Comal Springs dryopid beetle refugia population figures

	Beginning of Year Census	Incorporated 2025	End of Year Census	In Quarantine End of Year	Target Goal 2025 Work Plan	Percent Survival
SMARC	40	0	4	0	20	10
UNFH	0	77	25	0	20	32

Survival rate = (end of year census / (start of year inventory + # incorporated)))*100. Survival rate does not include any mortality during quarantine period.

COLLECTIONS

Refugia staff collected CSDB in concert with BIO-WEST research and biomonitoring in April, May, June and October. A total of 117 CSDB were retained by the EARP in 2025, all of which were transferred to the UNFH for incorporation.

QUARANTINE

Incoming CSDB were quarantined in the invertebrate refugia area at the UNFH. CSDB were acclimated to quarantine water conditions at a rate not exceeding one degree Celsius every hour. During the quarantine period, staff monitored for potential aquatic nuisance species that may have come in with the collection, the general health of the organisms, and any large die-offs that might indicate a disease. If none of those events occurred, CSDB joined the refugia population at the end of the 30-day quarantine period.

HUSBANDRY

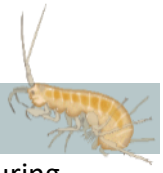
Square plastic containers were used as culture boxes for CSDB. Each container was fitted with a manifold to deliver water through a spray bar onto the side of the container, flowing down into the basin. Containers were kept dark to mimic the underground environment. All systems were checked daily for appropriate water temperature, adequate flow, and clear drain screens to maintain drainage and water level. Conditioned wooden dowels in the containers were checked for fungal growth, and if found were removed; CSDB may become entrapped in fungus and perish. CSDB refugia containers were not siphoned for debris because CSDB adults, larvae, or eggs could easily be discarded along with debris. As the CSDB feed on biofilm; leaves, wooden dowels, and cotton cloth containing biofilm were placed in containers and provided a constant food source. Inventories were conducted every other month, and new food items were added as needed. Obtaining census numbers during inventories, especially for larvae, were difficult at times as adult and larval dryopid beetles burrow under the surface of the wooden media used in the culture boxes.

SURVIVAL RATES

The small size of CSDB made it difficult to assess for mortality on a day-to-day basis. Mortalities were therefore calculated as inventories were conducted, where the number of dead or missing beetles equates to the number of mortalities for that time-period. During the inventory, the health condition of the dryopid beetles was assessed. The 2025 survival rates for CSDB in the refugia at the SMARC was 10% and 32% at the UNFH. Reasons for poor year to year survival are similar to CSRB. Most mortalities were observed 8 to 11 months post incorporation. Mortality rates were highest with SMARC's lots of CSDB from 2024 and UNFH lots from April and May 2025.

CAPTIVE PROPAGATION

Larvae were not observed or separated in refuge populations of CSDB this year.



PECK'S CAVE AMPHIPOD (*STYGOBROMUS PECKI*), ENDANGERED

Peck's cave amphipods (PCA) were collected from the Comal River by hand during collection events in February, April, June, Spetember and October. The refugia also received PCA caught as bycatch from Comal Spring riffle beetle lures set by BIO-WEST at 80 biomonitoring sites. Numbers incorporated, end of the year census, and survival rates can be found in Table 10.

Table 10. Peck's cave amphipod refugia population figures

	Beginning of Year Census	Incorporated 2025	End of Year Census	In Quarantine End of Year	Target Goal 2025 Work Plan	Percent Survival
SMARC	100	379	243	0	250	51
UNFH	168	350	353	0	250	68

Survival rate = (end of year census / (start of year inventory + # incorporated)))*100. Survival rate does not include any individuals in quarantine or mortality during quarantine period.

COLLECTIONS

Refugia staff conducted seventeen EARP-led collection events and one BIO-WEST lead biomonoriting event for PCA in 2025. These collection events took place around the Spring

Island of the Comal River, New Braunfels, Texas. A total of 1,074 PCA were captured in 2025. 1,068 were retained and transferred to the SMARC and UNFH for incorporation into the refugia.

QUARANTINE

Incoming PCA were quarantined in the refugia invertebrate areas in the quarantine rooms at the SMARC and UNFH. Peck's Cave amphipod were acclimated to quarantine water conditions at a rate not exceeding one degree Celsius every hour. During the quarantine period, staff monitored for potential aquatic nuisance species that may have come in with the collection, the general health of the organisms, or any large die-offs that might indicate a disease. If none of those events occurred, the PCA joined the Refugia population at the end of the 30-day quarantine period.

HUSBANDRY

All systems were checked daily for proper water temperature, adequate flow, and clear drain screens to maintain drainage and water level. Small amounts (ca. 10 ml) of fish flake slurry were added two times per week. Dried leaves from terrestrial sources were used as potential supplemental food and provided shelter within the systems. According to Nair (2019), PCA eat other smaller species of amphipods (Nair 2019), and Kosnicki and Julius (2019) reported that PCA are predators in their ecosystem and most likely prefer live feed in comparison to other *Stygobromus* amphipods (*S. flagellatus*; Kosnicki and Julius 2019).

Plastic totes were used as culture containers to house PCA, with PVC piping that delivered water in a manner to mimic upwellings. The systems did not have a cleaning or siphoning schedule, but rather were cleaned during inventory at which time staff checked water lines, hoses, and valves for functionality and cleaned or replaced them as needed.

SURVIVAL RATES

PCA are known to cannibalize smaller individuals, which lower survival rates. Mortalities were therefore calculated as inventories were conducted, where the number of dead or missing PCA equates to the number of mortalities for that time period. The 2025 survival rates for PCA in refugia at the SMARC was 51% and 68% at the UNFH. This year staff have observed a notable improvement in survival during incorporation and in between inventories. Staff experimented with reduced densities and feeding PCA live *Daphnia magna* as a secondary food source, but it is too early to tell if this is a contributing factor to increased survival.

CAPTIVE PROPAGATION

When counting PCA from refugia containers during inventory, each amphipod was carefully observed for brooding. PCA females hold their eggs and young in a brood pouch under the body. At the SMARC and UNFH, gravid females were noted and placed back into refugia wild stock. PCA juveniles were easily identifiable at the next inventory by their size. Biologists were confident, given observed growth rates, that juveniles that survived could be located, identified, and moved to an F1 container. To minimize the cannibalism from the mothers on their offspring, staff tested the potential of removing late-stage eggs from a gravid female and placing in a separate container to hatch. Although somewhat laborious, the eggs hatched successfully. The second half of 2025 saw continued success towards establishing methods to isolate neonates and prevent cannibalism (See Appendix E and Research Section). Staff have observed some success by isolating brooding females into chambers with mesh on the down flow side that allows neonates to separate from the adult PCA preventing cannibalism.

EDWARDS AQUIFER DIVING BEETLE (*HAIDEOPORUS TEXNUS*), UNDER REVIEW

No Edwards Aquifer diving beetles were collected during 2025. These beetles are rare, with little known about their native habitat, life history, or food requirements. Diving beetles have been previously collected from the Texas State Artesian Well, but these collections are only opportunistic, as beetles are ejected from the high-flow spring. There is an agreement with Texas State University to donate caught adults to the SMARC, at their discretion. Unfortunately, none were donated this year.

TEXAS TROGLOBITIC WATER SLATER (*LIRCEOLUS SMITHII*), NO LONGER PETITIONED

A non-lethal method to distinguish *L. smithii* from other species based on the characteristics of the pleotelson was discovered by Texas State University doctoral student Will Coleman. In 2019, using Coleman's method, we determined the refugia population consisted primarily of *Lirceolus hardeni* (no common name). Further, Mr. Coleman conducted extensive collections for his research and found *L. smithii* only in Texas State Artesian Well samples, and of those, very few live specimens. These live specimens were physically damaged, and Mr. Coleman was unable to keep them alive in captivity. This evidence suggests that *L. smithii* are a deep-aquifer species, like the Edwards Aquifer diving beetle, and are rarely found in surface waters; those that are found have likely suffered physical damage during the distance traveled to the surface.

No *L. smithii* were held in refugia in 2025. In the future, if *L. smithii* are collected from Texas Sate Artesian Well, the refugia will employ documented husbandry procedures that were successful at holding and propagating *L. hardeni*.

TEXAS BLIND SALAMANDER (*EURYCEA RATHBUNI*), ENDANGERED

The goal for Texas blind salamanders (TBS) is 500 standing-stock individuals distributed between the two facilities (SMARC and UNFH). Historically, Texas blind salamander catches were infrequent, and in 2017 projections indicated it would take up to 10 years to reach the standing stock goal. In 2019, there was a surge in the occurrence of small juvenile Texas blind salamanders collected from February to

Figure 4. Shawn Moore pulling up the Diversion Spring net in Spring Lake.

September from the Diversion Spring net in Spring Lake, San Marcos, Texas. This surge greatly and quickly increased refugia stock at the SMARC to over 250 animals with more than 50% of the refugia stock comprised of this age class. Some individuals of this age class were transferred to the UNFH. Numbers incorporated, end of the year census, and survival rates can be found in Table 11.

Table 11. Texas blind salamander refugia population figures

	Beginning of Year Census	Incorporated 2025	End of Year Census	In Quarantine End of Year	Target Goal 2025 Work Plan	Percent Survival
SMARC	105	14	97	1	250	82
UNFH	57	0	55	0	60	96

Survival rate = (end of year census / (start of year inventory + # incorporated)))*100. Survival rate does not include any mortality during quarantine period.

COLLECTIONS

Texas blind salamanders are collected from caves, wells, fissures, and driftnets on high flow springs. Traps are typically deployed quarterly in Primer's Fissure, Johnson's Well, Rattlesnake Cave, and Rattlesnake Well. Traps are checked two to three times weekly for two to three weeks before being removed from the site. To avoid over-sampling, only one third of salamanders observed are retained for refugia. Any gravid females are retained due to their rarity.

In 2025, Primer's Fissure and Johnson's Well were both sampled in February, March, May, July, and August. Only Johnson's Well was sampled in November due to low water during the month. In total, 24 TBS were captured from Primer's Fissure, 4 new individuals and 0 recaptures, with 3 (3 new individuals and 4 recaptures) retained and transferred to the SMARC for incorporation into the refugia. 20 TBS were captured from Johnson's Well, 16 new individuals and 4 recaptures, with 9 (1 new individual and 3 recaptures) retained and transferred to the SMARC for incorporation into the refugia. All newly encountered salamanders were tagged with a p-chip and tail clipped for genetic analysis. No movement has been observed between Johnson's Well and Primer's Fissure.

In 2025, the drift net over Diversion Spring was deployed from February to November. In total, 0 TBS were captured in the net. Neither Rattlesnake Cave nor Rattlesnake Well were sampled in 2025.

QUARANTINE

Texas blind salamanders were transported directly to the quarantine space at the SMARC after collection. The quarantine area is a separate, biologically secure area away from the refugia systems, preventing the spread of disease and aquatic nuisance species. Salamanders were acclimated to quarantine water conditions over the course of several hours after arrival. All newly collected larvae and juveniles were held in individual, isolated tanks at the SMARC. Each tank received its own flow of fresh well water and habitat items. Animals remained in isolation for at least 30 days. Healthy individuals measuring 30 mm or greater in total length (TL) were non-lethally cotton swabbed to test for disease. Weak, injured, or very small individuals were not swabbed until they had recovered and/or reached 30 mm TL. When animals resided in a group tank, representative swab samples were taken for the group and tested for the presence of *Batrachochytrium dendrobatidis* (Bd, commonly referred to as amphibian chytrid fungus) and *Batrachochytrium salamandrivorans* (Bsal, another type of lethal chytrid fungus). Bd is common in North America, but Bsal has not yet been observed here. Bsal is known to be lethal for at least one *Eurycea* species (*E. wilderae*; Martel et al 2014). Texas blind salamanders were housed in quarantine according to their collection location, collection date, and size.

HUSBANDRY

Texas blind salamanders from all collection locations were housed together and individuals were tagged via p-Chip tags so that individual identification was possible. Corbin (2020) completed a genetic analysis of wild-caught Texas blind salamanders and showed low genetic diversity and no genetic differentiation between sampling locations. Thus, Texas blind salamanders do not have to be separated in the refugia by collection site. Texas blind salamanders were housed in large, insulated fiberglass systems at the SMARC and the UNFH

with either flow-through or partial recirculation tanks. Water temperature and flow were checked multiple times daily. Total dissolved gas and pressure was checked immediately if salamanders begin showing symptoms of gas bubble disease, including the presence of trapped air bubbles underneath the skin, bloating, or an inability to stay submerged. Water quality parameters including dissolved oxygen, pH, and total gas pressure were checked weekly.

Habitat enrichment items, including natural and artificial rock, plastic plants, and mesh were placed throughout the tanks for salamanders to explore and seek refuge. Staff routinely siphoned tanks to remove waste and other debris and replaced habitat items with clean ones. Each tank system had dedicated equipment (nets, cleaning supplies) to prevent the potential spread of pathogens from system to system. If equipment was ever shared, it was cleaned and disinfected between systems. Upon reaching 30 to 40 mm in TL, juveniles were marked with p-Chip tags (for individual identification) under sedation and were combined with other individuals of equivalent sizes. The tags allow for identification of individuals, assess sex, and obtain collection information.

Adult salamanders were fed twice weekly and received either live amphipods, live blackworms, live red composting worms, live *Daphnia*, or frozen mysis shrimp. Juveniles were fed *Artemia* spp. nauplii or chopped blackworms as they increased in size.

SURVIVAL RATES

The survival of all Texas blind salamanders was 82% at the SMARC and 96% at the UNFH in 2025. Survival rates during quarantine period are not included in annual survival rates.

HEALTH MONITORING

Biologists monitored salamanders for changes in appearance and behavior including emaciation, bloating, lethargy, discoloration, development of external lesions or ulcers, mechanical damage, and abnormal swimming or walking. Salamanders that were sick or injured were removed from group housing and placed in isolated, individual hospital units with flow-

through well water. Mortalities were preserved in ethanol and a veterinarian was consulted, if needed, for investigation into the cause of death.

MAINTENANCE OF SYSTEMS

Salamander refugia systems were deep cleaned annually with 20-30% vinegar (SMARC) or muriatic acid (UNFH) to remove calcium carbonate deposits that formed within the tank, plumbing, chiller, or pump casing. Water lines, hoses, valves, and restrictors were frequently checked for degradation or occlusion. These were cleared, rebuilt, or replaced as needed.

CAPTIVE PROPAGATION

Due to a surplus of F1 animals from previous years, SMARC and UNFH did not propagate Texas Blind Salamanders this year. There are more Fx captive bred Texas blind salamanders in Refugia; approximately 200 salamanders. Staff will continue to assess whether or not there is a need for F1 animals and will resume propagation in the future when appropriate.

SAN MARCOS SALAMANDER (*EURYCEA NANA*), THREATENED

The Standing Stock goal for the San Marcos salamander (SMS) is 500 individuals, divided between the two facilities. In 2024, the number of collections for the refugia was reduced due to a mark-recapture study being conducted. The number of collection events was increased in 2025 to collect salamanders across the year instead of collecting large numbers in fewer events. Numbers incorporated, end of the year census, and survival rates can be found in Table 12.

Table 12. San Marcos salamander refugia population figures

	Beginning of Year Census	Incorporated 2025	End of Year Census	In Quarantine End of Year	Target Goal 2025 Work Plan	Percent Survival
SMARC	249	111	242	0	250	67
UNFH	134	191	249	0	250	77

Survival rate = (end of year census / (start of year inventory + # incorporated)))*100. Survival rate does not include any mortality during quarantine period.

COLLECTIONS

In 2025, refugia staff conducted seven collection events from Spring Lake and the Eastern spillway of the San Marcos River. The Eastern spillway was sampled four times, in May and October. 166 SMS were captured, retained, and transferred to the SMARC for incorporation into the refugia via dip net collections. San Marcos salamanders were passively collected from the drift net over Diversion Spring in Spring Lake, San Marcos, Texas. The drift net was installed from February to November in 2025. A total of 66 SMS were captured in the drift net, 61 of which were retained and transferred to the SMARC for incorporation into the refugia. USFWS dive team collected 74 SMS at Diversion Springs during a single collection event. In total, 306 SMS were collected in 2025. 85 were transferred to the SMARC and 208 to the UNFH for incorporation into the refugia.

QUARANTINE

Figure 5. Shawn Moore swabbing salamanders for testing.

Salamanders were transported directly to the quarantine areas of the respective facilities after collection. The quarantine areas are separate, biologically secure areas away from the refugia systems, preventing the spread of disease and aquatic nuisance species. Salamanders were acclimated to quarantine water conditions over the course of several hours after arrival. Healthy individuals collected from the wild were transported back to the SMARC where they were measured, and mucus samples were taken from those with a TL of 30 mm or greater with cotton swabs. Weak, injured, or very small individuals were not swabbed until they had recovered and/or reached 30 mm TL. For groups of salamanders, a representative sample was swabbed. Skin swabs were tested for presence of *Batrachochytrium dendrobatidis* (Bd, commonly referred to as amphibian chytrid fungus)

and *Batrachochytrium salamandrivorans* (Bsal). San Marcos salamanders were housed in quarantine according to their collection date and size. Individuals remained in quarantine for a minimum of 30-days under observation before being added to Standing Stock numbers.

HUSBANDRY

Genetic analysis (Lucas *et al*. 2009) determined that there is no population structure across sites sampled in the wild, so individuals from all collection locations were combined. San Marcos salamanders at both facilities were housed in large, insulated fiberglass systems with either flow-through chilled well water (SMARC) or partial recirculation through heater-chiller units (UNFH) to maintain water temperature at 21 ±1 ℃. Water temperature and flow were checked daily. Total gas pressure was checked immediately if salamanders began showing symptoms of gas bubble disease, including the presence of trapped air bubbles underneath the skin, bloating, or an inability to stay submerged. Water quality parameters including, but not limited to, dissolved oxygen, pH, and total gas pressure, were checked weekly.

Habitat enrichment items, including natural and artificial rock, plastic plants, and mesh were placed throughout the tanks for salamanders to explore and in which to seek refuge. Staff routinely siphoned tanks to remove waste and other debris and rotated habitat items to be cleaned. Each tank system had dedicated equipment (nets, cleaning supplies) to prevent the potential spread of pathogens from system to system. If equipment was ever shared, it was cleaned and disinfected between systems. Adult salamanders were fed twice weekly and received either live amphipods, live blackworms or frozen mysis shrimp. Juveniles were fed *Artemia* spp. nauplii or chopped blackworms as they increased in size. A detailed description of salamander care can be found in the USFWS Captive Propagation Manual for *Eurycea* spp., available upon request.

SURVIVAL RATES

The survival rate of San Marcos salamanders in the refugia population was 67% at the SMARC and 77% at the UNFH. Survival rates during their quarantine period are not included in

the annual survival rates. Declines in egg bound mortality were observed at both UNFH and SMARC.

HEALTH MONITORING

Biologists monitored salamanders for changes in appearance and behavior including emaciation, bloating, lethargy, discoloration, development of external lesions or ulcers, mechanical damage, and abnormal swimming or walking. Salamanders that became sick or injured were removed from group housing and placed in isolated, individual hospital units with flow-through well water. Mortalities were preserved in ethanol and a veterinarian was consulted, if needed, for investigation into the cause of death.

MAINTENANCE OF SYSTEMS

Salamander refugia systems at both UNFH and the SMARC were deep cleaned annually with muriatic acid to remove calcium carbonate deposits that formed within the tank, plumbing, chiller, and pump casing that can affect functionality. Water lines, hoses, valves, and restrictors were frequently checked for wear and clogs and were cleared, rebuilt, or replaced as needed.

CAPTIVE PROPAGATION

Neither UNFH nor SMARC propagated SMS to produce an F1 generation in 2025. Staff will continue efforts in the future when needed.

COMAL SPRINGS SALAMANDER (*EURYCEA PTEROPHILA*), NO LONGER PETITIONED

The Comal Springs salamander is a species covered in the EAHCP when it was designated as *Eurycea* sp. 8. At the time of writing the EAHCP, this species was undescribed yet petitioned for listing under the Endangered Species Act (ESA). Devitt et al. (2019) evaluated genetic markers and considered *Eurycea* sp. 8 at Comal Springs to be *Eurycea pterophila* (Blanco Springs salamander). Whether the Comal Springs population has unique standing is yet to be determined. The U.S. Fish & Wildlife Service no longer considers the Comal Springs salamander a petitioned species. Nevertheless, Congress defined ESA “species” to include subspecies,

varieties, and, for vertebrates, distinct population segments. For the purposes of the contract with the EAA, the Comal Springs population of *E. pterophila* will be considered as the Comal Springs salamander, and the refugia will continue to provide protection for this species as required under the EAHCP.

The Standing Stock goal for the Comal Springs salamander is 500 individuals, equally divided between the two facilities (SMARC and UNFH). Collections to augment the refugia population of Comal Springs salamanders have been limited by lower historical densities of Comal Springs salamanders in the currently used sampling locations as compared to sampling locations of San Marcos salamanders via observations of biologists and biomonitoring data. Lower densities in sampling locations should not be taken as a comment or speculation on overall population size. As total refugia population targets are approached, especially for Texas blind salamanders, opportunities to expand efforts to collect Comal Springs salamanders will increase. Numbers incorporated, end of the year census, and survival rates can be found in Table 13.

Table 13. Comal Springs salamander refugia population figures

	Beginning of Year Census	Incorporated 2025	End of Year Census	In Quarantine End of Year	Target Goal 2025 Work Plan	Percent Survival
SMARC	80	10	74	0	150	82
UNFH	64	1	1	0	135	2

Survival rate = (end of year census / (start of year inventory + # incorporated)))*100. Survival rate does not include any mortality during quarantine period.

COLLECTIONS

In 2025, staff conducted one sampling event for CSS in May. One CSS was captured during this event and was transferred to the UNFH for incorporation into the refugia population. Ten CSS were donated to the SMARC in July. These individuals were collected for behavioral studies but were never used in any studies. The lab was moving out of state and the salamanders donated to the Refugia. Historically low flows on the Comal River made finding salamanders difficult and led to a poor collection year for CSS.

QUARANTINE

In 2025, after collection all Comal Springs salamanders were transported directly to the quarantine facilities at the UNFH or SMARC. The quarantine areas are separate, biologically secure areas away from the refugia systems, preventing the spread of disease and aquatic nuisance species. Salamanders were acclimated to quarantine water conditions over the course of several hours after arrival. Individuals were measured and mucus samples taken from those with a TL of 30 mm or greater with cotton swabs. Weak, injured, or very small individuals were not swabbed until they had recovered and/or reached 30 mm TL. For groups of juveniles, a representative sample was swabbed. Skin swabs were tested for presence of *Batrachochytrium dendrobatidis* (Bd, commonly referred to as amphibian chytrid fungus) and *Batrachochytrium salamandrivorans* (Bsal). Comal Springs salamanders were housed in quarantine according to their collection date and size. Individuals remained in quarantine for a minimum of 30-days under observation before being counted towards Standing Stock numbers.

HUSBANDRY

Comal Springs salamanders at both facilities were housed in large, insulated fiberglass systems with partial recirculation through heater-chiller units to maintain the water temperature at 21℃ (ranging between 20 to 23 ℃). Water temperature and flow were checked daily. Total gas pressure was checked immediately if salamanders began showing symptoms of gas bubble disease, including the presence of trapped air bubbles underneath the skin, bloating, or an inability to stay submerged. Water quality parameters including dissolved oxygen, pH, and total gas pressure, were checked weekly.

Habitat enrichment items, including natural and artificial rocks, plastic plants, and mesh, were placed throughout the tanks for salamanders to explore and seek refuge. Staff routinely siphoned tanks to remove waste and other debris and rotated habitat items to be cleaned. Each tank system had dedicated equipment (nets, cleaning supplies) to prevent the potential spread of pathogens from system to system. If equipment was ever shared, it was cleaned and disinfected between systems. Adult salamanders were fed twice weekly and received either live amphipods, live blackworms or frozen mysis shrimp. Juveniles were fed *Artemia* spp. nauplii

or chopped blackworms as they increased in size. A detailed description of salamander care can be found in the USFWS Captive Propagation Manual for *Eurycea* spp.*,* available upon request.

SURVIVAL RATES

Survival rates of Comal Springs salamanders in 2025 were 82% at the SMARC and 2% at the UNFH. Poor survival at UNFH is directly related to the gasification event that occurred in May of this year. This led to the loss of the wild stock population of Comal Salamanders at UNFH.

HEALTH MONITORING

Biologists monitored salamanders for changes in appearance or behavior including emaciation, bloating, lethargy, discoloration, development of external lesions or ulcers, mechanical damage, and abnormal swimming or walking. Salamanders that became sick or injured were removed from group housing and placed in isolated, individual hospital units with flow-through well water. Mortalities were preserved in ethanol and a veterinarian was consulted, if needed, for investigation into the cause of death.

MAINTENANCE OF SYSTEMS

Salamander refugia systems at both UNFH and the SMARC were deep cleaned annually with muriatic acid to remove calcium carbonate deposits that have formed within the tank, plumbing, chiller, and pump casing that can affect functionality. Water lines, hoses, valves, and restrictors were frequently checked for wear and clogs and were cleared, rebuilt, or replaced as needed.

CAPTIVE PROPAGATION

During 2025 SMARC and UNFH did not hatch any F1 Comal Salamanders.

The standing-stock goal for Texas wild-rice (TWR) is 430 plants divided between the two facilities. Texas wild-rice is divided into alphabetical river segments (A-K) of the San Marcos River based on historical locations of bridges, dams and other structures (Richards et al. 2007). Richards *et al*. (2007) and Wilson *et al*. (2017) assessed the genetic diversity of TWR in the San Marcos River from samples taken in 1998, 1999, 2002, and 2012. They also evaluated genetic diversity of TWR plants held at the SMARC. Wilson et al. (2017) found three unique genetic clusters of TWR plants in the San Marcos River but found that each of these clusters were represented in all the sections sampled in the study. Both studies suggested follow-up genetic monitoring to ensure that refugia populations continue to represent wild populations. In addition, genetic monitoring of refugia population can determine if individual plants are genetically identical, thus calling for the removal of one of the clones and the collection of a genetically distinct wild plant. A follow-up genetic analysis of the TWR population in the San Marcos River and in the UNFH and SMARC refugia was completed in 2021. Results showed unique genetic clusters within the river and that the refugia populations were genetically similar to wild populations. The Refugia Program aims to preserve the genetic diversity of refugia TWR by collecting tillers from plants throughout the river so that the refugia populations reflect the wild population. Refugia staff specifically targeted plant stands that were not currently represented in the refugia population. Plant stands were selected after overlaying refugia plant locations (determined with GPS) onto GIS maps produced by the SMARC Plant Ecology Program during the 2019 annual Texas wild-rice Survey. Numbers incorporated, end of the year census, and survival rates can be found in Table 15.

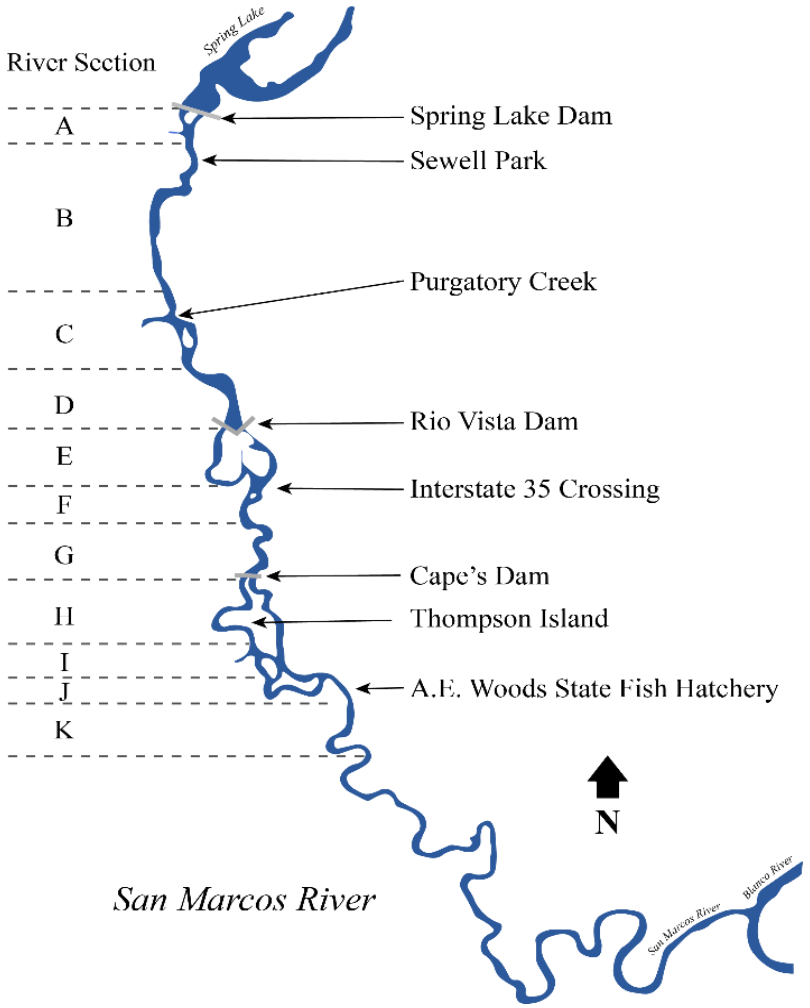


Figure 6. Lettered sections of the San Marcos River designating Texas wild-rice habitat established by Texas Parks and Wildlife Department.

Table 15. Texas wild-rice refugia population figures

	Beginning of Year Census	Incorporated 2025	End of Year Census	In Quarantine End of Year	Target Goal 2025 Work Plan	Percent Survival
SMARC	191	45	160	20	215	68
UNFH	132	205	205	15	215	61

Survival rate = (end of year census / (start of year inventory + # incorporated)))*100. Survival rate does not include any individuals in quarantine or mortalities during quarantine period.

COLLECTIONS

In 2025, refugia staff conducted eight collection events for TWR in April, May, July November and December. Staff collected 1585 tillers from 317 plants. Five tillers were collected from each plant. Of the 317 plants collected from, plants were transferred to the SMARC and 230 plants were transferred to the UNFH. USFWS staff collected tillers by hand from plant stands. During collection, the location of the TWR plant stand was recorded with a Global Positioning System (GPS) device. In addition, staff recorded the percent coverage and the river section for each plant stand collected. This information was collated in a central database maintained at the SMARC and UNFH. Tillers were placed in marked mesh bags and immersed in coolers filled with fresh river water for transport back to their respective facilities.

QUARANTINE

Upon arrival at each respective facility, tillers (still grouped by individual plant) were rinsed in fresh well water and inspected for any aquatic nuisance species. The Lead Biologist is in the process of standardizing the quarantine procedures between stations. Currently, staff start tillers in air pruning pots with the use of lava rock to allow for water circulation through the quarantine period. This year UNFH staff began using pipe insulating foam tubes to transport plants. Staff observed the foam tubes provide more support to the stems of the plant during transport and protect against damage. After the success of this transport method, the TWR SOP will be updated and this method will be used across stations to transport TWR. After transportation, staff pot the plants into air pruning pots with lava rock for a quarantine period. During the quarantine time, they were routinely checked for aquatic nuisance species, specifically the invasive snail *Melanoides tuberculata.* After 30 days, plants were un-potted and the full plant visually inspected for aquatic nuisance species, before the tillers were re-potted and incorporated into the standing stock population.

HUSBANDRY

The potting mix that is in use at both stations now is a 2 parts sand, 2 parts topsoil and 1 part gravel mix. Below the mix is a layer of lava rock and above is a layer of gravel. The lava rock protects against anoxia of the plant from the bottom while the gravel on top protects against algae growth. As in previous years, when plants were added to refugia tanks, the inventory and map of plants in the tank were updated. Hand-count inventory and tag checks were conducted twice annually.

SURVIVAL RATES

Overall survival rate of TWR plants at the SMARC was 68%. The overall survival rate of TWR plants at the UNFH was 61%. Algae continued to be the main concern in the summer months. UNFH has switched to cleaning tanks daily. This has improved overall rice survival. Tank pumps and water delivery were changed to better enhance the flow regime. The first four months of an individual plants time in the refuge is a difficult process as the plant establishes a root system and new growth. Not every plant will succeed in establishing in the Refugia and the result is lower survival rates (< 70%). Over time as the refuge population becomes dominated by more established plants survival rates should improve significantly as the plants become more robust.

MAINTENANCE OF SYSTEMS

Water flow in the tanks was checked daily and standpipe screens were cleaned to ensure that no debris blocked water flow through the pumps at both stations. TWR tanks at the SMARC had individual heater-chiller units on tanks with 2 HP main pumps and 1/3HP accessory pumps to circulate water through units and produce flow throughout the tanks. At the UNFH, 1/2 to 3/4 HP submersible pumps are used to facilitate flow throughout the tanks. At UNFH pumps are now placed in the center and back of each tank to promote a laminar flow regime to stimulate plant movement.

Staff removed filamentous algae from the leaf blades by gently running fingers or a mesh net across the surfaces of each plant. Algae was removed from tanks, pumps, stairs and

water intakes daily by scrubbing and floating debris was removed manually using mesh nets or siphons. TWR leaves were routinely trimmed to approximately 30 inches to prevent overcrowding and shading in tanks. Staff trimmed off emergent vegetation, so that the genetic integrity of each plant is maintained. Plants were housed very close together and it would be difficult to prevent cross-pollination between plants from different river sections if allowed to emerge and flower. Shade cloth was used over TWR tanks at the SMARC during the summer months to control algal growth in tanks.

CAPTIVE PROPAGATION

The EARP did not engage in propagation of TWR by sexual reproduction through seed production in 2025. However, the Plant Ecology and Restoration Program at the SMARC engaged in TWR plant propagation and continues to study and refine techniques.

RESEARCH

Research activities for the Refugia program (USFWS and sub-contractors) focused on genetic assessments of San Marcos salamander, expanding on a 2023-2024 mark-recapture study on the San Marcos Salamander, captive propagation of Peck's cave amphipod and refining a devise that will allow for thermal tolerance testing of covered species. Much of this research was built on knowledge gained in previous studies. Below are summaries for each project approved within the 2025 Work Plan (Appendix A).

MARK AND RECAPTURE OF SAN MARCOS SALAMANDERS USING PHOTOS

The objective of this study is to build on the p-Chip mark and recapture project that examined the recapture rate, movement rate, and estimated population size of wild San Marcos salamanders. This effort using pictures of the unique markings on their heads as a "tag" instead of a physical tag (i.e. p-Chips). In May and June 2023, 453 San Marcos salamanders were tagged with p-Chips and released back to their collection locations at three sites in San Marcos, Texas, just downstream of the eastern spillway of the Spring Lake Dam, around the Diversion Springs pipe in Spring Lake, and at the headwaters area of Spring Lake. During this effort, photos were taken of all tagged and recaptured salamanders. Recapture collections occurred 1-2 times each month at each of the sites for a year (May 2023-May 2024). 3,469 salamanders were collected during this time. A full body photo and two closeup photos of the head were taken for all salamanders. In total, 10,407 photos were collected. The p-Chip tagging effort resulted in a recapture rate across sites of 14%, varying 10-21%. No movement was detected across sites. Research efforts in 2025 focused on editing the photos to isolate the head, excluding the gills. A subset of the photos (880 photos) from recapture collections with known recaptured p-chipped

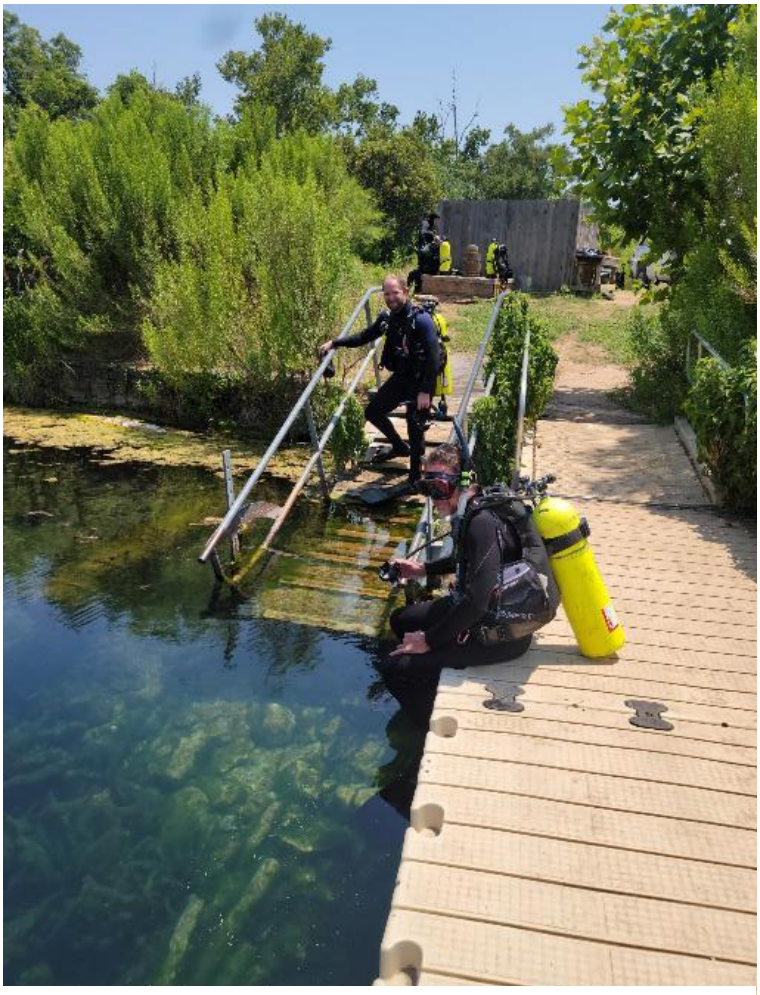


Figure 7. Justin Crow and Randy Gibson (SMARC biologists) preparing to dive to collect San Marcos salamanders in Spring Lake.

individuals were run through Wild.ID to determine if the unique markings on the salamanders' heads would identify the same recaptured p-chipped individuals (Figure 8).

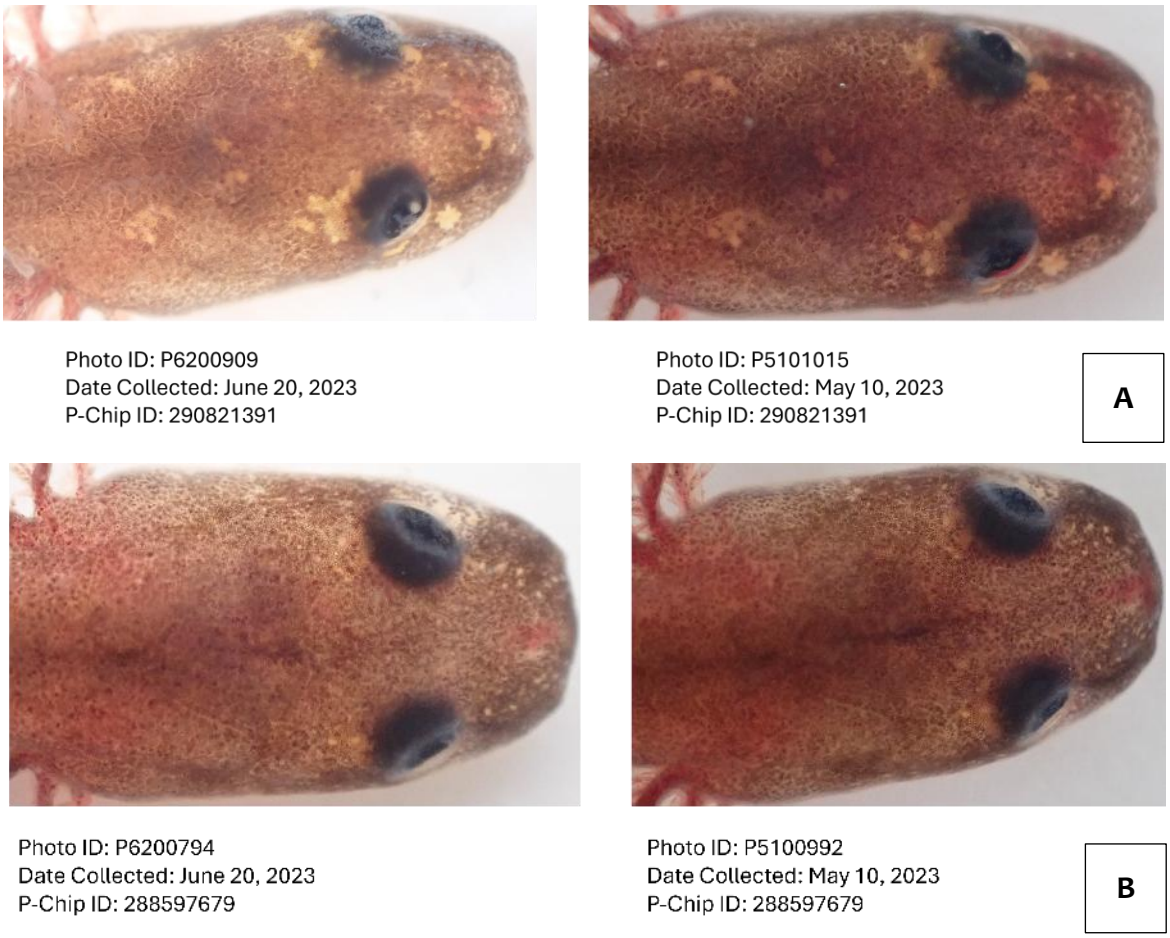


Figure 8. Photos of San Marcos salamanders used to identify recaptured individuals at Diversion Springs, Spring Lake, San Marcos, TX. A and B are two different individuals. Each individual was tagged with a p-chip in May 2023 and recaptured one month later in June 2023.

The p-chip tagged salamanders were identified from the photos, showing individuals can be identified using unique markings on their heads. In addition to validating the method and identifying known recaptured salamanders, new untagged salamanders were identified as recaptures. Some p-chip salamanders did lose their tags throughout the 2023-2024 collections but the photos were able to identify them as recaptures (Figure 9).

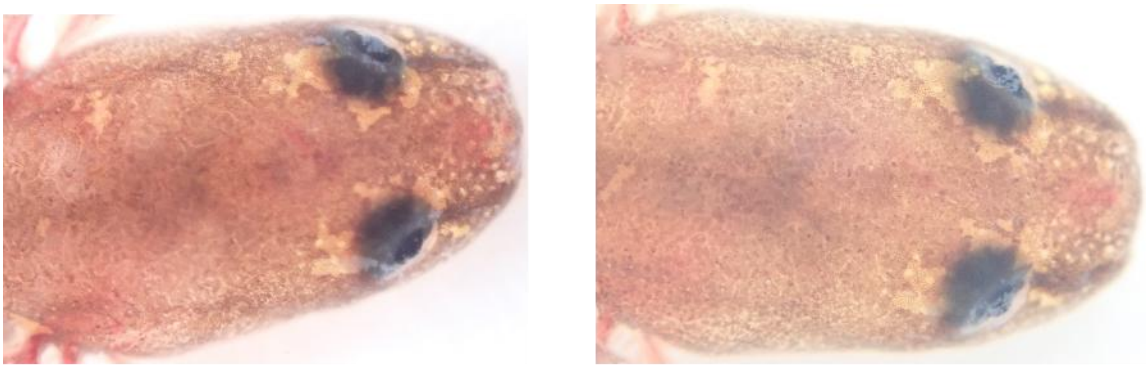


Photo ID: P6200890
Date Collected: June 20, 2023
P-Chip ID: 290711507

Photo ID: P3130161
Date Collected: March 13, 2024
P-Chip ID: N/A

Figure 9. Photo of a p-Chip tagged San Marcos salamander used to identify this individual salamander as a recapture even after losing their p-Chip. This individual was tagged in May of 2023, recaptured in June of 2023 with their p-chip and then recaptured 9 months later in March 2024 without their p-Chip.

This research will continue into 2026 to expand the sample size of the p-chip study from 453 salamanders to over 3,000 salamanders. The drastic increase in sample size will provide significant statistical power to provide the best chance of detecting movement between locations and to further refine population size estimates. The interim report is in Appendix B.

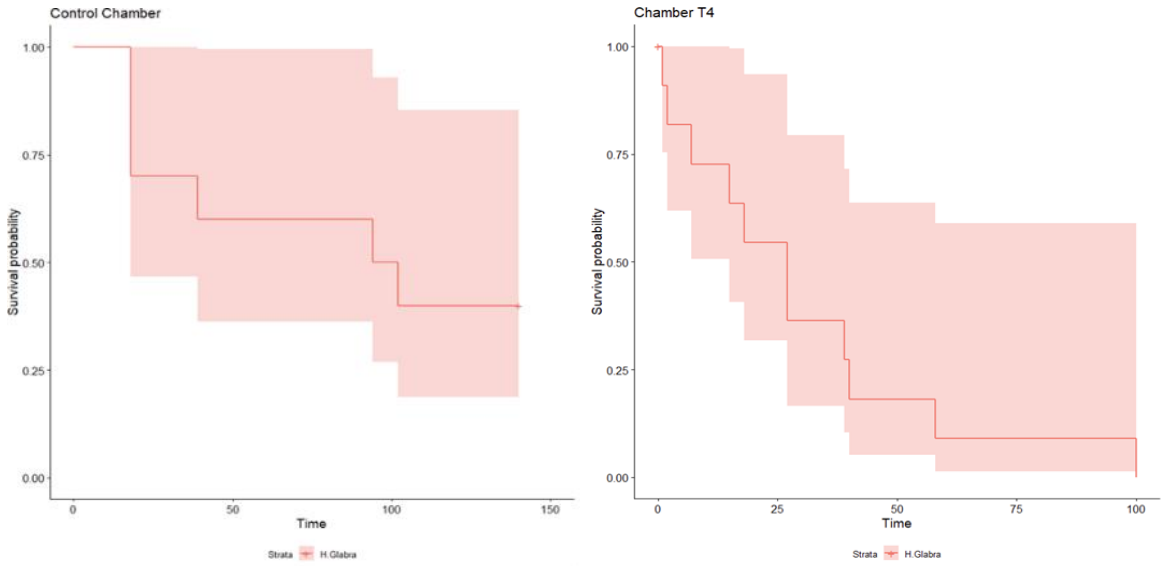
TAGGING AQUATIC INVERTEBRATES

Figure 10. A Comal Springs riffle beetle tagged with a p-Chip.

Determining tagging methodology for unique species is important for conducting research to inform the refugia and reintroduction methods. Dr. Shannon Brewer of the U.S. Geological Survey, Alabama Cooperative Fish and Wildlife Research Unit led this cooperative effort where the objectives were to: 1) evaluate the attachment of p-Chips and short-term tag retention on Comal Springs riffle beetle and Peck's cave amphipod and 2) determine longer-term retention of the tag and survival of the tagged animals. Tagging for PCA was largely unsuccessful. Hyalella texana was used as a surrogate species when testing BEEtags. They

initially recovered quickly from tagging and moved around without much trouble but ultimately did not survive post tagging. Most of this effort focused on CSRB where initial testing showed a greater potential for success. A tagging protocol was designed for Comal Springs riffle beetle by chilling the beetle for two minutes and using superglue to affix the tag to the elytra of the beetle. The beetle quickly regained activity as it was warmed by the microscope light and was able to walk with no obvious hindrance from the tag. *Heterelmis glabra* was used as a surrogate species for this study. Beetles were placed in passive

Figure 11. Image of the tagged housing with two flow through tubes connected by a clear laser field where p-chip tagged beetles are scanned as they move from one chamber to the other.



monitoring tubes with a p-chip scanner that would record beetles as they moved across the laser field (Figure 11). Survival was recorded over 100 days. P-chip tagged beetles died at a significantly faster rate than non-tagged control beetles (Figure 12). The final report, which also includes information on PCA and CSRB tagging using BEEtags, is in Appendix C.

DEVELOPING TOOLS TO DETERMINE THERMAL TOLERANCES AND PREFERENCE OF COVERED HCP SPECIES

In 2025, Enzo Silvagni and Dr. David Huffman (Texas State University) worked to modify and refine a Thermal Biology Assessment Devise (TBAD) that will allow USFWS to test thermal maximum and minimum tolerances as well as preferred thermal ranges for Edwards Aquifer Habitat Conservation Plan covered species. The Thermal Biology Assessment Device is a novel apparatus designed to generate a linear, stable, and rapidly responsive thermal gradient in a straight trough of water, enabling simultaneous assessment of thermal preference, critical thermal minimum, (CTmin), and maximum (CTmax) for a variety of aquatic species. The TBAD employs counter-current heat exchange (CCHE) principles, using submerged conduit assemblies with heated and cooled fluids flowing in opposite directions to establish a predictable temperature gradient along the length of the trough. The whole trough is separated into two functional sections; the bottom half of the trough holds the CCHE conduits

Figure 12. Heterelmis glabra Kaplan—Meier survival curves, post tagging. 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. Control group (left) vs Tagged group (right). Both groups were handled the same but the control group did not receive tags while the tagged group did. X-axis is number of days of the trial, y-axis is probability of survival measured by passive scanning (tagged group) and physical counts (control group).

Figure 13. Image of the TBAD while under construction.

and drives the gradient, the upper half consists of a testing arena where organisms are held and observed during the thermal biology assessment. Most species of interest are local spring dependent and spring associated organisms. The ecological research collected with the TBAD on these species of interest could provide critical conservation or captive management improvement strategies. Thermal tolerance testing on HCP covered species is planned for 2026. A final report for the construction of the TBAD is in Appendix D.

PECK'S CAVE AMPHIPOD CAPTIVE PROPGATION THROUGH PHYSICAL NEONATE REMOVAL AND PASSIVE EXCLUSION

Successful captive propagation of PCA has been historically rare in the Refugia. PCA release around 10 eggs per brood and may reproduce multiple times throughout their lives. Female PCA are known to be cannibalistic when held in captivity, even when fed in excess. Recent observations suggest that gravid female amphipods may extract newly hatched offspring (neonates) from their marsupium utilizing their extended pereopods. The cannibalistic behavior of adult PCA presents challenges to a successful captive breeding program.

Preliminary experimentation of manual separation began at the Uvalde National Fish Hatchery at the start of 2024 to determine manual separation warranted a larger research effort. Preliminary results suggested more works should be done in 2025. Gravid females were discovered during quarterly inventories and housed separately. Females were checked weekly under the microscope to monitor the

Figure 14. Manual removal of neonates from a gravid female. Panel A shows microdissection tools being used to brace the gravid female with a blunt end probe while a blunt microhook is used to sweep the neonates out of the brooding pouch. Panel B shows the freshly removed neonate and the adult female. Panel C shows the neonate one month post removal.

development of the neonates. Once neonates are visibly moving in the egg and measuring around 0.95-1mm they were removed using dull probes and housed separate from the mother to avoid predation. During this study, one neonate was successfully separated from the female and monitored. As of the conclusion of this study, the neonate is active and growing (Figure 14). Unfortunately, the female died two weeks post removal.

Along with the manual removal method, a passive exclusion chambers was designed, with three designated chambers. Gravid female PCA were placed in the first chamber and neonates had the option to move into the next Chamber where they are physically separated from the adult by mesh screen too small for the adult to pass through. Two trials with 5 replicates each were completed. The number of eggs and neonates for each female was monitored. Overall, no neonates were collected via passive exclusion. The number of eggs and neonates reduced over time until there were none remaining. The mean number of days female PCA remained gravid was 19 days but ranged between 2 – 55 days. This effort will continue in 2026 under general husbandry work. The final report is located in Appendix E.

GENETIC ASSESSMENT OF SAN MARCOS SALAMANDERS

In 2024, DNA was collected from wild San Marcos salamanders that were collected during the 2023-2024 p-Chip mark and recapture study. 453 salamanders were sampled across three sites in Spring Lake and San Marcos River (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 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. 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). An interim report for this effort was included in the 2024 annual report. 2025 research plans included collecting additional tail clips from the Refugia population and Fx captive bred individuals to include in the full genetic assessment. Data analysis and reporting was to be conducted by Dr. Chris Nice and Dr. Kate Bell (Texas State University). Unfortunately, Texas State University and USFWS were unable to establish a cooperative agreement to fund these efforts. Negotiations were carried out concerning significant increases in Texas State University's overhead rate. Data analysis will now occur in 2026 using the same methods used for the 2024 PCA and CSRB genetic assessments. No interim report is included in the 2025 annual report for this project.

REINTRODUCTION

The EARP conducted its first salvage and reintroduction operation for fountain darters in the Comal River on May 24, 2025. Spring flows for the Comal River were dropping consistently over the month of May and fountain darter habitat declined (Figure 15). Dr. Katie Bockrath, Mike Montagne (SMARC), Randy Gibson (SMARC), Garrison Engstrom (Student Conservation Association), and Braden West collected 1604 fountain darters in danger of being

stranded by receding water levels (Figure 16) at the Spring Island area of the Comal River, New Braunfels, TX.

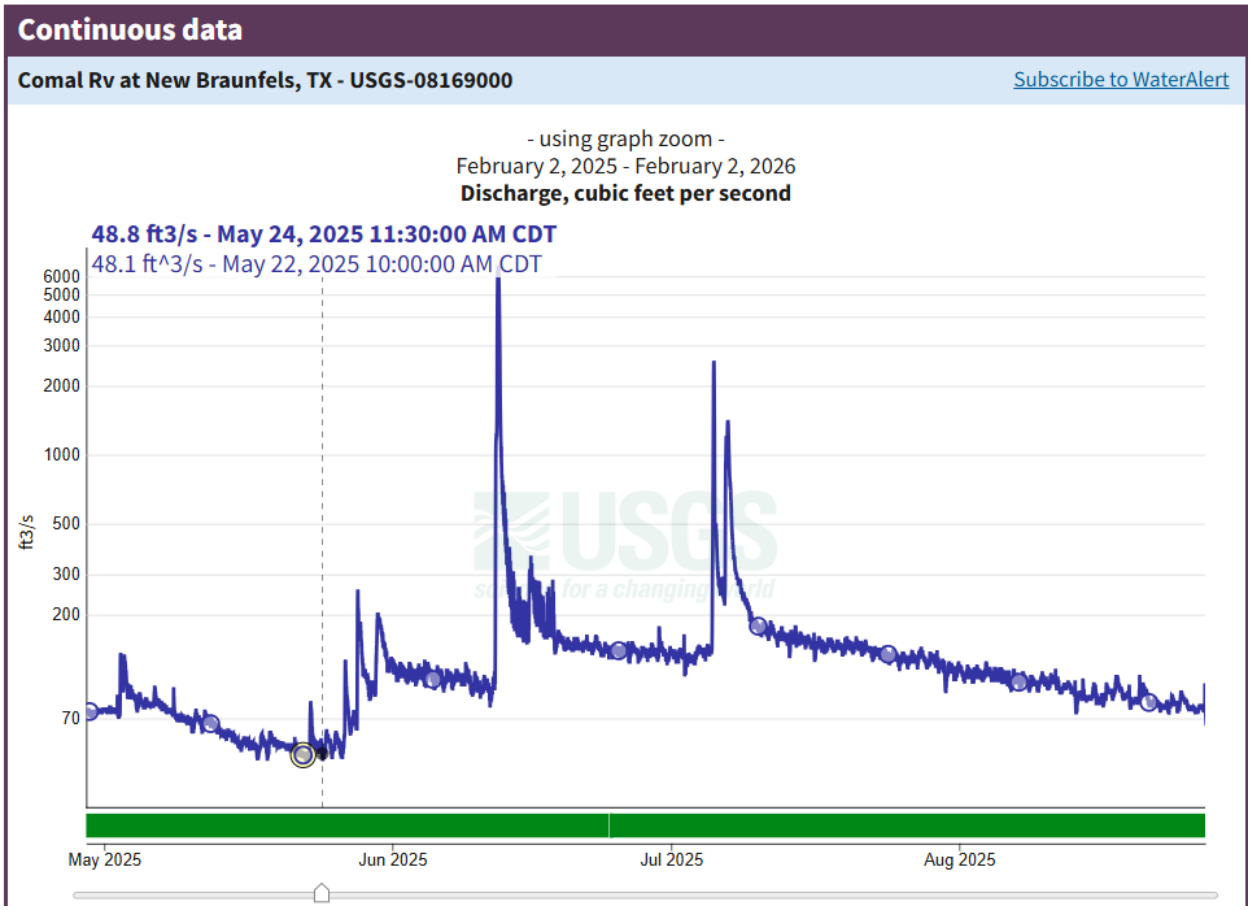


Figure 15. USGS River Gauge 08169000 showing 48.8 ft3/s on May 24, 2025 when fountain darters in the Comal River were salvaged due to extremely low flow.

Staff used long-handled dip nets to collect fish from fragmented pools of water in the swimming area (Figure 16). Due to heat and unfavorable stream conditions, staff collected fish in the morning to minimize additional thermal stress to fountain darters. Once collected, staff transported fish to the SMARC using fish transport bags supplied with supplemental oxygen. Fish were packed into transport bags that were limited to a density of 100 fish per bag.

Figure 16. The swimming area at Spring Island experienced severely reduced water levels in May 2025.

The fountain darters were transported to the SMARC in coolers and were placed in pre-constructed partially recirculating raceway systems with chillers set to 21°C ±1°C. These chillers were necessary to maintain ideal temperatures in the outdoor raceways during the summer months. Several volunteers assisted in this project by constructing in-tank habitat from PVC pipe, while EARP staff administered salt to the fountain darters as a therapeutic treatment. EARP staff administered 3 g/L salt treatments for periods of one hour daily for three days following collection.

These fountain darters were cared for and fed a diet consistent with established EARP animal husbandry protocols. Mortalities were promptly removed and preserved in 95% ethanol for potential future analysis. The fountain darters were fed multiple times daily to enhance feed delivery and provide feeding opportunities to all individuals in the tanks. Their diet consisted of *Artemia* nauplii, *Daphnia magna*, and *Lumbriculus* worms. Excess food and waste were removed daily via siphoning, and tank walls were scrubbed weekly to remove algal buildup.

These fountain darters were held in captivity at the SMARC from May to November. Staff monitored the United States Geological Survey stream gauge 08169000 and air temperatures in order to evaluate habitat quality at the collection sites for potential reintroduction dates. Staff gathered weekly water quality data such as dissolved oxygen, temperature, salinity, and pH using a YSI ProDSS multiprobe sonde at the Spring Island sites to monitor for further reductions in suitable fountain darter habitat.

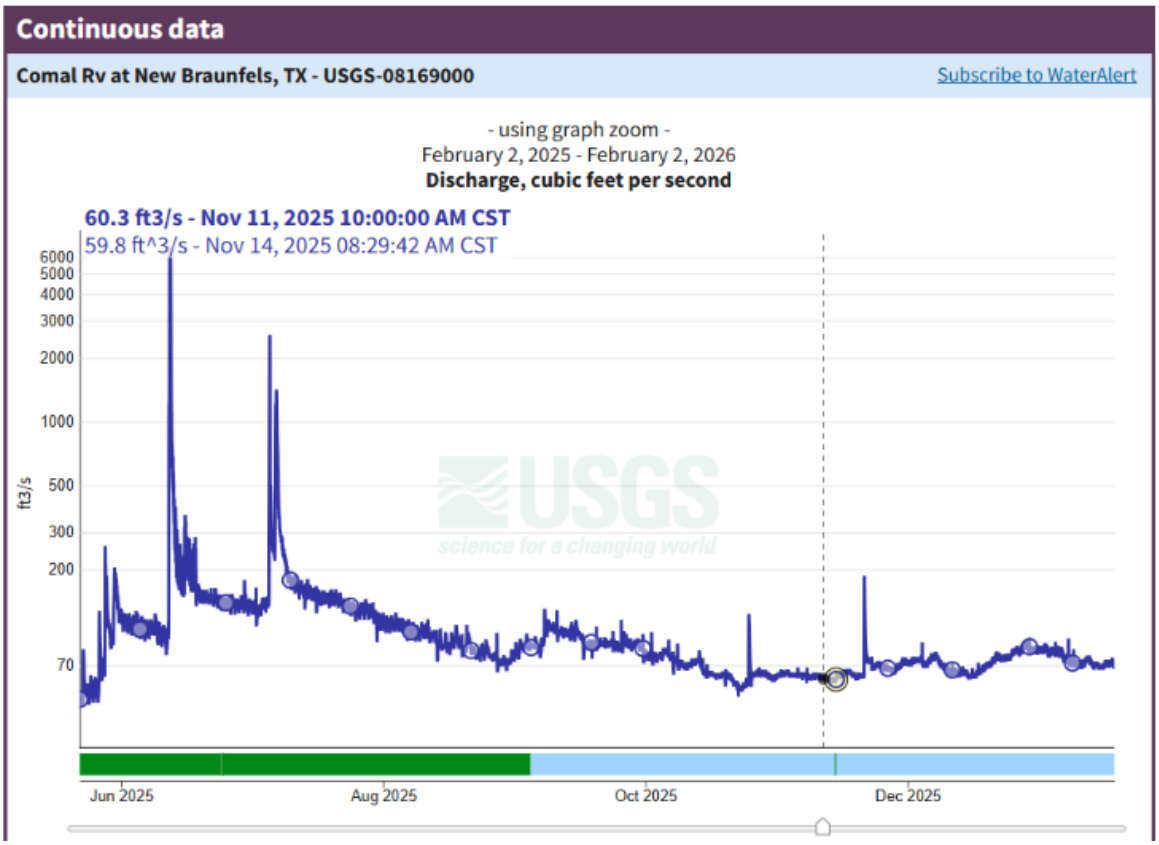


Figure 16. USGS River Gauge 08169000 showing 60.3 ft3/s on May 24, 2025 when fountain darters were reintroduced to the Comal River. On site flow measurements showed the flow was 63.3 ft3/sec at the Spring Island reintroduction site.

The USFWS EARP reintroduced fountain darters (Texas Parks and Wildlife Department Permit to Introduce Fish, Shellfish or Aquatic Plants into Public Waters No. INT 25 07-01e) on November 11, 2025, following periods of stable discharge from Comal Springs as well as little to no sign of continued habitat degradation (Figure 16). In conjunction with Megan Bean (U.S. Fish and Wildlife Service, Austin Ecological Services Field Office), Kristy Kollaus Smith, EARP staff

transported fountain darters collected in May back to the Spring Island site (Figure 17). fountain darters were transported in fish transport bags using SMARC well water supplied with supplemental oxygen as well as over the counter aquarium water conditioner to reduce transport stress. A total of 500 fountain darters were placed in transport bags that were limited to a density of 100 fish per bag and stocked at the Spring Island area.

The bags of fountain darters were transported to the Spring Island site in coolers. Staff tempered water in the transport bags with water from the Comal River to align bag water temperature and dissolved oxygen with local water conditions and chemistry (Figure 18). Using a YSI ProDSS multiprobe sonde to monitor water chemistry in the transport bags, staff determined that when water chemistry in the bags matched the destination, and the water temperature was within ±1°C of the destination that staff could reintroduce of fountain darters back into the Comal River. Transport bags were released by hand one at a time (Figure 19). Staff carried bags of fish into the river and slowly emptied the contents into the river. There were no observed mortalities during the reintroduction event.

Figure 17. EAA and USFWS staff reintroducing 500 fountain darters to the Comal River. Shown from right to left: Kristy Smith (EAA), Dr. Katie Bockrath (USFWS), Braden West (USFWS), Marisol Farias (SCA) and Megan Bean (USFWS).

Figure 17. Dr. Katie Bockrath and Braden West acclimating fountain darters before releasing them to the Comal River.

Figure 18. Braden West released fountain darters back into the Comal River by immersing the transport bags in river water and slowly releasing all fish in the bag.

US. Fish and Wildlife Service 2025			
Task	Description	2025 Work Plan Amounts	2025 Actuals
1	**Refugia Operations**		
	SMARC Refugia & Quarantine Bldgs		
	Building Maintenance	$5,000.00	$ -
	Utilities	$10,500.00	$ 6,729.77
	UNFH Refugia & Quarantine Bldgs		
	Building Maintenance	$5,000.00	$ -
	Utilities	$30,000.00	$ 32,602.68
	Salary		
	SMARC Species Husbandry and Collection	$130,000.00	$ 125,528.69
	UNFH Species Husbandry and Collection	$287,000.00	$ 306,568.45
	Purchases		
	Fish Health	$8,000.00	$ 2,179.26
	SMARC Reimbursables for Husbandry and Collections	$104,000.00	$ 51,195.44
	UNFH Reimbursables for Husbandry and Collections	$121,484.00	$ 100,461.83
	Subtotal	$700,984.00	$ 625,266.12
	Admin Cost Subtotal	$168,237.00	$ 137,558.55
	Task 1 Total	**$869,221.00**	**$ 762,824.67**
2	**Research**		
	Partnered Research		
	Texas State University: SMS Genetics	$34,000.00	$0.00
	Texas State University: Thermal Tolerance	$12,390.00	$12,390.38
	Auburn University: Invertebrate Tagging	$19,000.00	$18,705.90
	Texas State University: PCA 2024 Rollover	$8,790.00	$8,789.18
	University of Texas: Gene Expression 2024 Rollover	$49,704.00	$49,704.00
	BIO-WEST Drypoid 2024 Rollover	$9,602.00	$9,601.83

	FWS Research			
	FWS Salary	$170,000.00		$139,699.31
	Materials	$5,000.00		$9,631.51
	Subtotal	$308,486.00	$	239,854.11
	Admin costs for Task 2 (23% Overhead)	$74,037.00	$	52,767.91
	Task 2 Total	**$382,523.00**	**$**	**292,622.02**
5	**Reporting**			
	SMARC Staff	$17,600.00		$6,288.75
	UNFH Staff	$13,000.00		$11,793.00
	Subtotal	$30,600.00	$	18,081.75
	Admin costs for Task 5	$7,344.00	$	3,977.99
	Task 5 Total	**$37,944.00**	**$**	**22,059.74**
6	**Meetings and Presentations**			
	SMARC Staff	$8,000.00		$5,520.65
	UNFH Staff	$6,000.00		$2,390.74
	Subtotal	$14,000.00	$	7,911.39
	Admin costs for Task 6	$3,360.00	$	1,740.51
	Task 6 Total	**$17,360.00**	**$**	**9,651.90**
	Totals	**$1,307,048.00**	**$**	**1,087,158.31**

ACRONYMS AND ABBREVIATIONS

Bd	*Batrachochytrium dendrobatidis*
Bsal	*Batrachochytrium salamandrivorans*
CSDB	Comal Springs dryopid beetle
CSRB	Comal Springs riffle beetle
EAA	Edwards Aquifer Authority
EAHCP	Edwards Aquifer Habitat Conservation Plan
ESA	Endangered Species Act
FAC	Fish & Aquatic Conservation Program
GIS	Geographic information system
GPS	Global positioning system
HP	Horsepower
ITP	Incidental take permit
JGI	Joint Genome Institute
LHRH	Luteinizing hormone releasing hormone
LMBV	Largemouth bass virus
PCA	Peck's cave amphipod
PIT	Passive integrated transponder
PVC	Polyvinyl chloride
USFWS	U.S. Fish & Wildlife Service
SCUBA	Self-contained underwater breathing apparatus
SFHU	Southwestern Fish Health Unit
SMARC	San Marcos Aquatic Resources Center
TL	Total length
TWR	Texas wild-rice
TXST	Texas State University
UNFH	Uvalde National Fish Hatchery
VIA	Visible implant alpha-numeric
VIE	Visible implant elastomer
WAAS	Wide area augmentation system

WORKS CITED

Corbin, A. B., 2020. Population genomics and conservation of Texas cave and spring salamanders (Plethodontidae: *Eurycea*). Dissertation. University of Texas at Arlington.

Devitt, T. J., Wright, A. M., Cannatella, D. C., and Hillis, D. M., 2019. Species delimitation in endangered groundwater salamanders: Implications for aquifer management and biodiversity conservation. Proceedings of the National Academy of Sciences, 116(7), 2624–2633. doi:10.1073/pnas.1815014116.

Hall, R., 2016 Comal Springs riffle beetle SOP work group: attachment 2: existing CSRB cotton lure SOP.

Kosnicki, E., and E. Julius. 2019. Life-history aspects of *Stygobromus pecki*. Report for U.S. Fish and Wildlife Service, San Marcos Aquatic Resources Center, San Marcos, Texas.

Lee NK, K. W., Paik HD. 2019. Bacillus strains as human probiotics: characterization, safety, microbiome, and probiotic carrier. Food Science and Biotechnology 28(5):1297-1305.

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

Martel, A., M. Blooi, C. Adriaensen, P. Van Rooij, W. Beukema, M. C. Fisher, R. A. Farrer, B. R. Schmidt, U. Tobler, K. Goka, K. R. Lips, C. Muletz, K. R. Zamudio, J. Bosch, S. Lotters, E. Wombwell, T. W. J. Garner, A. A. Cunningham, A. Spitzen-van der Sluijs, S. Salvidio, R. Ducatelle, K. Nishikawa, T. T. Nguyen, J. E. Kolby, I. Van Bocxlaer, F. Bossuyt, and F. Pasmans. 2014. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science 346(6209):630–631. doi:10.1126/science.1258268.

McDonald, D., T. H. Bonner, E. L. Oborny, and T. M. Brandt. 2007. Effects of Fluctuating Temperatures and Gill Parasites on Reproduction of the Fountain Darter, *Etheostoma fonticola*. Journal of Freshwater Ecology 22:2, 311-318.

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

Richards, C. M., M. F. Antolin, A. Reilley, J. Poole, and C. Walters. 2007. Capturing genetic diversity of wild populations for ex situ conservation: Texas wild-rice (*Zizania texana*) as a model. Genetic Resources and Crop Evolution 54:837–848.

U.S. Fish and Wildlife Service. 2021. Endangered and threatened wildlife and plants; removal of 23 extinct species from the lists of endangered and threatened wildlife and plants. Federal Register 86(187):54298-54338.

Wilson, W. D., J. T. Hutchinson, and K. G. Ostrand. 2017. Genetic diversity assessment of in situ and ex situ Texas wild-rice (*Zizania texana*) populations, an endangered plant. Aquatic Botany 136:212-219.

PUBLISHED MANUSCRIPTS

Dobbins B.A., Ruben U. Tovar, Braden J. Oddo, Thomas J. Devitt, David M. Hillis, Dana M. García. 2024. "PAX6 protein in neuromasts of the lateral line system of salamanders (Eurycea)." PLoS One. 2024 Aug 30;19(8):e0293163. DOI: 10.1371/journal.pone.0293163

Moore, D.M, Gillis, M.D., Funk, T.S. 2024. Evaluation of p-Chip microtransponder tags on small-bodied salamanders (Eurycea spp.). Amphibian & Reptile Conservation. 18(1&2):10-19(e330).

Nice CC, Bell KL, Gompert Z, Lucas LK, Ott JR, Tovar RU, Crump P, Diaz PH. Extensive Admixture Among Karst-Obligate Salamanders Reveals Evidence of Recent Divergence and Gene Exchange Through Aquifers. Ecol Evol. 2025 Jan 9;15(1):e70785. doi: 10.1002/ece3.70785. PMID: 39803198; PMCID: PMC11717725.

PROFESSIONAL PRESENTATIONS FROM STAFF AND COLLABORATORS

Alvear, D.A. 2024. It is getting hot out here: So out come all the parasites?!. Third Annual Fish and Aquatic Conservation Science Symposium, Region 2, USFWS. - Awarded Early Career Award

Bockrath, K.D. 2024. Genetic Assessment of Comal Springs Riffle Beetles. EAHCP Comal Springs Riffle Beetle Work Group Meeting. Edwards Aquifer Authority, San Antonio, Texas.

Bockrath, K.D. 2024. Genetic Assessment of Comal Springs Riffle Beetles. EAHCP End of Year Joint Committee Meeting. Edwards Aquifer Authority, San Antonio, Texas.

Moore, S.E. 2024. The historical Fountain Darter tissue archive. Texas Conservation Symposium, Georgetown, Texas.

Moore, S.E. 2024. The historical Fountain Darter tissue archive. Third Annual Fish and Aquatic Conservation Science Symposium, Region 2, USFWS.

West, B. 2024. 2024 State of Groundwater Invertebrate Culture in the EARP. Texas Groundwater Invertebrate Forum, San Marcos, Texas.

Ruben U. Tovar, Brittany Dobbins, Thomas J. Devitt, Dana M. Garcia, David M. Hillis. Independent Subterranean Invasions and Parallel Sensory Compensation in Texas Groundwater Salamanders (Eurycea): A Rising System for Evo-Devo. UT-EEB EcoLunch Seminar.

Kattan, GN, Sledge, RY, Tovar, RU, Devitt, TJ, Hillis, DM, García, DM. Eye loss during development in the Texas blind salamander. Research, Inquiry, and Creative Expression Poster Showcase, San Marcos, TX. (July, 27)

Kattan, GN, Sledge, RY, Tovar, RU, Devitt, TJ, Hillis, DM, García, DM. Eye loss during development in the Texas blind salamander. I.D.E.A Center student presentations, San Marcos, TX. (Aug. 8)

Sheena A. Leelani, Ruben U. Tovar, Thomas J. Devitt, Dana M. García, and David M. Hillis. Eye development in surface and subterranean Fern Bank salamanders (E. pterophila). Undergraduate Research Forum. (Aprl, 8)

Dan A. Tatulescu, Qainaat Merchant, Ruben U. Tovar, John J. Jacisin, Thomas J. Devitt, Dana M. García, and David M. Hillis. Quantifying disparate craniofacial morphology between the Texas blind salamander (E. rathbuni) and the San Marcos salamander (E. nana). Longhorn Research Poster Session. (April, 18)

Braden Oddo, Nisa Sindhi, Brittany Dobbins, Ruben U. Tovar, Thomas Devitt, David Hillis, Dana M. García. Characterization of neuromasts and PAX6 labelling in subterranean and surface salamanders (Eurycea nana, Eurycea sosorum, Eurycea rathbuni). Texas Conservation Symposium. (Jan. 18-19, 2024)

Dan A. Tatulescu, Qainaat Merchant, Ruben U. Tovar, John J. Jacisin, Thomas J. Devitt, Dana M. García, and David M. Hillis. Quantifying disparate craniofacial morphology between the Texas blind salamander (E. rathbuni) and the San Marcos salamander (E. nana). Texas Conservation Symposium. (Jan. 18-19, 2024)

Ryan Y. Sledge, Ruben U. Tovar, Thomas J. Devitt, David M. Hillis, and Dana M. García. Apoptosis in the retina and lens of E. rathbuni during development. Texas Conservation Symposium. (Jan. 18-19, 2024)

Miranda A. Contello, Katherine Bockrath, Ruben U. Tovar, Thomas J. Devitt, David M. Hillis, Dana M. García. Comparisons in development between salamanders of the genus Eurycea. Texas Conservation Symposium. (Jan. 18-19, 2024)

Sheena A. Leelani, Ruben U. Tovar, Thomas J. Devitt, Dana M. García, and David M. Hillis. Eye development in surface and subterranean Fern Bank salamanders (E. pterophila). Texas Conservation Symposium. BEST UNDERGRADUATE PRESENTATION. (Jan. 18-19, 2024).

Brittany A. Dobbins, Ruben U. Tovar, Thomas J. Devitt, David M. Hillis, and Dana M. García. Possible link between Pax6 expression and expansion of the lateral line system in salamanders of the genus Eurycea. Texas Conservation Symposium. (Jan. 18-19, 2024)

Ruben U. Tovar, Brittany Dobbins, Thomas J. Devitt, Dana M. Garcia, David M. Hillis. Society for Integrative and Comparative Biology (SICB). "Evolution underground: Sensory Compensation Parallels Eye Loss in Neotenic Salamanders (Eurycea)": ID # 1335 (Jan.2-7). Competing in: Division of Vertebrate Morphology Best Student Presentation.

APPENDICES

A. 2025 EA Refugia Work Plan
B. Mark and recapture of San Marcos salamanders using photos - Interim Report
C. Evaluating survival and tag retention of cave amphipods and Comal Spring Riffle Beetles - Final Report
D. Thermal Biological Assessment Devise (TBAD) – Final Report
E. Peck's cave amphipod captive propagation through passive exclusion and manual removal – Final Report
F. Monthly reports
G. USFWS Southwestern Native Aquatic Resources and Recovery Center Fish Health Unit reports

Edwards Aquifer Authority
2025 Work Plan

Amendment #4; approved by the Implementing Committee on May 22, 2025

2025 Edwards Aquifer Authority Work Plan Budget

EAHCP Section	Conservation Measure	Table 7.1	Estimated 2025 Budget[a]
5.1.1	Refugia	$1,678,597	$1,307,048
5.1.2	VISPO	$4,172,000[b]	$8,954,150[c]
5.1.3	RWCP	$493,250	$0
5.1.4	Stage V	NA	NA
5.5.1	ASR Leasing & Forbearance	$4,759,000	$5,689,162
	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	$2,111,508[e]
Total		$14,671,847	$19,132,642

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, 2024, the VISPO program was triggered, resulting in suspension payments totaling $8,954,150.
d. Includes Critical Period Monitoring if required.
e. Funding increase for additional programmatic costs

2025 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	5/19/2024	Original Work Plan	NA	NA	NA	Original Work Plan
1	10/13/2024	VISPO, Water Quality Monitoring, and Program Management	N	N	11/8/2024	Updated amount for VISPO suspension payments as well as updated Water Quality Monitoring and Program Management with known activities and 2025 costs
0	10/13/2024	Original Funding Application	NA	NA	11/8/2024	Original Funding Application
2	12/19/2024	Refugia	N	N	NA	Updated Refugia with known activities and updated 2025 research project costs
3	2/6/25	Refugia, ASR, and Applied Research	N	N	N	Updated Refugia and Applied Research sections with known activities and research efforts and ASR section with updated program numbers
4	5/22/25	Refugia, Applied Research, and Program Management	Y	$50,000	6/10/25	Updated Refugia and Applied Research sections with revised activities and research efforts as well Program Management section with updated costs
1	5/22/25	Funding Application	Y	$50,000	6/10/25	Increased Funding Application by $50,000 per Work Plan Amendment #4

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 2025, which provide for the maintenance of a refugia population of the Covered Species (Table 1), including salvage, propagation, and restocking of the species (if species-specific habitat triggers occur and species are extirpated), plus research conducted on the Covered Species.

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

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

Long-term Objective

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

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

2025 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 2025 (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 2025)	Anticipated SMARC census (Dec 2025)	Anticipated UNFH census (Jan 2025)	Anticipated UNFH census (Dec 2025)
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 2025, the USFWS will collect Covered Species as required to reach and maintain target standing and refugia stock numbers as shown in Table 2. If possible, the USFWS will avoid collections during July and August when temperatures are high and flow is low, resulting in increased stress for priority species during collections, with the exception of Texas blind salamanders and San Marcos salamanders collected from the Diversion Spring net in Spring Lake. 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 2025 collection, maintenance, and propagation efforts for each species.

Fountain Darters:

Collection: In 2025, the USFWS will collect fountain darters from the San Marcos River and the Comal River in coordination with the Spring and Fall Biomonitoring events. This will be more efficient than separate collection events and will reduce habitat disturbance. For refugia purposes, USFWS staff will retain fountain darters collected by biomonitoring staff via drop nets. Staff will collect fish proportionally from the three sections of the San Marcos River: 1) Upper = Spring Lake, 2) Middle = Spring Lake dam to Rio Vista dam, and 3) Lower = below Rio Vista dam to Cape's Dam. The USFWS will thoroughly investigate unusual mortality events. The USFWS will include summary reports to the EAA as part of the monthly reports. Collections will target sufficient fish so to account for regular, expected mortality, such that the captive population should remain at or above the target.

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

As part of quarantine procedures, the USFWS will send a subset of fish (maximum of 60 per river) to the Southwestern Fish Health Unit or equivalent facility for pathogen (bacteria, virus, and parasite) testing prior to incorporating collected animals into the general refugia population.

The USFWS will follow standardized methods outlined within USFWS and AFS-FHS (2016) and AFS-FHS (2005) protocols and provide Fish Health reports to the EAA.

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

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

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 fare well due to heat stress. In 2025, 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 genetic assessments of wild and refugia populations (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.

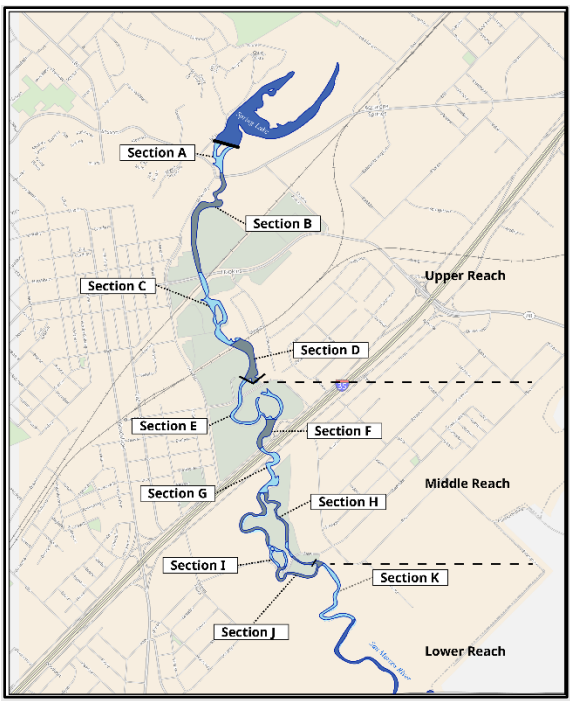


Figure 1. Letters define designated San Marcos River reaches where Texas wild-rice is collected for refugia populations.

Maintenance: USFWS staff will tag and maintain plants 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 plants collected from each geographical location. During reintroduction, staff will transplant 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 and Johnson's well (Table 5). To avoid oversampling these habitats, staff will only collect 1/2of salamanders observed from each of these locations during quarterly sampling events Texas blind salamanders will be checked for tail clips and/or p-Chips to collect reoccurrence data. Staff will also collect salamanders from a driftnet on Diversion Springs in Spring Lake throughout the year during times when we are not actively trapping in caves and wells. We will retain all specimens from this site, under the assumption that any Texas blind salamander leaving a spring orifice that enters a stream or lake environment will ultimately succumb to predation. We will check these sites up to three times per week when applicable. Staff will transport all specimens alive and

maintain them in the SMARC or UNFH refugia. Texas State University staff may check drift nets on Texas State University Artesian Well a few times a year for 14 consecutive days. 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 for no more than 5 years 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 culture systems to produce *Daphnia* on site.

Propagation: Staff will maintain standing and refugia stocks to encourage reproduction. Staff will maintain all progeny separately by generations. If reintroduction is warranted, an attempt will be made to produce offspring from adults collected at each geographical location and offspring will be reintroduced back to the geographic location once they are 30mm total length.

San Marcos salamanders:

Collection: USFWS staff will collect San Marcos salamanders in the Spring and Fall, avoiding breeding season and the hot summer months, from Hotel Springs, below Spring Lake dam

(Eastern Spillway) 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 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, 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 of adults from each geographic location to produce offspring. Staff will initiate stocking to each geographic once juveniles have reached 30 mm total length.

Comal Springs salamanders:

Collection: USFWS staff will collect Comal Springs salamanders monthly except July and August, from Spring Island and surrounding areas (Table 5) by hand, with dipnets, using snorkelers. Comal Springs salamanders are more difficult to collect than San Marcos salamanders and require more frequent collections to reach Standing Stock goals. We will coordinate with the HCP biological monitoring program to ensure to the degree practicable, refugia collections do not overlap with specific EAHCP long-term monitoring locales. If overlap 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, 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 incorporation into the general refugia population. Staff will retain duplicate swabs for no more than 5 years 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 culture systems to produce *Daphnia* on site.

Propagation: USFWS staff will maintain salamander standing stock 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 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 from 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 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 and geographic area. 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: USFWS staff will maintain Comal Springs riffle beetle standing stock in flowthrough tubes to encourage reproduction. If warranted, captive propagated larva will be reintroduced to each geographic area.

Peck's cave amphipod:

Collection: USFWS will conduct Peck's cave amphipod collection for standing stock seven times annually, if needed (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). EARP staff will avoid collecting the summer months.

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 conduct 10 dedicated collections for Comal Springs dryopid beetles, often coinciding with Peck's cave amphipod or Comal Springs salamander collections. Opportunistic collections will occur if dryopid beetles are present during Comal Springs riffle beetle lure checks. Drypoid beetles will be collected 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 2025. 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 2025			
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
January	1-3 day sampling event	Spring Island and Comal Springs	Comal Springs salamander
January	1-day sampling event	Spring Island	Peck's cave amphipods and Comal Springs dryopid beetle
January	3 days	Spring Lake, Eastern Spillway and Rio Vista dam	San Marcos fountain darters

Edward's Aquifer Species Collection Plan 2025			
Date (month)	**Interval**	**Location**	**Target Species**
January	3 days	Land Lake, Spring Island and Old Channel	Comal Springs fountain darters
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
February	1-3 day sampling event	Spring Island and Comal Springs	Comal Springs salamander
February	1 day sampling event	Spring Island	Peck's cave amphipod Comal Springs dryopid beetle
March	Collect Lures and reset	Spring Runs, Spring Island, Western Shore	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod
March	1 day sampling event, hand pick	Landa Lake	Peck's cave amphipod Comal Springs dryopid beetle
March	1-3 day sampling event	Spring Island	Comal Springs salamander
March	1 day sampling event, hand pick from downed wood	Landa Lake	Comal Springs dryopid beetle
April	Check 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
April	Collect Lures and check logs	Spring Runs, Spring Island, Western Shore	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod

Edward's Aquifer Species Collection Plan 2025			
Date (month)	**Interval**	**Location**	**Target Species**
April	1-2 day sampling event	Spring Lake and Eastern Spillway	San Marcos salamander
April	1 day sampling event	San Marcos River	Texas wild-rice
April	Throughout, coincide with bio-monitoring	San Marcos River	Fountain darters
April	Throughout, coincide with bio-monitoring	Spring Island and Landa Lake	Fountain darters
April	Drift net checked every 24-48 hours over two weeks.	Artesian Well	Texas troglobitic water slater, Edwards Aquifer diving beetle, Texas blind salamanders
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
May	1-3 day sampling event	Comal Springs	Comal Springs dryopid beetle and Comal Springs salamander
May	Drift net, donated from bio-monitoring	Comal Springs	Peck’s cave amphipod
May	Set lures	Spring Runs, Spring Island, Western Shore	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck’s cave amphipod
May	1-3 day sampling event	San Marcos River	San Marcos Fountain Darters
May	1-3 day sampling event	Spring Island and Landa Lake	Comal Springs Fountain Darters

Edward's Aquifer Species Collection Plan 2025			
Date (month)	**Interval**	**Location**	**Target Species**
June	1 day sampling event, hand pick	Landa Lake	Peck's cave amphipod Comal Springs dryopid beetle
June	1-3 day sampling event	Spring Island	Comal Springs salamander
June	Retrieve lures	Spring Runs, Spring Island, 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
September	Check nets M and Th every week	Diversion Springs	Texas blind salamander, San Marcos salamander
September	2 day sampling event	Hotel Springs and Eastern Spillway	San Marcos salamander
September	1 day sampling event, hand pick	Landa Lake	Peck's cave amphipod Comal Springs dryopid beetle
September	1-3 day sampling event	Comal Springs	Comal Springs salamander
September	Set lures	Spring Runs, Spring Island, 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	Throughout, coincide with bio-monitoring	Spring Island and Landa Lake	Fountain darters

Edward's Aquifer Species Collection Plan 2025			
Date (month)	**Interval**	**Location**	**Target Species**
October	Drift net, donated from bio-monitoring	Comal Springs	Peck's cave amphipod
October	Check nets M and Th every week	Diversion Springs	Texas blind salamander, San Marcos salamander
October	1 day sampling event	San Marcos River	Texas wild-rice
October	Retrieve Lures	Spring Runs, Spring Island, Western Shore	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod
October	1 day sampling event, hand pick from downed wood	Spring Runs, Landa Lake	Comal Springs dryopid beetle
October	1-3 day sampling event	Spring Island	Comal Springs salamander
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 Comal Springs dryopid beetle
November	Check nets M and Th every week	Diversion Springs	Texas blind salamander, San Marcos salamander
November	1-3 day sampling event	Comal Springs	Comal Springs salamander
November	Drift net, donated from bio-monitoring	Comal Springs	Peck's cave amphipod
November	Set lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod

Edward's Aquifer Species Collection Plan 2025			
Date (month)	**Interval**	**Location**	**Target Species**
December	Check nets Mand Th 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

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 2025.

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 and 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.

San Marcos Aquatic Resource Center and Uvalde National Fish Hatchery are committed to maintaining high quality captive husbandry systems to meet and maintain the contractual agreement of the Edwards Aquifer Refugia Program. Wear and tear on system components, such as chiller units, leads to failure over time. Additionally, the EARP will finish adding the Whalchem automatic monitoring controller units (purchased in 2024) to existing captive husbandry systems. The EARP will use unspent funds from Tasks 2, 5 and 6 to replace failing system components and to finish adding the controller units to existing systems. The total 2025 EARP budget remains within the contractual agreement of $1,307,048.

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.

Significant staffing changes have occurred between 2024 – 2025. Desiree Moore took another position within the USFWS, resulting in unspent 2024 funds in Task 2, 5 and 6. During the presidential mandate to reduce the government work force, Deputy Director Jennifer Howeth, Shawn Moore and Richelle Jackson took a Deferred Resignation Program offer and left the USFWS. Their resignation will result in less funds needed to cover EARP work in tasks 2, 5 and 6. With a government issued hiring freeze in place, theses vacant position will not be filled in 2025. Due to staffing changes, the EARP will be using underspend funds from Tasks 2, 5 and 6 to cover partial salary costs for Dr. Scott Walker (UNFH), Justin Crow (SMARC) and Somerley Swarm (SMARC) to cover EARP husbandry and collections duties while dedicated EARP staff were temporarily separated from the USFWS and after other dedicated EARP staff resigned from the USFWS. The total 2025 EARP budget remains within the contractual agreement of $1,307,048.

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:

Refining fountain darter prorogation protocol and determining expected propagation output - A fully functioning captive assurance population should have reliable and predictable captive propagation methods and projections. Previous work established captive propagation protocols for fountain darters, but this protocol had evolved and expectations for how many

offspring can be produce in a year and the minimum number of adults required to produce those offspring. EARP staff will work with a Student Conservation Association (SCA) intern to test the existing fountain darter propagation protocol and to update the protocol as needed. The SCA intern will monitor breeding fountain darters for the presence of eggs, remove eggs from the adults and grow out the eggs to fry stage. Fry feeding SOPs will also be tests and fry survival will be assessed.

Task 2. Research

The Research Plan for 2025 will, in part, be a continuation of 2024 research projects with the addition of new research efforts. Genetics assessments for the San Marcos will be concluded in 2025. Additional research will focus on advancements in captive propagation of Peck's cave amphipods as well as developing tools to investigate thermal tolerance/preference of current and future EAHCP covered species.

A reduction in work force and a hiring freeze across the Department of the Interior resulted in a staffing shortage in 2025. To ensure the Refugia husbandry and collections duties are sufficiently covered, some Refugia Research was necessarily pushed to 2026. This includes the Texas blind salamander genetic assessment started in 2024 which was anticipated to be completed in 2025.

Due to reduced staff and research load of 2025, the total cost for proposed 2025 research will be under the contractually agreed amount of $492,519 and will be $328,523. The total 2025 EARP budget remains within the contractual agreement of $1, 307,048. The following section describes the basic components of each of these proposed 2025 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	4	3	4
Texas wild-rice	5	5	5	5	5
Texas blind salamander	4	5	4	3	1
Peck's cave amphipod	5	4	2	4	1
San Marcos salamander	5	5	3	3	3
Comal Springs salamander	5	5	3	3	1
Comal Springs riffle beetle	5	5	4	4	3
Comal Springs dryopid beetle	4	3	2	2	1
Texas troglobitic water slater	1	1	0	1	1
Edwards Aquifer diving beetle	1	0	0	0	1

Project 1:

Title: Developing tools to determine thermal tolerances and preferences of current and future covered HCP species.
Species: *multiple*
Principal: Dr. David Huffman and Enzo Silvagne (Texas State University)
Overview: A few studies have investigated temperature tolerance in EAHCP covered species. Unfortunately, these studies do not use standard practices, are not very precise and have only investigated high temperatures. A variable temperature gradient prototype was developed by the Huffman laboratory that allows for the stable establishment of a very precise and customizable temperature gradient to investigate thermal tolerances. In 2025, funds will be used to build version two of the apparatus to conduct future thermal tolerance studies.
Budget:

- Texas State University: $12,390
- USFWS: $40,000
- Total: $53,390

Benefit to the Refugia: Establishing thermal tolerances improves refugia housing conditions by determining the extent of acceptable fluctuation in water temperatures maintained by heater/chiller units, especially during extreme outdoor temperature conditions. Additionally, investigating the extent of each species thermal tolerances can provide information about how species may tolerate low flows and extreme temperature conditions in the wild.
Expected Results: A report will be presented to the EAA, and a peer-reviewed publication will be generated, if appropriate.

Project 2:

Title: Continuation of Genetic Assessment of San Marcos salamanders
Species: *Eurycea nana*
Principal: Dr. Chris Nice and Dr. Kate Bell (Texas State University)
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 2023-2024 Mark Recapture tagging study. These tail clips were used to assess wild genetic diversity. Tail clips were also collected from standing stock and captive propagated salamanders in the refugia and 2025 research efforts will focus on collecting and analyzing genetic data for captive held and propagated individuals. High-throughput sequencing will be used to assess genetic variation of wild caught and Fx captive breed Texas blind salamanders.
Budget:

- Texas State University: $34,000
- USFWS support: $40,000
- Total: $74,000

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 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 3:

Title: Assessing the effectiveness of using pictures for mark and recapture of San Marcos salamanders.
Species: *Eurycea nana*
Principal: USFWS
Overview: in 2023-2024, the EARP conducted a year long mark and recapture effort using pChip transponders in the San Marcos salamander. The study was very successful and produced information on recapture rates, movement, population demographics, and estimates of population size. 453 salamanders were tagged with pChips and over 3000 salamanders were collected during the study. Pictures are taken of Barton Springs salamander heads for mark and recapture efforts, but this method has yet to be tested for San Marcos salamanders and it is unknown if variation in pigmentation/markings on their heads are suitable or consistent enough for reliable mark and recapture studies. Due to the relatively small number of individuals who were tagged with p-chip, population estimates may be underestimated and movement between locations may have gone undetected. This study aims to expand on the pChip mark and recapture study and analyze the photos to determine if this method is effective and if movement can be identified and population estimates can be improved.
Budget: $96,500
Benefit to the Refugia: Population estimates can inform how many individuals are required to maintain in the Refugia to ensure the reintroduction efforts are successful, if they were to occur. Isolation, or lack of movement, between sites informs collection efforts and breeding pairs for maintaining diversity in the refugia and reintroductions.
Expected Results: A report will be presented to the EAA, and a peer-reviewed publication will be generated, if appropriate.

Project 4:

Title: Peck's Cave Amphipod Captive Propagation
Species: *Stygobromus pecki*
Principal: USFWS
Overview: A fully functioning captive assurance population should have reliable and predictable captive propagation methods and projections. The biggest challenge with captive propagation of Peck's cave amphipods is the high rate of cannibalism of offspring by adults, including the female brooding the offspring. Small pilot studies have investigated means to reduce cannibalism and exclude offspring from adults, some with promising results. This study expands on these pilot studies to fully test exclusion setups and the efficacy of physical extraction of offspring from the brood pouch.
Budget: $90,000
Benefit to the Refugia: Reliable captive propagation is the first step to establishing a

means to reintroduce a species if necessary, which is especially important for short-lived species like Peck's cave amphipods. By establishing protocols and/or housings that allow offspring to avoid cannibalism, EARP staff can begin to assess reproduction rates and offspring survival in captivity, which will lead to establishing a predictive captive propagation program.

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

Project : 5

Title: Tagging Aquatic Invertebrates

Species: *Microcylloepus pusillus or Heterelmis vulnerata* (surrogate for *Heterelmis comalensis*)

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 Comal Springs riffle beetle through external attachment.

Budget:

- Auburn University Support: $19,000
- FWS Support: $40,000
- Total: $59,000

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.

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

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 and improvements to captive holding and propagation practices,

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 2025: 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 2025 EAHCP Annual Program reporting– A year-end report of 2025 activities will be provided to the EAA no later than 1/31/2026.
5.5 Program reporting as required by ITP and TPWD. TPWD Scientific Research Permit Report will be filed July 31, 2025.
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:

US. Fish and Wildlife Service 2025		
Task	Description	2025 Work Plan Amounts
	Refugia Operations	
	SMARC Refugia & Quarantine Buildings	
	Building Maintenance	$5,000.00
	Utilities	$10,500.00
	UNFH Refugia & Quarantine Buildings	
	Building Maintenance	$5,000.00
	Utilities	$30,000.00
	Salary	
1	SMARC Species Husbandry and Collection	$130,000.00
	UNFH Species Husbandry and Collection	$287,000.00
	Purchases	
	Fish Health	$8,000.00
	SMARC Reimbursables for Husbandry and Collections	$104,000.00
	UNFH Reimbursables for Husbandry and Collections	$121,484.00
	Subtotal	$700,984.00
	Admin Cost Subtotal (24% Overhead)	$168,237.00
	Task 1 Total	**$869,221.00**

2	**Research**	
	Partnered Research	
	Texas State University: SMS Genetics	$34,000.00
	Texas State University: Thermal Tolerance	$12,390.00
	Auburn University: Invertebrate Tagging	$19,000.00
	BIO-WEST Dryopid 2024 Rollover	$9,602.00
	Texas State University: PCA Genetics 2024 Rollover	$8,790.00
	University of Texas: Gene Expression 2024 Rollover	$49,704.00
	FWS Research	
	FWS Salary	$170,000.00
	Materials	$5,000.00
	Subtotal	$308,486.00
	Admin costs for Task 2 (24% Overhead)	$74,037.00
	Task 2 Total	**$382,523.00**
5	**Reporting**	
	SMARC Staff	$17,600.00
	UNFH Staff	$13,000.00
	Subtotal	$30,600.00
	Admin costs for Task 5 (24% Overhead)	$7,344.00
	Task 5 Total	**$37,944.00**
6	**Meetings and Presentations**	
	SMARC Staff	$8,000.00
	UNFH Staff	$6,000.00
	Subtotal	$14,000.00
	Admin costs for Task 6 (24% Overhead)	$3,360.00
	Task 6 Total	**$17,360.00**
	Totals	**$1,307,048.00**

Projected (2025) Budget Summarized by Task:

Task 1: $869,221
Task 2: $382,523
Task 3: $0
Task 4: $0
Task 5: $37,944
Task 6: $17,360

Projected (2025) Subcontractor Expenses Summarized by Task

Task 1: $0
Task 2: Texas State SMS Genetics: $34,000
Task 2: Texas State Thermal Tolerance: $12,390
Task 2: Auburn University: $19,000
Task 2: Texas State PCA Genetics 2024 Rollover: $8,790
Task 2: University of Texas Gene Expression 2024 Rollover: $49,704
Task 2: BIO-WEST Dryopid 2024 Rollover $9,602
Task 3: $0
Task 4: $0
Task 5: $0
Task 6: $0

Timeline of 2025 Milestones

January	Subcontracted research awards executed
	2025 Specific Research Study Plans finalized
July	Submit and renew TPWD permit
September	Draft Research Reports
December	Draft Annual report

Literature Cited

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

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

Corbin, A. B. 2020. Population genomics and conservation of texas cave and spring salamanders (Plethodontidae: Eurycea). University of Texas, Arlington, Texas.

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

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

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

5.1.2 Voluntary Irrigation Suspension Program Option

Long-term Objective:

The goal of VISPO is to enroll 41,795 acre-feet (AF) of permitted irrigation rights (base and/or unrestricted) that will remain unused in years of severe drought based on the USFWS approved 2019 amendment. Permit holders are enrolled in five-year and ten-year VISPO agreements and will be compensated based on the amount of water enrolled and the program selected. Permit holders enrolled in 10-year agreements are paid a standby fee of $70.20/ac-ft per year every year of the term regardless of aquifer conditions and an additional fee of $210.60/ac-ft per year will be paid for each year when temporary pumping suspensions are required. Permit holders enrolled in 5-year agreements are paid a standby fee of $54/ac-ft per year every year of the term regardless of aquifer conditions and an additional fee of $160/ac-ft per year will be paid for each year when temporary pumping suspensions are required. On December 31, 2025, over 3,161 acre-feet in VISPO agreements will expire and those permit holders are currently being offered the opportunity to renew their VISPO forbearance agreements prior to their expiration.

If the water level at the J-17 index well in San Antonio is at or below 635 feet on October 1 of any year, program participants are contractually obligated to suspend the use of their enrolled water for the following year - beginning on January 1. On October 1, 2024, the J-17 index well was reported to be at 629 feet msl, therefore triggering suspension of use of enrolled water in VISPO by participating permit holders in year 2025. Annual VISPO payouts through 2024 are reflected in Table 5.1.2-1.

Table 5.1.2-1: VISPO Total Payout by Year

Year	Payment Type	Total Enrolled (AF)	Total
2014	Stand-by	22,388	$1,201,938
2015	Stand-by + Suspension	40,921	$8,677,262[a]
2016	Stand-by	40,921	$2,208,723

2017	Stand-by	40,921	$2,228,299
2018	Stand-by	40,921	$2,320,309
2019	Stand-by	39,646	$2,341,927
2020	Stand-by	39,803	$2,508,070
2021	Stand-by	41,795	$2,509,975
2022	Stand-by	41,795	$2,509,975
2023	Stand-by + Suspension	41,795	$9,987,533[b]
2024	Stand-by + Suspension	41,795	$8,936,926[c]
		Grand Total	$45,430,937

a. 2015 payment breakdown: Standby $2,169,315; Suspension $6,507,947
b. 2023 payment breakdown: Standby $2,509,975; Suspension $7,477,576
c. 2024 payment breakdown: Standby $2,331,876; Suspension $9,253,167

Target for 2025:
The total volume goal of 41,795 ac-ft in VISPO agreements will continue to be maintained and managed by EAA staff. Throughout 2025, staff will continue to work on renewing 28 VISPO agreements totaling 3,161 acre-feet that will expire on December 31, 2025. VISPO suspension payments will be made to program enrollees by March 1, 2025.

Budget:
Table 7.1:
$4,172,000

2025 budget for Suspension:
$8,954,150

5.1.3 Regional Water Conservation Program

Long-term Objective:
Conservation measures will be implemented to conserve 20,000 acre-feet of water to reduce withdrawals from the Edwards Aquifer by 10,000 acre-feet. The concept is to reduce aquifer withdrawals by 10,000 acre-feet using a Regional Water Conservation Program (RWCP).

Several entities within the Edwards Aquifer Authority (EAA) jurisdictional area agreed to make Initial Commitments to the EAA Groundwater Trust to provide an immediate benefit to the aquifer and springflow. The EAA maintains contracts with three communities to conserve water under the RWCP through 2028. The City of Uvalde began implementing its toilet replacement program in 2013 to conserve 57.450 ac-ft of water. In 2014, the City of Universal City began implementing its leak detection program to conserve 163.684 ac-ft of water and in 2016, SAWS began implementing a five-year Leak Detection and Repair Program. The SAWS Leak Detection and Repair Program satisfies the total remaining RWCP goal for water committed into the EAA Groundwater Trust for the remainder of Incidental Take Permit (TE-63663A-1).

The estimated total savings of 20,053 ac-ft of conserved water was achieved from all three communities in 2020. One-half of the conserved water (10,027.13 ac-ft) has been placed in the EAHCP Groundwater Trust through the RWCP to remain unpumped through 2028.

Target for 2025:
None. This conservation measure was achieved in 2020 and 10,027.13 ac-ft has been placed in the EAHCP Groundwater Trust.

Budget:

Table 7.1:
$493,250

Estimated 2025 budget:
$0

5.1.4 Edwards Aquifer Authority Stage V Critical Period Management

Stage V Critical Period Management was developed to help decrease withdrawals and maintain adequate springflows at both Comal and San Marcos Springs during times of drought. On February 14, 2012, the Edwards Aquifer Authority (EAA) Board of Directors voted to amend its Critical Period Management (CPM) Program to include the new emergency Stage V. Implementation of Stage V results in a reduction of 44% to municipal, industrial and irrigation permit holders in both pools of the Edwards Aquifer who are authorized to withdraw more than 3 ac-ft per year. Stage V became effective as a rule on March 18, 2013 when the Incidental Take Permit was issued by the U.S. Fish and Wildlife Service.

2025 Implementation:

EAA staff monitors daily aquifer levels in both the San Antonio and Uvalde Pools of the Edwards Aquifer Region, and if at any time, the 10-day average for aquifer or springflow levels in either pool reaches the designated trigger for Stage V, the EAA General Manager will issue a Notice of Commencement for implementation in five newspapers within the EAA jurisdiction. Notice will also be posted at the EAA's office and on the EAA website. All affected permit holders will also be provided written notice of implementation of Stage V and the requirement to reduce pumping by 44%.

Permit Holder Assistance:

The EAA provides an online Critical Period Calculator to assist permit holders in calculating CPM reductions as they apply to each individual permit holder's total authorized withdrawal amount throughout the year. EAA staff also assists permit holders through "one-on-one" customer service offerings as may be necessary.

Triggers:

The triggers for Stage V in the San Antonio Pool are as follows: the 10-day average at the J-17 index well in San Antonio falls below 625 mean sea level (msl); or the 10-day average at Comal Springs falls below 45 cubic feet per second (cfs); or the 3-day average at Comal Springs falls below 40 cfs. In the Uvalde Pool, Stage V is triggered when the 10-day average at the J-27 index well falls below 840 msl.

Reporting:

By rule, permit holders are required to report their annual groundwater use to the EAA by January 31 for all groundwater used the preceding year. Permit holders who use more Edwards groundwater than authorized annually are subject to enforcement action.

5.5.1 Edwards Aquifer Authority and San Antonio Water System Aquifer Storage and Recovery Work Plan

Section 5.5.1 of the Edwards Aquifer Habitat Conservation Plan (EAHCP) assigns acquiring leases of water permits for use in the San Antonio Water System (SAWS) Aquifer Storage and Recovery (ASR) to the Edwards Aquifer Authority (EAA). SAWS will operate the ASR infrastructure and retain control of day-to-day operations of the ASR facility related to EAHCP water injection and recovery. The EAA will ensure compliance with EAHCP requirements through management of the Interlocal Contract between the EAA and SAWS for the Use of the Twin Oaks Aquifer Storage and Recovery Project for Contribution to Springflow Protection, which became effective August 14, 2013. The contract outlines the responsibilities of both parties, including administration and implementation.

Long-term Objective:
The objective of SAWS Twin Oaks ASR (ASR now runs out of H_2O Oaks facility) system is to deliver 126,000 acre-feet of Edwards Aquifer groundwater. This water is best managed to offset pumping from Edwards Aquifer wells during a repeat of a drought similar to the drought of record and acquire an additional 50,000 acre-feet of agricultural, municipal, industrial groundwater withdrawal rights that will be unpumped during a repeat of the drought of record.

Target for 2025:
The ASR contract between EAA and SAWS will continue to be implemented. EAA is the agent for ASR enrollments and in year 2020 issued its final notice of availability of EAHCP groundwater to SAWS for injection resulting in the completion of the storage goal of 126,000 acre-feet. Effective in 2021, a total of 50,000 acre-feet of groundwater rights was secured by EAA staff to be used as forbearance water and will go unpumped during a repeat of a drought of record. Future water acquired by the EAA through contractual agreements will be necessary to maintain the 50,000 ac-ft balance due to expiring leases occurring annually. The 50,000 ac-ft balance will be utilized for forbearance purposes during a repeat of a drought of record as outlined in the EAHCP. During a drought of record, the stored ASR water may be used by SAWS to offset forbearance and the EAA will also forbear the use of the 50,000 acre-feet of groundwater under its control.

ASR Program:

Description of the SAWS ASR: The SAWS H_2Oaks ASR is an underground storage reserve in the Carrizo Aquifer in southern Bexar County. As a SAWS water management project, it is designed to store Edwards Aquifer water when demand is less than available supply. The stored water is returned to San Antonio for use when demand is high and Edwards supply is restricted by Critical Period Management and other drought-related limitations.

The capacity and capabilities of the SAWS ASR are such that it can be used to meet SAWS ratepayer expectations and, if operated as described in the EAHCP, will play a significant role protecting the Covered Species at Comal and San Marcos springs.

Operations: The EAHCP Program Interlocal Contract between the EAA and SAWS for the Use of the Twin Oaks Aquifer Storage and Recovery Project for contribution to Springflow Protection, effective August 14, 2013, takes elements of the EAHCP's ASR flow protection strategy and places them into an operations contract.

Injection: Storage of EAHCP groundwater shall be at the discretion of SAWS and will be dependent on operating conditions. All EAHCP groundwater made available to SAWS before June 30th, 2020, was physically stored or credited as if stored, and will be used to meet any forbearance from the Aquifer should triggers defined in the Interlocal Contract occur in 2025.

Forbearance and Recovery: Forbearance of Edwards Aquifer pumping from certain wells will occur when the ten-year rolling recharge average is less than 500,000 acre-feet and the ten-day average of aquifer levels measured at the J-17 index well drop below 630 feet mean sea level (MSL). The annual amount of water to be recovered from the ASR during a repeat of the drought of record is outlined in Exhibits E & F of the Interlocal Contract. Changes to the Presumptive Forbearance Schedule outlined in Exhibit E may be approved as outlined in Section 5.3 of the Interlocal Contract. The ten-year rolling recharge average reported April 5, 2024 was 549,660 acre-feet and the ten-day average of aquifer levels measured at the J-17 index well as of April 5, 2024 was 639.4 ft msl.

Leasing: In 2018, EAA staff began marketing long-term (ten-year) forbearance agreements with regional permit holders and in 2020 completed the enrollment goal for years 2021 through 2028. In 2025, the total amount of water available under long-term leases is 11,486.018 acre-feet and 38,412.982 acre-feet in forbearance agreements for a total of 49,899 acre-feet. On December 31, 2025, a total of 5 ASR leases in the amount of 722.120 acre-feet will expire and will be re-enrolled as a forbearance agreements by the end of 2025. EAA staff will continue to maintain and manage 50,000 acre-feet of groundwater withdrawal rights under leases and forbearance agreements. This water will remain unused during a repeat of drought of record conditions.

Monitoring:
The EAA will actively manage the Interlocal Contract with SAWS. Status reports and updates will be provided regularly to the Implementing Committee.

ASR Regional Advisory Group: Per Section 5.5.1 of the EAHCP, a 12-person SAWS ASR Regional Advisory Group will meet to advise SAWS as SAWS makes the decisions relating to the operation of the ASR facility relevant to the EAHCP. Membership on the Regional Advisory Group will include: four representatives from the San Antonio Water System, the EAHCP Program Manager; one representative each from the EAA, EAA permit holder for irrigation purposes, small municipal pumpers, the spring cities, environmental interests, industrial pumpers, and downstream interests.

Budget:
Table 7.1:
$4,759,000 – Lease Options
$2,194,000 – O&M
$6,953,000 – Total
Estimated 2025 budget:
$5,689,162 – Lease & Forbearance Options

$0 – O&M $5,689,162 – Total

5.7.2 Water Quality Monitoring Program Strategy for Comal Springs and San Marcos Springs

This work plan details the sampling strategy and protocols for water quality monitoring in 2025 for the Edwards Aquifer Habitat Conservation Plan (EAHCP) (Section 5.7.2) implemented by the Edwards Aquifer Authority (EAA). Water quality monitoring of the Comal and San Marcos springs complexes and their associated surface waters has occurred since 2013 under implementation of the EAHCP. During this time period, the program has employed a variety of sampling strategies: stormwater, surface water, sediments, fish tissue, and passive samplers aimed at a range of environmental contaminants.

The water quality monitoring program underwent a formal review as part of the *National Academy of Sciences (NAS) Report 1* (2015) containing recommendations for EAHCP's Monitoring, Modeling and Applied Research programs, including the Expanded Water Quality Monitoring Program. Subsequently, a work group was formed in 2016 to assess recommendations presented in the NAS report. The result was a scope of work that was executed from 2017 – 2020.

Beginning in 2021, additional refinements to the program are being implemented. The primary changes from the previous implementation include discontinuing stormwater and passive sampling, adding surface water sampling, and modifying the analyte list. Table 1 presents an overview of the core activities comprising the EAHCP Water Quality monitoring program. Additionally, as needs arise, other water quality sampling activities may occur as developed through the EAHCP committees and included in the Annual Work Plan.

Target for 2025:

Water quality monitoring activities for 2025 include sampling activities for surface water, groundwater, and fish tissue in addition to operation of the real-time network. Specific actions for each sample type are discussed below. Analyte lists and maps follow this discussion. All samples will be collected following the EAA's *Field Sampling Plan* and analyzed by a NELAP accredited contract laboratory.

Groundwater sampling:

Groundwater samples will be collected from Spring 1, Spring 3, Spring 7 (Comal), Deep and Hotel (San Marcos) springs during the Spring and Fall under normal flow conditions (Figures A1 and A2). Groundwater samples will be collected by directly filling a bottle or using a previously decontaminated peristaltic pump with the intake portion of the pump placed in the spring orifice to minimize surface water contamination. Samples will be submitted to a contract laboratory for analysis of cations, anions, nutrients, metals, VOCs, SVOCs, herbicides and pesticides, bacteria, TOC, PCBs, and PPCPs. The analyte list for laboratory analyses along with the methods are

shown in Table 4. During the collection event, field parameters will be collected that include dissolved oxygen, pH, conductivity, temperature, and alkalinity.

In addition to the biannual groundwater sampling, sucralose will be measured on a monthly basis at Spring 3 and Hotel, and PPCPs will be measured on an every other month basis at Spring 3 and Hotel. These samples will be collected by directly filling bottles at the source of spring flow. During the collection event, field parameters will be collected that include dissolved oxygen, pH, conductivity, and temperature.

Table 1. EAHCP Water Quality monitoring program core activities.

Sample Type	Activity
Surface water	Twice annual sampling in conjunction with Biological Monitoring activities
	Laboratory analyses are focused on bacteria and nutrients
	Locations include upper and lower stations at each spring system
Groundwater	Twice annual sampling in conjunction with EAA springs sampling activities
	Laboratory analyses are focused on geochemical analytes and industrial, commercial, and emerging contaminants. The analytes include cations, anions, nutrients, metals, VOCs, SVOCs, herbicides, pesticides, bacteria, TOC, PCBs, and PPCPs
	Locations include Spring 1, Spring 3, Spring 7 (Comal), Hotel, and Deep (San Marcos)
Sediment	Every other year sampling in even numbered years
	Laboratory analyses are focused on PAHs
	Locations include 6 San Marcos and 5 Comal stations
Fish Tissue	Every other year sampling in odd numbered years
	Laboratory analyses are focused on metals and PPCPs in two fish species
	Locations include upper and lower stations at each spring system
Real-time network	Continuous, telemetered measurements
	Analytes include temperature, dissolved oxygen, and conductivity
	Locations include 3 San Marcos and 3 Comal stations

Surface water sampling:

Surface water samples will be collected from upper and lower river stations at both systems. For Comal Springs, Landa Lake near Spring Island will serve as the upper location, and the lower station is downstream of the Old and New Channel confluence. In San Marcos, Spring Lake near Hotel spring will serve as the upper location, and the downstream location is located at the most downstream real-time water quality monitoring station. Samples at each location will be collected on a biannual basis during normal flow conditions in conjunction with the Biological

Monitoring program (Spring and Fall). Water samples will be taken from flowing parts of the stream on the upstream side of the sample collector. A previously decontaminated Kemmerer or similar device will be used to collect samples at approximately mid-depth in the water column. Samples will be submitted to a contract laboratory for analysis of nutrients (Table 5). During the collection event, field parameters will be collected that include dissolved oxygen, pH, conductivity, and temperature.

Fish Tissue sampling:

Fish collections from the Comal and San Marcos rivers will be conducted during the spring Biological Monitoring survey. For both systems, fish will be collected at locations near the surface water sampling locations described above.

At each site, gambusia and largemouth bass will be collected. For each sample, whole body organisms will be combined to create a composite sample for tissue analysis. The length, weight, and sex of the individual fish will be recorded prior to creating the homogenate. Tissue samples will be submitted to a contract laboratory and analyzed for metals and PPCP contaminants listed in Table 6.

Real Time Instrument Water Quality Data Logging:

Continuous water quality monitoring stations will continue in 2025 at three locations in the Comal and three locations in San Marcos. The network consists of Insitu AquaTroll sondes measuring dissolved oxygen, conductivity, temperature, and turbidity (Sessom Creek only). Measurements are collected every fifteen minutes and telemetered in real-time. The Sessom Creek site logs data on five-minute intervals to support turbidity measurements at this location.

Quality control procedures:

Field collection methods and quality control procedures for the discrete sampling types are guided by the EAA's Field Sampling Plan. The anticipated number of samples and field quality control samples sent for analyses in 2025 are shown in Table 2. Brief descriptions of the intent of the quality control tests are described below.

Table 2. Sample amounts for 2025 water quality activities.

Sample type	Field Samples	Equipment blank	DI blank	Lab duplicate	Field duplicate	Total samples
Groundwater	18	2	2			22
Sucralose	24		4		2	30
Surface water	10	2			4	16
Fish Tissue	10					10

Both equipment blanks and DI blanks use reagent grade ASTM II deionized water to assess external contamination of environmental samples. Equipment blanks examine the contamination introduced through the sampling procedure. These are conducted by transferring the deionized water through equipment that has been decontaminated for field use. DI blanks consist of deionized water sent directly to the laboratory and are designed to examine sample containers and other laboratory contamination.

Lab and field duplicates are intended to assess the precision and repeatability of the analytical procedure and homogeneity of the environmental sample type. Laboratory duplicates consists of a single well-mixed sample split into two samples for analysis. Field duplicates consists of a second sample collected immediately after an initial sample.

Additionally, all laboratory quality control data including matrix spikes and surrogate blanks will be reported.

Monitoring:
A summary report presenting the 2025-year findings will be prepared by EAA staff and included in the EAHCP annual report. The report will include an evaluation of the analytical data and its quality, discussions of results, and a description and rationale for any deviations from the Work Plan described here. The report will be completed by March 2025.

Data collected as part of the 2025 EAHCP Water Quality monitoring program will be kept electronically with the EAA. Data from quality controlled discrete sample types (surface water, groundwater, sediment, and fish tissue) will be housed by EAHCP staff in delimited file types that include all discrete measurements from the program beginning in 2013. Quality controlled time series data associated with the real-time network are housed with existing aquifer time-series data by the EAA.

Cost Estimate:
Costs for laboratory analyses are shown in Table 3 and are based on estimates provided by commercial laboratories in 2023. Field supplies costs in Table 3 cover field collection and analysis equipment including calibration standards and Kemmerer device.

Table 3. 2025 EAHCP Water Quality monitoring program costs.

Sample type	Total samples	Cost per sample	Total Costs
Groundwater	22	$1,1174	$25,828
Sucralose	30	$232	$6,960
Surface water	16	$250	$4,000

Fish Tissue	10	$602	$6,020
Fillet homogenization	5	$31	$155
Field Supplies			$5,000
		Total	**$47,963***

*This amount does not include surplus monies made available for additional Water Quality Monitoring needs but will not exceed the $65,000 listed in the funding table on Page 2.

Sample location maps and analyte lists

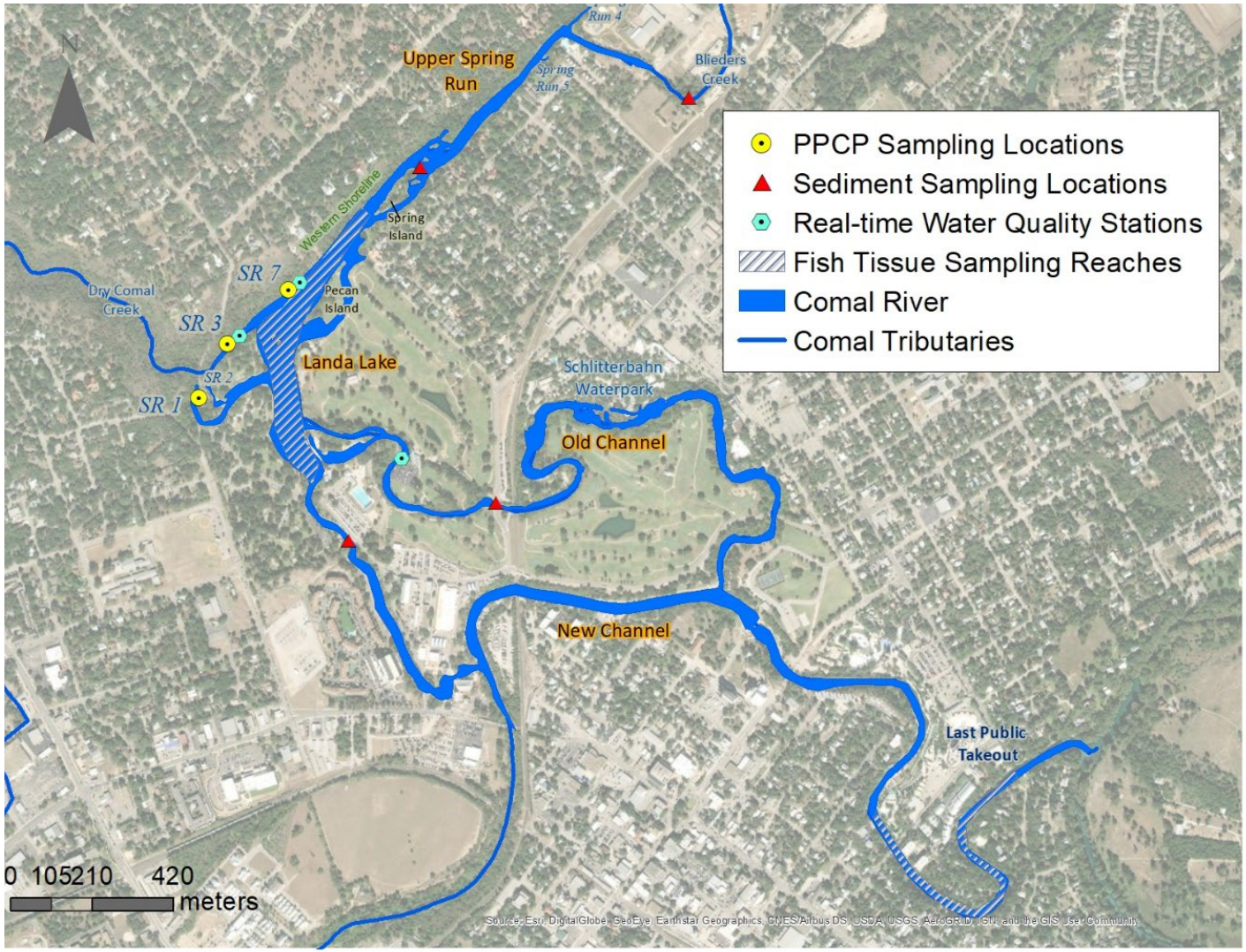


Figure 1. Water quality sampling locations for the Comal system.

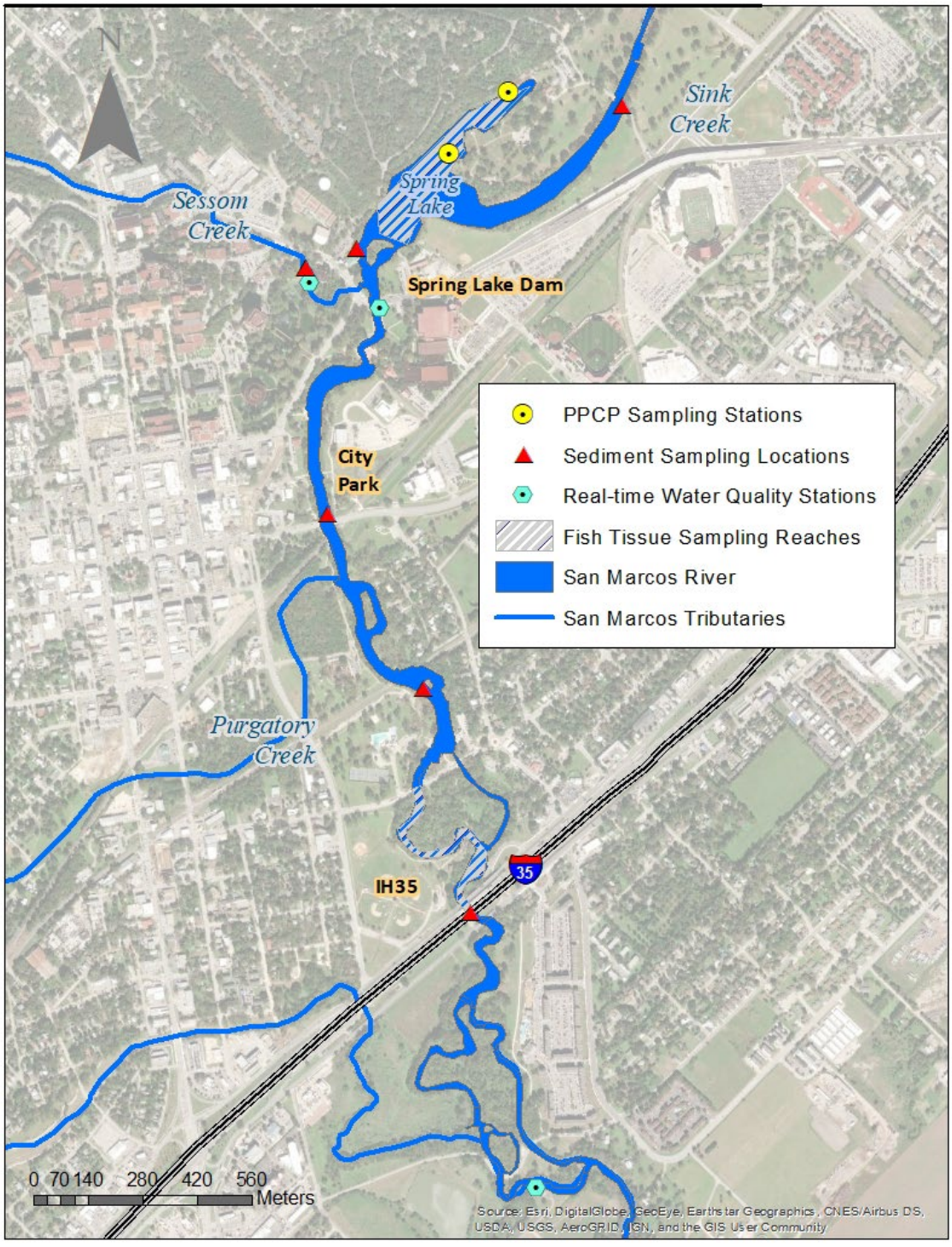


Figure 2. Water quality sampling locations for the San Marcos system.

Table 4. Analytical parameters for groundwater samples.

Analyses
Volatile Organic Compounds (VOCs)
Semi-volatile Organic Compounds (SVOCs)
Organochlorine Pesticides
Polychlorinated Biphenyls (PCBs)
Organophosphorous Pesticides
Herbicides
Metals (Al, Sb, As, Ba, Be, B, Cd, Cr (total), Cu, Fe, Pb, Mn, Hg, Ni, Se, Ag, Tl, V, and Zn)
General Chemistry (GWQP) Total Alkalinity (as CaCO3), Bicarbonate Alkalinity (as CaCO3), Carbonate Alkalinity (as CaCO3); (Cl, Br, NO_3, SO_4, Fl, pH, TDS, TSS, Ca, Mg, Na, K, Si, Sr, CO_3,)), and Total Suspended
Phosphorus (total)
Total Organic Carbon (TOC),
Dissolved Organic Carbon (DOC)
Kjeldahl Nitrogen
Bacteria Testing (*E coli*)
PPCPs

Method	**Method Description**	**Protocol**
8260B	Volatile Organic Compounds	(GC/MS) SW846
8270C	Semivolatile Organic Compounds	(GC/MS) SW846
8081B	Organochlorine Pesticides	(GC) SW846
8082A	Polychlorinated Biphenyls (PCBs)	by Gas Chromatography SW846
8141A	Organophosphorous Pesticides	(GC) SW846
8151A	Herbicides	(GC) SW846
6010B	Metals	(ICP) SW846
6020	Metals	(ICP/MS) SW846
7470A	Mercury	(CVAA) SW846
300.0	Anions,	Ion Chromatography
340.2	Fluoride	MCAWW
365.4	Phosphorus,	Total EPA
9040C	pH	SW846
9060	Organic Carbon,	Total (TOC) SW846
SM 2320B	Alkalinity	SM
SM 2540C	Solids,	Total Dissolved (TDS) SM
SM 2540D	Solids, Total Suspended (TSS)	SM
351.2	Nitrogen, Total Kjeldahl	MCAWW
1694	PPCPs	LC-MS/MS

Protocol References:
EPA = US Environmental Protection Agency
MCAWW = "Methods For Chemical Analysis Of Water And Wastes", EPA-600/4-79-020, March 1983 And Subsequent Revisions.
SM = "Standard Methods For The Examination Of Water And Wastewater",
SW846 = "Test Methods For Evaluating Solid Waste, Physical/Chemical Methods", Third Edition, November 1986 And Its Updates.

Table 5. Analytical parameters for surface water samples

Analyses
Soluble Reactive Phosphorous
Phosphorus (total)
Total Organic Carbon (TOC),
Dissolved Organic Carbon (DOC)
Kjeldahl Nitrogen
Nitrates and Ammonium

Method	Method Description	Protocol
365.4	Phosphorus,	Total EPA
9060	Organic Carbon,	Total (TOC) SW846
351.2	Nitrogen, Total Kjeldahl	MCAWW
445.0	Chlorophyll a	Fluorescence
8141a	Organophosphates	SW846
353.2	Nitrates	
350.3	Ammonia	

Protocol References:
EPA = US Environmental Protection Agency
MCAWW = "Methods For Chemical Analysis Of Water And Wastes", EPA-600/4-79-020, March 1983 And Subsequent Revisions.
SM = "Standard Methods For The Examination Of Water And Wastewater",
SW846 = "Test Methods For Evaluating Solid Waste, Physical/Chemical Methods", Third Edition, November 1986 And Its Updates.

Table 6. Analytical parameters for fish tissue samples

PPCP List
Acetaminophen
Azithromycin
Caffeine
Carbadox
Carbamazepine
Cefotaxime
Ciprofloxacin
Clarithromycin
Clinafloxacin
Cloxacillin
Dehydronifedipine
Digoxigenin
Digoxin
Diltiazem
Diphenhydramine
Enrofloxacin
Erythromycin-H2O
Flumequine

Fluoxetine
Lincomycin
Lomefloxacin
Miconazole
Norfloxacin
Norgestimate
Ofloxacin
Ormetoprim
Oxacillin
Oxolinic Acid
Penicillin G
Penicillin V
Roxithromycin
Sarafloxacin
Sulfachloropyridazine
Sulfadiazine
Sulfadimethoxine
Sulfamerazine
Sulfamethazine
Sulfamethizole
Sulfamethoxazole
Sulfanilamide
Sulfathiazole
Thiabendazole
Trimethoprim
Tylosin
Virginiamycin M1
1,7-Dimethylxanthine

6.3.1 Biological Monitoring Program for the Comal and San Marcos Aquatic Ecosystem

Long-term Objective:
Since 2000, the Edwards Aquifer Authority (EAA) has undertaken biological monitoring of the Comal and San Marcos spring systems. In 2013, the elements of the program were incorporated into the Biological Monitoring Program (BioMP) for the Edwards Aquifer Habitat Conservation Plan (EAHCP).

The purpose of the BioMP is "to monitor changes to habitat availability and population abundance of the Covered Species that may result from Covered Activities" (EAHCP § 6.3.1). The BioMP includes: (1) Comprehensive Sampling, (2) any triggered Critical Period Monitoring, (3) any high flow triggered monitoring (4) and any EAHCP-specific sampling required by Section 6.4.

Target for 2025:
The 2025 BioMP for the Comal and San Marcos aquatic ecosystems will continue to include Baseline and Critical Period Monitoring along with a Net Disturbance impact assessment and overall Take Determinations. The 2025 BioMP will continue to use the standard operating procedures adopted in 2016 because of the Biological Monitoring Work Group (EAHCP 2016) in addition to what is noted in this document. These standard operating procedures were instituted for the BioMP beginning in 2017.

Monitoring:
Aquatic Vegetation Mapping: The contractor will conduct aquatic vegetation mapping in the four long-term monitoring reaches in the Comal Springs system and in the three long-term monitoring reaches in the San Marcos Springs system. The comprehensive mapping is conducted using a GPS unit with real-time differential correction with sub-meter accuracy.

Zebra Mussel Monitoring: The contractor will conduct zebra mussel monitoring using passive techniques in both the Comal and San Marcos rivers.

Texas wild-rice Mapping: The contractor will map all Texas wild-rice from Spring Lake downstream to the confluence of the Blanco River on an annual basis. The annual mapping will occur during the summer (July-August). The location of every stand of Texas wild-rice will be recorded using a GPS unit with real-time differential correction with sub-meter accuracy.

Fountain Darter Sampling: The contractor will conduct drop and dip netting and visual aquatic surveys with SCUBA during the Spring and Fall sampling events. Additional dip net sampling will be conducted during the Summer sampling event. Aquatic vegetation will be mapped in the reaches prior to drop and dip net activities.

Drop Net Sampling: Drop netting will be used to sample fountain darters in identified reaches of the rivers among dominant aquatic vegetation species that have been selected through stratified random sampling. Fountain darters will be identified, counted, measured, examined for

condition, and returned to the river at the point of collection. Other fish will be identified and released, or preserved, and identified in a laboratory. Live rams-horn snails will be counted, measured, and destroyed. Exotic Asian snails and Asian clam will be identified, general abundance recorded, then destroyed. The number of crayfish and grass shrimp per drop net will be noted. Furthermore, vegetation species, vegetation height, vegetative areal coverage, substrate type, water depth, mean column velocity, velocity at 15 centimeters (cm) above the bottom, water temperature, conductivity, pH, and dissolved oxygen levels will be recorded for each drop net.

Dip Net Sampling: The contractor will conduct dip net timed surveys, as well as presence/absence surveys in specified sections throughout the spatial extent of both systems. Fountain darters collected by dip net monitoring will be examined for gill condition. Additionally, total length of collected individuals will be measured during timed dip net surveys. Timed surveys will be conducted in all habitat types up to a depth of 1.4 m, within each section, moving upstream during the sampling process with prime darter habitat receiving the most effort.

Presence/absence surveys will be conducted by taking 4 dip net sweeps at 50 random sample site locations within the 4 representative reaches at Comal Springs (Upper Spring reach [5 locations], Landa Lake reach [20 locations], Old Channel reach [20 locations], and New Channel reach [5 locations]), and the 50 random sample site locations within the three representative reaches in San Marcos Springs (Spring Lake Dam reach [15 locations], City Park reach [20 locations], and I-35 reach [15 locations]).

Visual Fountain Darter Survey: Visual aquatic surveys will be conducted using SCUBA in a fixed location in Landa Lake to identify fountain darters at depths deeper than conventional sampling methods allow.

Comal Springs Invertebrate Sampling: The contractor will conduct sampling for Comal Springs invertebrates during the Spring and Fall sampling events.

One drift net each will be placed over the main spring orifice of Spring Run 1, Spring Run 3, and Spring Run 7 at Comal Springs. All endangered invertebrates will be identified and counted in the field and returned to the orifice they were collected upon completion of the 24-hour sample period. All other invertebrates will be preserved and transported to an off-site laboratory for taxonomic classification. Coordination with the USFWS San Marcos Aquatic Resources Center (SMARC) will take place each time to assist with refugia collections when needed.

The Comal Springs riffle beetle (CSRB) cotton lure standard operating procedure, or a suggested (and EAHCP staff approved) alternate method, and quantitative survey methods will be utilized to conduct Comal Springs riffle beetle sampling in three locations (i.e., Spring Run 3, western shoreline of Landa Lake, and Spring Island area). Ten springs within each of the three locations will be identified for sampling by the contractor. If possible, the same ten springs from the previous year will be sampled.

The CSRB cotton lure standard operating procedure, cotton lure quantitative survey method, and recommendations generated during the CSRB workgroup describe the appropriate protocols for

CSRB to be identified, counted, and returned to their spring of origin. Other spring invertebrates collected on the lures will also be noted including the Comal Springs dryopid beetle (*Stygoparnus comalensis*) and Peck's cave amphipod (*Stygobromus pecki*).

Salamander Visual Observations: The contractor will conduct salamander sampling during each Spring and Fall sampling event. Comal salamander surveys will be timed and conducted by observation from the surface or dive mask and snorkel at Spring Run l, Spring Run 3, Spring Island spring runs, and at the eastern outfall at Spring Island.

San Marcos salamander surveys follow the quantitative sampling method described in Nelson, J. (M.S. Thesis, Texas State University, 1993). Observations for the San Marcos salamander will be done by dive mask and snorkel or SCUBA for three, 5-minute timed surveys per area. San Marcos salamanders will be counted, measured and the overall substrate where they were found documented.

In both systems, sampling will require turning over rocks in the sample site for set periods of time in order to expose the salamanders and obtain a visual count. Whenever possible, all rocks will be returned to their original location. For this monitoring, salamanders will only be observed, and no collections will occur.

Comal Springs Discharge Measurements: The contractor will conduct discharge measurements on Comal Springs during the Spring and Fall sampling events. Discharge measurements will be conducted at Spring Runs 1, 2, and 3, Upper Spring Run Reach, and the Old Channel below Elizabeth Street and will be used to establish the contributions of each major spring run to total discharge in the river and to establish the relative proportion of water flowing in the Old and New Channels.

Water Quality Sampling: The contractor will maintain and download existing thermistors located throughout each system. Standard water quality parameters (water temperature, conductivity compensated to 25°C, pH, dissolved oxygen [mg/l], water depth at sampling point, and observations of local conditions) will be sampled during drop net sampling and fish community sampling activities.

Fixed Station Photography: The contractor will photo document each established, fixed station photograph site. Photographs involve an upstream, across, and downstream picture of the reach and capture key changes in the habitat in the reach.

Macroinvertebrate Community Assessment: The macroinvertebrate community assessment will be conducted using rapid bioassessment (RBA) protocol as described in "Surface Water Quality Monitoring Procedures, Volume 2: Methods for Collecting and Analyzing Biological Assemblage and Habitat Data." TCEQ RG-416. 2014. The RBAs will be conducted in 5 reaches in the Comal and 4 reaches in the San Marcos at the drop-net fountain darter sites. One composite sample will be collected from each reach (i.e. 9 samples total across both systems). Macroinvertebrate community assessments will be conducted during Comprehensive Sampling and Critical Period Monitoring events.

Fish Community Sampling:

SAN MARCOS SYSTEM—Fish will be sampled at two locations within Spring Lake associated with San Marcos salamander surveys (Big Riverbed and Hotel Area) and one location just upstream of the eastern spillway. Two different SCUBA techniques will be used to document the fish within the three locations, mesohabitat and microhabitat surveys. Three additional SCUBA survey locations will occur in the San Marcos River (Upper, Mid, and Lower), located in representative deep areas where seining has proven to be inefficient. The exact location of the SCUBA sampling within each section may change slightly based on conditions at the time of the sampling event.

In addition to SCUBA, fish in the San Marcos River will be sampled among five sites within three reaches (Upper: Sewell, Veteran's Park, Middle: Crook's Park, and Lower: San Marcos Wastewater Treatment plant and Smith property) via seines within wadeable habitats. Multiple seine hauls will occur along a river transect perpendicular to the flow. Within each seine haul, fish will be identified, measured, examined for disease, and native fish returned to the river. Exotics will be removed from the system as per scientific permit. In addition to fish data, habitat data will be collected for each seine haul including current velocity, water depth, substrate composition, in-stream coverage, climatic conditions, and mesohabitat type.

COMAL SYSTEM—Fish will be sampled at three locations within Lake via SCUBA surveys. In particular, one of the SCUBA survey locations in Landa Lake will be in the same as the ongoing fountain darter belt transect survey. In addition, SCUBA surveys will be conducted within the Upper Spring Run, Old Channel, and New Channel sections of the Comal River. Two different SCUBA techniques will be used to document the fish within the three locations, mesohabitat and microhabitat surveys..

In addition to SCUBA surveys, three locations (Upper Spring Run, New Channel, and Old Channel) will be sampled via seines among wadeable habitats to evaluate and track fish populations in the Comal River. Multiple seine hauls will occur along a river transect perpendicular to the flow. Within each seine haul, fish will be identified, measured, examined for disease, and native fish returned to the river. Exotics will be removed from the system per scientific permit requirements. In addition to fish data, each seine haul will include habitat measurements (i.e. current velocity, water depth, substrate composition, in-stream coverage, climatic conditions, and mesohabitat type).

EAHCP Habitat Baseline and Disturbance Determination: This determination is intended to fulfill Section M 1a and 2a of the Incidental Take Permit (ITP).

DOCUMENT BASELINE HABITAT CONDITIONS—The contractor will use January 1 of the contract year GIS mapping, biomonitoring data and other existing sources to establish occupied habitat for the EAHCP Covered Species. Specific to Item M (la and 2a) of the ITP, only occupied habitat within the Comal and San Marcos springs/river ecosystems will be included.

DOCUMENT EAHCP MITIGATION AREAL EXTENT PER PROJECT—The contractor will work with staff and contractors from the City of New Braunfels, City of San Marcos and Texas State University, coordinating through EAA staff, to describe in GIS map form, representing a snapshot in time on December 31 of the contract year, the areal extent of all direct EAHCP mitigation and restoration activities in the Comal and San Marcos springs systems.

If GIS files of the project/affected areas are unavailable, the contractor will either: l) map those areas directly with high grade GPS in real-time, or 2) use existing areal imagery to pinpoint and outline locations with subsequent, supplemental GPS ground truth mapping. The contractor will ensure that areas represented on all maps are representative of actual mitigation, not concept areas.

Assessment of Net Disturbance: The contractor will evaluate the baseline maps versus the EAHCP project maps and quantify the area of direct disturbance that may have potential effects from mitigation and restoration activities as described in Item M (la and 2a) of the ITP. The focus will be on quantifying the direct impacts (removal of non-native vegetation, etc.) via areal coverage of habitat, but will also describe potential indirect impacts (turbidity, etc.) qualitatively. This analysis will not extend beyond comparisons of areal coverage of occupied habitat.

Annual "Take" Estimate: The contractor shall estimate Take for each of the Covered Species utilizing the information generated by the BioMP, the information and guidance in Chapters 4 and 6 of the EAHCP, the Biological and Conference Opinion issued by USFWS, and any other relevant information. The purpose of this Take estimation is to ensure compliance with Section H of the ITP.

Critical Period Monitoring: The Critical Period Monitoring component will be performed on both systems and be based upon established flow trigger levels for each system. The type and extent of sampling conducted is dependent on the respective trigger level and is designed to be duplicative of full biomonitoring sampling and will include species-specific sampling based on the flow triggers.

HIGH/LOW FLOW MONITORING—The contractor will conduct high flow Critical Period Monitoring only after the following triggering criteria are met:

a) The daily average flow exceeds 385 cubic feet per second (cfs) in the San Marcos aquatic ecosystem or 500 cfs in the Comal aquatic ecosystem (total flow through the ecosystem as measured at the USGS gauging station located immediately downstream of the ecosystem); and
b) After conducting a joint visual inspection of the aquatic ecosystem with the contractor, EAA staff determines that high flow Critical Period Monitoring is warranted and approved.

Before high flow Critical Period Monitoring is conducted, the sampling parameters must be recommended by the contractor and pre-approved by EAA staff, based on professional judgment,

and may include any parameter from the full biomonitoring sampling, with the exception of gill net sampling.

The Comal and San Marcos springs systems flow-based triggers are associated with specific sampling parameters.

SAN MARCOS SYSTEM SAMPLING—Low flow Critical Period Monitoring for the San Marcos River triggers at 120 cfs, with Texas wild-rice vulnerable stand monitoring as described in Task 3 of the Comprehensive Sampling Program. Monitoring will occur at 5 cfs declines or a maximum of once per week. The first Full Sampling Event is triggered at 100 cfs, with subsequent declining Full Sampling Events triggering at 85, 60, 25, and 10-0 cfs for a total of five declining Full Sampling Events. In addition, two recovery Full Sampling Events would be conducted as the system rebounds from the low flow period. Between Full Sampling Events, habitat evaluations, per every 5 cfs decline, would be conducted again not to exceed weekly monitoring.

COMAL SYSTEM SAMPLING— Low flow Critical Period Monitoring for the Comal River triggers at 200 cfs. This triggers the first Full Sampling Event with 4 subsequent Full Sampling Events being triggered at 150, 100, 50, and 10-0 cfs, respectively. Two recovery Full Sampling Events are scheduled as the flows rebound and stabilize from drought conditions. The Comal system also has habitat evaluations scheduled between Full Sampling Events; however, at 10 cfs increments again not to exceed weekly observation. An additional component for the Comal system is the detailed riffle beetle habitat evaluation and spring orifice condition documentation that is triggered at 120 cfs and continued at 10 cfs increments during decline. Flow split monitoring between the Old and New Channel will also occur during the riffle beetle evaluation and spring orifice condition documentation.

A review of historic flow records indicates that the lower the flow, the lower the chance an even lower flow event will occur, thus reducing the chances of a complete decline and recovery as outlined above. Typically, both systems rebound from drought conditions due to a tropical depression rainfall event or some other weather pattern that produces a large amount of rainfall over the watershed. Flows typically come up rapidly and require a period of stabilization before the collection of biological data is meaningful.

Gill Net Evaluation: In addition to the full sampling activities, the contractor will conduct gill net evaluations in the immediate vicinity of the fountain darter SCUBA surveys in Spring Lake and Landa Lake. The Spring Lake evaluation will be triggered at 85 cfs and lower triggers. The Landa Lake assessment will be triggered at 100 cfs and lower triggers. The survey is designed to examine exotic fish concentrations and stomach content analyses with respect to predation of listed species. The number of each species (native and non-native) collected in the gill net and the data will be recorded and converted to catch per unit effort.

Water Quality Grab Sampling: The contractor will collect water quality grab samples at the established triggers at 18 stations longitudinally distributed in the San Marcos system and 12

stations longitudinally distributed in the Comal system. The samples will be from the surface, mid-depth and near bottom.

EAHCP Low Flow Sampling: To protect the Covered Species, Chapter 6 of the EAHCP contains specific flow requirements for both systems that trigger sampling events. This sampling is in addition to the Comprehensive Sampling and Critical Period Monitoring components and consists of an increased frequency of sampling for aquatic vegetation, Texas wild-rice mapping, as well as additional sampling of fountain darters, Comal Springs riffle beetles, and salamanders.

Cost estimate:
Table 7.1:
$400,000

Estimated 2025 cost:
$755,774*

*Includes Critical Period Monitoring if required

6.3.3 Ecological Modeling

Long-term Objective:
The development of a mechanistic ecological model (Ecomodel) is assigned to the Edwards Aquifer Authority per section 6.3.3 of the EAHCP. The purpose of the Ecomodel is to evaluate potential adverse effects to Covered Species and their critical habitat, and to the extent such effects are determined to occur, quantify their magnitude, and develop alternate strategies.

Target for 2025:
No Ecological Modeling work is anticipated in 2025.

Budget:
Table 7.1:
$25,000

Estimated 2025 budget: *
$0

*There is no proposed budget for 2025.

6.3.4 Applied Research

Long-term Objective:
Applied research adds a valuable component to the EAHCP to better understand the ecological dynamics for all Covered Species.

Target for 2025:
Savings from past years will be applied to perform research to support a better understanding of existing Conservation Measures, EAHCP Covered Species, and other aspects of the EAHCP program. A study will be performed to test a new sampling methodology to assess population trends in the San Marcos salamander. The study is being conducted at the recommendation of the EAHCP Biological Objective Subcommittee and should aid in the development of new Biological Objectives for the new Incidental Take Permit starting in 2028. Additionally, an aquatic vegetation maintenance study will occur concurrently with the San Marcos salamander study to aid in assessing the effects of Spring Lake aquatic vegetation maintenance efforts on the salamander and evaluating the habitat based EAHCP Biological Objective for the salamander.

The multi-year Comal Springs riffle beetle population study effort will continue into 2025 with a final report produced by the end of February 2025.

A genetic study on the Comal Springs riffle beetle was recently completed by the EA Refugia program in 2024. The results of the study suggested recent declines in genetic diversity and limited connectivity between the beetle populations found in the spring runs and the main body of Landa Lake (i.e., Westernshore and Spring Island) of the Comal system. A follow up study examining genetics from Comal Springs riffle beetles collected prior to the EA Refugia study would be useful to further evaluate the genetic history of the species. The study would start in fall of 2025 and be completed by end of 2026.

One of the main goals of the EAHCP is to maintain sufficient habitat for Covered Species to ensure the long-term persistence of the species. To accomplish this goal, it's imperative to identify the habitat for each Covered Species and to monitor any changes in the species associated with changes in habitat. Presently, only basic knowledge on the Comal Springs dryopid beetle's spatial distribution and habitat preferences is known and no methods specifically focused on monitoring the beetle are in the EAHCP. A multi-year study on the Comal Springs dryopid beetle will start in late summer of 2025 with the main study objectives being to develop field sampling protocols that can reliably detect the beetle in Landa Lake and spring runs of the Comal system and evaluate analytical protocols that can effectively monitor changes in the beetle population. The goal is to use the sampling and analytical protocols developed from this study to add a monitoring plan for the species into the EAHCP biomonitoring program.

The EAA in 2025 will initiate a contract focused on environmental data management, specifically aimed to improve the organization and synthesization of the EAHCP biomonitoring data, making it easier to analyze and data share.

Applied Research in 2025 will also include a Spring Lake aquatic vegetation and springs mapping study. Spring Lake aquatic vegetation mapping is currently not included as part of the Biological Monitoring program. However, it is considered critical habitat for the fountain darter, Texas wild-rice, and the San Marcos salamander. Spring Lake aquatic vegetation was last surveyed in 2020 and the 2025 study survey will serve as the 5-year assessment to evaluate changes in Spring Lake's aquatic habitat. The Spring Lake survey will include acquisition of high-resolution aerial imagery, mapping of aquatic vegetation (floating and submerged), and mapping of major springs locations within Spring Lake. The surveys and study results will be completed during 2025 and will be summarized in a final report produced by the end of December 2025. Final data produced will include total estimated vegetation coverage, georeferenced polygon shapefiles that contain attribute data for vegetation species composition, georeferenced point shapefile with locations of major springs in Spring Lake, and high-resolution, geo-referenced aerial imagery of Spring Lake.

Budget:

Table 7.1:
$0

Estimated 2025 budget:
$250,000

FMA § 2.2 EAHCP Program Management

Section 2.2 of the Funding and Management Agreement (FMA) assigns "general management and oversight" of the EAHCP to the Edwards Aquifer Authority (EAA). Section 5.6.5 of the FMA allows the EAA to use EAHCP funds for administrative costs and employee salaries, so long as all incurred costs and salaries are 100% related to "general management and oversight" of the EAHCP.

Long-term Objectives:
To manage and oversee day-to-day operations and administration, in coordination with the Applicants, of the EAHCP; resulting in a valid and continued Incidental Take Permit (ITP) from the USFWS for designated Covered Activities.

Program Activities in 2025:
EAHCP staff will continue to coordinate and monitor habitat protection measures completed by the City of New Braunfels and City of San Marcos/Texas State University in their respective 2025 Work Plans. The springflow and supporting measures are described in this 2025 EAA Work Plan.

The EAHCP Program Manager will execute duties as assigned in the FMA and:

- Manage EAHCP day-to-day activities;
- Facilitate program correspondence with the USFWS;
- Manage program activities in support of a 2028 ITP renewal;
- Serve on the ASR Advisory Committee;
- Facilitate the Adaptive Management Process (AMP) for all Routine and Nonroutine decisions; and
- Facilitate and coordinate all meetings of the EAHCP Implementing and Stakeholder committees and possible Subcommittees and Work Groups as created by the Implementing, Science and Stakeholder committees.

EAHCP Chief Science Officer and support staff will continue the following activities:

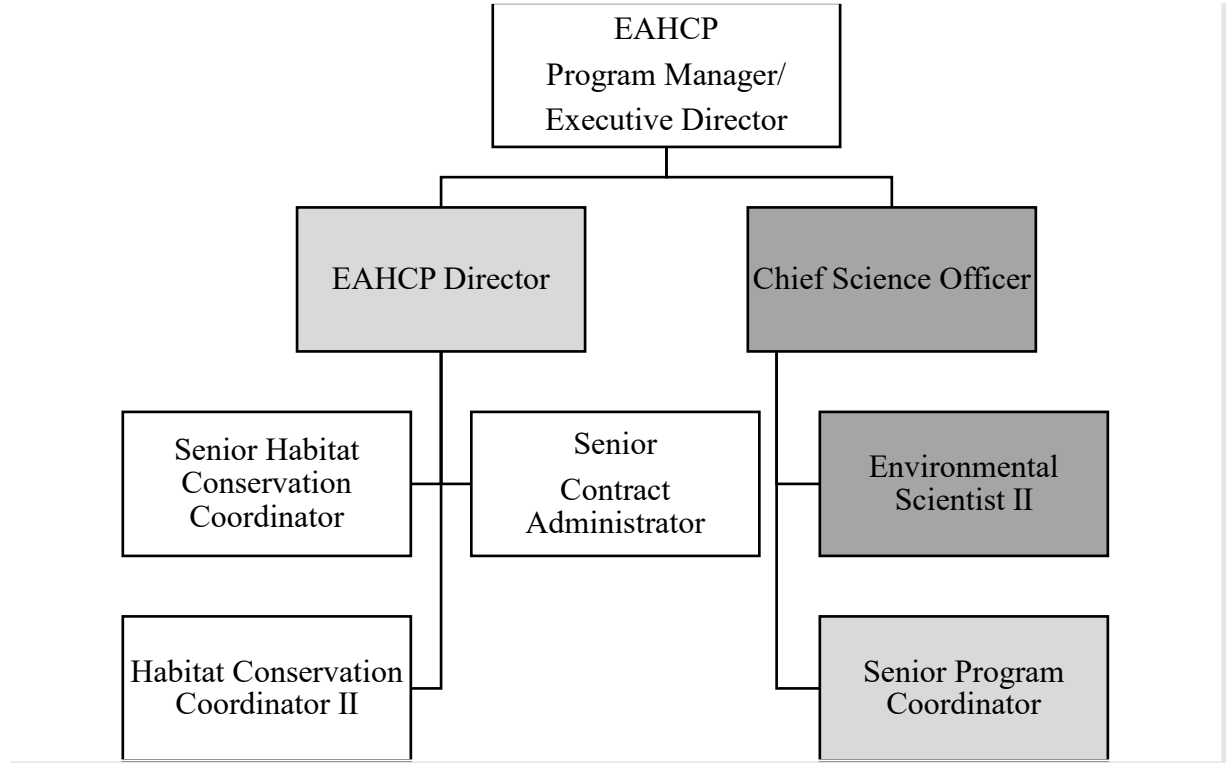
- Manage Refugia Work Plan activities including operations and research;
- Manage applied research;
- Manage biological monitoring;
- Manage and perform water quality monitoring;
- Update and maintain biological and water quality monitoring databases;
- Prepare for all meetings of the EAHCP Science Committee and EAHCP Implementing, and Stakeholder committees at the request of the Program Manager; and
- Prepare for all meetings of the Comal Springs Riffle Beetle Work Group, Research Work Group, and other possible Subcommittees and Work Groups as created by the Implementing, Science and Stakeholder committees at the request of the Program Manager.

EAHCP Administrative staff will continue the following activities:

- Oversee the City of New Braunfels and San Marcos/Texas State University Work Plan activities;
- Coordinate 2025 Work Plan and funding application amendments for the EAA, City of New Braunfels, and San Marcos/Texas State University;
- Coordinate the development of 2025 Work Plans and funding applications for EAA, City of New Braunfels, and San Marcos/Texas State University;
- Process City of New Braunfels and San Marcos/Texas State University reimbursement's from EAA for habitat protection measures;
- Procure and execute contracts for support measures and program administration;
- Oversee EAA contract tracking and compliance;
- Process EAA contractor's invoices for support measures and program administration;
- Coordinate and prepare for all meetings of the EAHCP Implementing, Science, and Stakeholder committees, (and possible Subcommittees and Work Groups as created by the Implementing, Science and Stakeholder committees);
- Coordinate and prepare correspondence with all EAHCP Implementing, Science, and Stakeholder committee members and Work Groups members under the direction of the EAHCP Program Manager;
- Prepare materials for all AMP activities consistent with Article 7 of the FMA and under the direction of the EAHCP Program Manager;
- Support the EAHCP Program Manager in correspondence to the USFWS including informational memorandums, clarifications, and amendments to the ITP and EAHCP;
- Participate in public outreach initiatives;
- Coordinate and publish the monthly EAHCP Steward newsletter and podcast;
- Maintain the content of the EAHCP website;
- Prepare and compile all Permittees' information for the annual report to USFWS; and
- Track and assist EAHCP Permittees with maintaining compliance with secondary implementation permits, such as: U.S. Army Corps of Engineers, Texas Parks and Wildlife Department, Texas Commission on Environmental Quality, General Land Office, and Texas Historical Commission permits.

Staffing in 2025:
The EAHCP staff consists of the Program Manager, EAHCP Director, Senior Contract Administrator, Senior Habitat Conservation Coordinator, and Habitat Conservation Coordinator II. EAA funds the Chief Science Officer and the Environmental Scientist II positions. Two positions remain vacant but could be filled in 2025. The structure of the existing EAHCP staff positions and EAA-funded positions – **the Threatened and Endangered Species Team** - are illustrated in the chart on the next page.

Threatened and Endangered Species Team



Positions Paid from EAA General Budget
Vacant Position

Budget:

EAHCP Program Management Budget for 2025

Description of Expense	Estimated 2025 Costs
Salaries and Fringe Benefits	$ 846,008
Office Supplies	$ 1,500
Non-Capital Assets	$ 6,000
Meeting Expenses	$ 20,000
Conferences, Seminars, and Training	$ 22,000
Memberships	$ 2,000
Printing	$ 23,000
Hosting, SAAS and Support Agreements	$ 2,000
Professional Contracted Services	
Annual Report	$ 50,000
Historical/Archeological Consultation	$ 35,000
Permit Oversight	$ 50,000
Outreach/Newsletter	$ 40,000
Science Committee Compensation	$ 25,000
ITP Renewal	$ 904,000
Other	$ 85,000
Estimated 2025 Total	$2,111,508

Table 7.1:
$750,000

Estimated 2025 budget:
$2,111,508

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

2025 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Dr. Katie Bockrath

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Background

A fully functioning refugia program must be capable of successfully reintroducing individuals produced from the captive-assurance population to the wild following a catastrophic event. Successful reintroduction depends on the survival of released individuals after release. Mark–recapture studies are commonly used to determine whether reintroduced individuals remain present in the wild (Canessa et al. 2016); however, expected recapture rates cannot be interpreted without baseline data describing recapture probabilities of tagged salamanders.

Mark–recapture approaches can also be used to evaluate how long reintroduced salamanders persist in the wild and to determine the optimal size or life stage for release. Repeated sampling following reintroduction is necessary to confirm long-term persistence, as reintroduction can only be considered successful if released individuals survive. When all released individuals are tagged, the duration of persistence can be quantified. Establishing this duration prior to a catastrophic event also allows estimation of the minimum number of salamanders required for effective reintroduction.

The Edwards Aquifer Refugia Program conducted a year-long mark recapture study of the San Marcos salamander (*Eurycea nana*). A total of 453 individuals were tagged with p-chips, and recapture surveys were conducted twice monthly across three sites. More than 3,000 salamanders were collected during the study, with relatively high recapture rates averaging 15%. Variation in snout-to-vent length was observed among sites, and no movement between sites was detected. Population size was also estimated using the ratio of tagged recaptures to untagged captures (see the 2024 research report for full details). Although this effort produced valuable demographic information for refugia management and conservation, the number of tagged individuals remained limited.

Photographic identification has been successfully used as a noninvasive “tagging” method in Barton Springs salamanders to estimate population size and infer emigration events (Bendik et al. 2023) and was shown to out perform physical tagging (Bendik et al. 2013). However, this method has not been evaluated for mark recapture monitoring of *E. nana*. The demonstrated success of photographic tagging in related salamanders suggests it may also be applicable to *E.*

nana. During the 2023–2024 p-chip mark recapture effort, photographs were collected for all captured individuals to enable future comparison with p-chip identifications. The present study continues the *E. nana* mark recapture work while also investigating alternative monitoring approaches by analyzing these photographic records.

The objective of this study was to evaluate recapture rates of *E. nana* using photographs of unique head markings for individual identification. Due to staff loss and the program's first Salvage and Reintroduction event, the original study scope was reduced. Rather than fully replicating the 2023–2024 photographic analysis, 2025 efforts focused on method validation and confirmation that photographs can reliably identify tagged salamanders.

Methods

While salamanders were anesthetized, three photographs were taken of each individual: one full-body image and two close-up images of the head. For each photograph, the photo number, p-chip number (if present), snout-to-vent length, and sex were recorded. All photographs were evaluated, and the highest-quality images for each individual were selected. Ideal images were in focus, showed the head in a consistent, non-tilted orientation, and were free of debris or air bubbles that could bias the photo-identification algorithm in Wild.ID. Each selected image was rotated to a standardized orientation with the tip of the nose pointing to the right. Images were then cropped from the tip of the nose to the base of the first gill to remove unnecessary visual information.

Processed images were uploaded to Wild.ID (Bolger et al. 2012), where they were systematically compared and scored for potential matches based on identifying features. All algorithm-generated matches were visually reviewed to confirm or reject the proposed identification. The photo numbers of confirmed matches were recorded and used to determine the date, location, and p-chip status of each individual. Photographs of p-chip tagged salamanders served as positive controls and were expected to match their corresponding reference images. Unmarked individuals with confirmed photographic matches were classified as recaptures.

Recapture rates for both p-chip tagged and photographically identified individuals were

calculated by dividing the number of recaptured individuals by the total number of individuals captured after tagging and multiplying by 100 to obtain a percentage.

Results

In total, 3,469 salamanders and 10,407 photographs were collected between May 2023 and May 2024. To validate the photo identification method, 880 photographs from May 2023 through September 2023 and March 13, 2024, were selected and processed. The dates were selected because they contained a relatively high number of p-chip recaptured individuals to compare to photo recapture data. During this period, 453 salamanders were tagged with p-chips. A total of 532 salamanders were captured following tagging, including 18 recaptures.

There is significant variation in the markings found on the heads of *E. nana*. Some individuals display high contrast and numerous markings while others are more uniform in pigmentation and pattern (Figure 1.) After processing in Wild.ID, 2,101 photo comparisons were completed, resulting in 24 photographic matches. Of these matches, 16 corresponded to confirmed p-chip tagged salamanders, three of which had lost their p-chip over the course of the year. Eight matches represented recaptures of previously untagged salamanders. Two p-chip tagged individuals were not recovered through photographic matching, yielding an initial error rate of 11%. Within this subsample, the recapture rate was 3% for p-chip tags and 5% for photographic identification.

Figure 1. **Eurycea nana** ***photographs showing both variation in unique markings across individuals and matched photos identifying specific individuals. Three*** **E. nana** ***individuals are shown.***

Discussion

The primary aim of this study was to determine whether photographs of the unique head markings of *E. nana* could be used to reliably identify individuals and therefore serve as a viable method for recapture monitoring. Physically tagged salamanders provided a control for validating photographic identification. Overall, our results indicate that photographic identification is a suitable and promising method for identifying individual *E. nana*, although some limitations remain. Sixteen of the eighteen p-chip tagged individuals included in the subsample were correctly identified using photographs, demonstrating a high level of agreement between physical and photographic identification methods. The two tagged individuals that were not recovered through photographic matching likely reflect limitations in photo quality, algorithm performance, or user confirmation error rather than true failure of the method. Poor image focus, debris obscuring markings, or inconsistent head orientation may have reduced the ability of Wild.ID to detect distinguishing features, especially in individuals with few markings and more uniform coloration. Additionally, because all automated matches require visual confirmation, human error in accepting or rejecting potential matches may have contributed to the observed discrepancy.

Photographic identification resulted in a higher recapture rate than p-chip tagging within the analyzed subsample. Photo identification also outperformed physical tagging *E. sosorum* (Bendik et al. 2013). This increase likely reflects the ability of photographs to detect recaptures among individuals that were never physically tagged, thereby expanding the observable dataset beyond the tagged cohort. As a result, photographic methods may provide a more complete representation of population persistence and encounter probability than reliance on physical tags alone.

There are several notable advantages to using photographs instead of physical tags. Photographic identification is noninvasive and avoids the potential stress, injury, or tag loss associated with physical tagging. The method is also comparatively inexpensive, requires less specialized training, and allows a larger volume of biological and spatial data to be collected during routine surveys. These characteristics make photographic identification especially attractive for monitoring programs or for use by personnel with varying levels of technical skill and experience.

Despite these benefits, photographic methods introduce distinct challenges. The workflow is more susceptible to human error during image capture, processing, and confirmation of algorithm-generated matches. Misidentification can occur if photographs are of insufficient quality, if duplicate or incorrect images are uploaded, if unique marking change over time or if database management errors arise. Bendik et al. (2013) showed that 5% of falsely rejected photo pairs (error) were due to changes in unique markings, 85% of errors were due to poor photo quality and 11% was due to both changes in unique markings and poor photo quality. In addition, reliance on digital equipment introduces the possibility of data loss, such as SD card failure or file corruption. These factors highlight the importance of standardized imaging protocols, redundant data storage, and quality control procedures when implementing photographic mark recapture approaches.

Taken together, the findings support the use of photographic identification as a practical alternative to physical tagging for *E. nana* monitoring. Preliminary photo recapture rates for the *E.nana* were lower than what was observed in *E. sosorum* (Bendik et al. 2023). Using the same recapture calculations utilized in this study, photo recapture rates were 21% in *E. sosorum* while the photo recapture rates for E. nana were 5%. This could be due to the different time scales of the study. The *E.nana* effort spanned one year while the Bendik study was over five years, allowing for more recapture events to occur. If the Bendik study was truncated to a year, photo recapture rates may be very comparable. While refinement of image collection procedures, database management, and validation workflows will improve accuracy, photographic identification offers substantial logistical and biological advantages. Future work should expand the analysis to the full dataset, quantify detection probabilities across sites and seasons, and evaluate how photographic recapture rates compare to traditional tagging methods for estimating survival, movement, and population size. Such efforts will clarify the role of photographic identification within long-term conservation and refugia management strategies for the San Marcos salamander.

References

Bendik NF, Morrison TA, Gluesenkamp AG, Sanders MS, O'Donnell LJ. 2013. Computer-assisted photo identification outperforms visible implant elastomers in an endangered salamander Eurycea tonkawae. PLOS ONE 8:e59424

Bendik NF, Chamberlain DA, Devitt TJ, Donelson SE, Nissen B, Owen JD, Robinson D, Sissel BN, Sparks K. Subterranean movement inferred by temporary emigration in Barton Springs salamanders (Eurycea sosorum). PeerJ. 2021 Apr 23;9:e11246. doi: 10.7717/peerj.11246. PMID: 33981501; PMCID: PMC8074841.

Bolger, D. T., T. A. Morrison, B. Vance, D. Lee, and H. L. I. 2012. A computer-assisted system for photographic mark–recapture analysis. Methods in Ecology and Evolution 3: 273–285

Canessa, S., P. Genta, R. Jesu, L. Lamagni, F. Oneto, S. Salvidio, and D. Ottonello. 2016. Challenges of monitoring reintroduction outcomes: insights from the conservation breeding program of an endangered turtle in Italy. Biological Conservation 204:128-33.

Sugiura, N. 1978. Further analysts of the data by akaike's information criterion and the finite corrections. Communications in Statistics-Theory and Methods 7:13-26.

Evaluating survival and tag retention of cave amphipods and Comal Spring riffle beetles

Report by: Brian De La Torre, Auburn Cooperative Fish and Wildlife Research Unit, Auburn University

USFWS Partners: Desiree Moore, M.S., and Dr. Katie Bockrath

Project period: January 1, 2023-December 2025

Importance of the research

Populations of Peck's cave amphipod (*Stygobromus pecki*) and the Comal Spring riffle beetle (*Heterelmis Comalensis*) are maintained at the San Marcos Aquatic Resources Center and the Uvalde National Fish Hatchery under the Edwards Aquifer Refugia Program 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, Edwards Aquifer 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 refugia. Tagged individuals would also allow biologists to conduct controlled laboratory studies to better understand the *H. comalensis* 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

S. pecki 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. *S. pecki* 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 *H.comalensis* 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 *H. comalensis* is uniquely adapted to spring obligates with highest occurrence around spring openings and are rarely found outside of a close proximity to spring flow.. 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 *S. pecki* is < 11-mm long (estimated maximum size) and the adult *H. comalensis* 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 *S. pecki* and the *H. comalensis* 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 *H. comalensis* and *S. pecki*. 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 H. comalensis 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 *S. pecki* 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 and replicating water quality and condition in a remote lab would have introduced additional sources of environmental error. Because *S. pecki* 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 *H. comalensis* using pChips

The beetles used in our experiments were raised in a hatchery or collected from the wild by USFWS biologists. Adult *H. comalensis* 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 *H. comalensis* 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 four 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 2017); 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 °C. 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 *H. comalensis*. 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 the *H. comalensis*. 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 *H. comalensis*.

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).

Kaplan-Meier

We constructed Kaplan-Meier (KM) time-to-event curves (Goel et al. 2010) to estimate beetle survival over time in control chambers. These non-parametric curves provide survival probabilities at successive time intervals, defined here as days post-introduction into a control chamber. We designed control chambers to allow for panoramic visual access, facilitating accurate and consistent monitoring of individual beetles. We defined an "event" as a visually confirmed death, indicated by prolonged immobility or an inverted posture, and the date of each

observed death was recorded. We applied right-censoring to individuals that survived past the end of the trial (e.g., 50 days, Barrajon and Barrajon 2020). Because mortality checks were conducted weekly, the exact day of death was typically unknown. This introduces interval censoring since deaths were only known to have occurred in the week in which they were observed (Turkson 2021). Standard KM methods assume exact event times; however, their use here is still justified given the adherence of USFWS schedules. Regardless, KM curves still provide an informative approximation of survival trends in this context. We used KM survival curves for control groups as baselines for comparison with experimental groups modeled using a Bayesian framework. These comparisons allowed assessment of whether experimental conditions (e.g., tag attachment, tag weight) influenced beetle survival relative to the known mortality in controls. All KM analyses were performed using the *survival* package (version 3.3-1; Therneau 2023) in R version 4.4.0 (R Core Team 2022).

Multistate survival analysis

To estimate survival probabilities while accounting for imperfect detection in experimental beetle chambers, we used a robust design multi-state model with latent imputation, based on a state-space extension of the Cormack-Jolly Seber (CJS) framework (Schaub et al. 2004. Traditional CJS models estimate survival from detection histories assuming a single "alive" state (White and Burnham 1999). In contrast, multi-state models allow individuals to transition among multiple states, providing a better understanding of biological processes. These models improve biological interpretation by separating the underlying state process (e.g., survival) from the observation process (i.e., whether an individual was detected) (Horton et al. 2011, Le-Rademacher et al. 2022). This state-space structure helps to account for uncertainty due to non-detection and enables inference when deaths are not directly observed.

We developed a Bayesian multistate survival model in JAGS using passive detections of beetles recorded via p-Chip scanners. Each detection was automatically timestamped with an exact date and time, allowing for the construction of individual detection histories over the course of each trial. We then used these detection histories to estimate survival rates in captive populations, where ongoing detection indicated continued survival, and gaps in detection required probabilistic inference. We chose a multistate modeling approach due to the imperfect detection inherent in passive monitoring systems (Zhang et al. 2023). Individuals could remain

alive yet undetected because of behavioral variation, limited scanner coverage, or intermittent tag failure. By probabilistically resolving these uncertainties, the model yields more accurate survival estimates under experimental conditions. Understanding survival patterns in tagged *H. comalensis*, is important for evaluating how tagging may influence individual viability and for informing conservation strategies for this endangered species.

We defined three biological states to represent each beetle's condition during a sampling period: (1) Alive but undetected, (2) Alive and detected, and (3) Dead. These latent states capture the true, unobserved status of individuals and form the basis of the state-space modeling framework. Transitions between these states were governed by two key parameters: survival (ϕ) and detection (p). Survival (ϕ) determined the probability of remaining alive between sampling occasions and controlled transitions from either alive state (1 or 2) to the dead state (3). Detection (p) governed transitions between undetected and detected states (1 <–> 2) during closed periods. The model uses patterns in observed detections, especially repeated detections, to probabilistically infer the latent states of individuals with incomplete or uncertain detection histories. Because multistate models require a known initialization state, all individuals were assigned to a known state (i.e., alive but undetected) at the first sampling occasion if not already detected (Hougaard 1999). By linking these biological processes to observed detection data, the model probabilistically reconstructed individual survival histories, even when detections were intermittent or missing.

To align the model with the biological and observational processes of the study, we discretized each experimental detection period into temporal bins. Because beetle detections occurred intermittently and without consistent daily patterns, bins were constructed by grouping days with similar detection frequencies, resulting in periods of unequal duration (Fig. 12 & 13). Our model follows a robust design framework with alternating closed and open periods (Kendall et al., 1997; Kendall and Bjorkland, 2001). Closed periods corresponded to intervals during which detections could occur; during these intervals, the latent biological state was assumed to remain unchanged. This structure allowed estimation of detection probabilities (p) independently of survival, thus reducing bias from short-term variability in detection (Pollock 1982). In contrast, open periods were defined as intervals without detections, during which individuals were allowed to transition between alive and dead states according to the survival probability (ϕ).

In the model, we implemented a mapping vector (safe_idx) that linked each interval to a specific survival estimate.

All multistate analyses were analyzed by means of a Bayesian framework with Markov Chain Monte Carlo (MCMC) methods using JAGS called from the R package 'jagsUI' (Kellner 2024). All priors were uninformative to express our current lack of knowledge regarding each parameter (Hobbs and Hooten 2015). Weakly informative normal priors were used to aid initial convergence of latent state chains. Posterior distribution for each parameter were estimated based on the 5th sample from 20,000 iterations across three parallel chains with a burn in period of 8,000 iterations per chain. Model convergence was assessed based on visual inspection of trace plots, the Gelman -Rubin statistic (R), and density plots for each parameter. Convergence was considered successful if trace plots showed good mixture of MCMC chains, if was less than 1.1 (Gelman and Rubin 1992) and density plots showed similar shapes for each chain.

Results

Evaluation of tagging amphipods

Hyalella azteca were used as a surrogate species for *S. pecki*. We initially tagged eight *H. azteca* using pChips. After 24 h, all of the tags remained on the H. azteca except for one. We did not assess survival of the *H. azteca* (see beetle survival) and felt that pChips might be an option for further testing with S. pecki, if available in the refugia population. Unfortunaly, *S. pecki* numbers in the refugia were insufficient to justify potential mortality associated with this tagging effort. .

We also tagged 28 *H. azteca* 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 two 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 *H. comalensis* 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 seven living beetles in our treatment chambers at the end of the first experiment (all *H. glabra*). Alternatively, we had 10 living *H. comalensis* in chamber one and 14 living *H. glabra* beetles in control chambers two and three. All of the living beetles retained their p-Chips, indicating high tag retention. Data collected from our scanners indicated detection (n = seven 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 two 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 two beetles

alive in the treatment chambers and 4 beetles alive in the control chamber at the end of this experiment.

We started our third series of experiments in October 2024. We successfully 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 induced stress. We maintained our tagging procedure similar to previous trials only swapping out the superglue for aquarium glue (Cyanoacrylate glue, WoldoClean) because it does not tend to run when it touches water. For this trial, we randomly assigned p-Chips to a treatment chamber prior to tagging. This allowed us to minimize handling time as we could tag beetles in 15 count batches and then immediately place them in their respective chamber. Due to the high number of mortalities discovered in trial two, we increased the number of control beetles to 15.

We ended Trial three in December 2024 with the experiment lasting 50 days. We chose this duration based on results from Trial two, where less than half of the beetles presumably survived beyond day 50 based on detection results. Notably, there were no instances of the high mortality rates observed in Trial one. At the end of the trial, across all three experimental chambers containing p-Chip tagged beetles, we recovered nine surviving H. glabra from the initial 45 tagged individuals (T2 = five, T3 = two, T4 = two). In the p-Chip control chamber, 13 beetles remained alive.

We began Trial 4 in March 2025 and successfully tagged 34 *H. comalensis* beetles using p-Chips. Due to the inability to collect *H. glabra* from the Devil's River springs, we relied on an already collected stock of *H. comalensis* beetles available at SMARC. Due to concerns that chamber conditions may influence survival outcomes, we implemented an interspersion design to assign both tagged and untagged beetles across experimental and control chambers. This design aims to minimize potential chamber effects by distributing beetles more evenly across treatments. We used three experimental chambers (T2, T3, T4), placing 6 p-Chip *tagged H. comalensis* beetles and five untagged beetles in each chamber. The only exception was T4, which contained three untagged beetles, supplemented with two *H. glabra* individuals due to limited beetle availability. Similarly, we used three control chambers with an interspersion design, placing five tagged and five untagged beetles in each. The only exception was control chamber

one, which contained six tagged beetles, (one of which had an unreadable tag). Notably, we observed that these *H. comalensis* were much less active compared to previous trials when using H. glabra beetles. This allowed for much quicker tagging and the majority of beetles did not require placing them in freezer conditions for tagging to happen. After all beetles were tagged, they were placed in their respective chambers and connected to flow set to 15m/s to maintain consistent conditions.

We concluded Trial four in May 2025 after a 50-day experimental period. Detection using p-Chip scanners was notably poor, as only one tagged beetle was detected on a single day in April and no additional detections were recorded. Survival outcomes reflected this limited detection: among tagged beetles in experimental chambers, only 6 beetles were recovered alive (T1 = one, T3 = five, T4 = one). Similarly, in control chambers, only three beetles were recovered alive (one tagged, two untagged), all from control one.

We suspect that beetle mortality in Trial four was primarily driven by natural senescence rather than tagging effects. This interpretation is supported by the unusually low detection rate across the 50-day period, which contrasts with patterns observed in previous trials (2 and 3). Further evidence comes from the early mortality observed in control chambers, during the first weekly check, 26 beetles were reported as either dead or missing (i.e., due to carapace degradation and washout), with only four individuals confirmed alive. Lastly, during the tagging process, many beetles appeared sluggish and less responsive compared to earlier trials, suggesting physiological decline consistent with end-of-life behavior (Stroustrup et al. 2016).

Survival analyses of pChipped and control beetles

Survival analysis was conducted to compare p-chip tagged (experimental) and untagged (control) beetles. Control beetle survival was estimated using Kaplan-Meier (KM) methods, whereas experimental beetle survival was modeled using a Bayesian multistate survival model that accounted for imperfect detection. Because multistate modeling requires sufficient sample size, only Trials two and three were analyzed. The trials differed in total duration (Trial two: 150 days; Trial three: 50 days) but for comparability, only the first 50 days of Trial two were used.

In Trial two, control beetle survival declined gradually, with an estimated probability of 0.60 (95% CI: 0.45 – 0.78) by day 50 (Fig. 14). Experimental beetles exhibited lower survival (with quite a bit of uncertainty), with a posterior mean of 0.55 (95% CrI: [0.14–0.89]) at the last open interval (days 35-47). The Bayesian survival curve paralleled the KM trajectory, though trended lower with modest overlap in credible and confidence intervals throughout the trial. Note that credible and confidence intervals are not directly comparable but should provide reasonable estimates of the differences in curves while including uncertainty. In short, confidence intervals result from a frequentist approach and describe the reliability of the procedure, whereas credible intervals describe the probability of the parameter itself under a Bayesian model.

Interval-specific survival probabilities (ϕ) were estimated for four open periods: $\phi_1 = 0.75$ [95% CrI: 0.40–0.94] (days 4–7); $\phi_2 = 0.69$ [0.33–0.93] (days 11–21); $\phi_3 = 0.60$ [0.18–0.91] (days 25–31); and $\phi_4 = 0.55$ [0.14–0.89] (days 35–47). These values represent the conditional probability of surviving each interval. Across all intervals, the cumulative survival probability was 0.17 by day 50, indicating ~17% of individuals were expected to survive the full trial duration. Based on scanner detections, at least 6 of 60 individuals (10%) were confirmed alive at the end of the 50-day trial, which seems reasonably in line with the model predictions.

In Trial three, survival patterns diverged more strongly between groups (Fig. 15). Control beetles maintained a high average survival at 0.87 (95% CI: 0.71-1.0) at day 50, with only two deaths recorded (i.e., at day 49). In contrast, average experimental beetle survival was lower at 0.58 (95% Crl: 0.16 -0.90) for the final open interval (i.e., days 35-47). Unlike Trial two, the credible and confidence intervals did not overlap, suggesting there may be a larger difference between the two groups (but note that the two uncertainty intervals are not interpreted the same way).

As expected, interval-specific survival estimates declined slightly across the four open periods: $\phi_1 = 0.66$ [95% CrI: 0.26–0.92] (i.e., days 4–7), $\phi_2 = 0.62$ [0.22–0.92] (i.e., days 11–21), $\phi_3 = 0.59$ [0.17–0.91] (i.e., days 25–31), and $\phi_4 = 0.58$ [0.16–0.90] (i.e., days 35–47). The cumulative modeled survival probability by day 50 indicated that only ~14% of individuals were expected to survive the full duration of the experiment. Consistent with this prediction, six of 45 individuals (13%) were confirmed alive at the end of the trial.

Comparison of survival curves

To better assess the degree of similarity between Bayesian survival trajectories for Trial two and Trial three, the proportional overlap of 95% credible intervals was quantified (Fig. 16). Values closer to 1.0 indicate complete agreement between the curves, whereas a value closer to 0.0 indicates no overlap. Overlap proportions ranged from 0.86 at day 7 to 0.95 at day 50, indicating high overlap between curves; thus, relatively consistent survival dynamics between trials.

Similarly, control (KM) curves were compared using a log rank test as a standard way to measure difference between the two groups (Fig. 17). Although Trial 3 beetles had higher average survival (Trial 2: 60%; Trial 3: 83%), survival did not differ significantly between the control groups ($\chi^2 = 2.9$, $p = 0.09$).

Model Diagnostics

Convergence of the Bayesian survival models was evaluated using posterior density plots, trace plots, and Gelman – Rubin statistics ($\hat{R}$). Posterior density estimates for interval-specific survival parameters (ϕ) were generally unimodal, with most of its mass concentrated between 0.5 and 0.8. For one interval, the density displayed a slightly flattened peak between ~0.55 and 0.75 and a mild left skew, suggesting some uncertainty or weak identifiability. However, the distribution remained within the expected range (0-1) and centered on biologically plausible values. Trace plots for all ϕ parameters indicated adequate mixing of chains. $\hat{R}$ values for ϕ_1–ϕ_4 (1.004, 1.027, 1.002, 1.014) were all close to 1.0, supporting adequate model convergence. The slightly elevated $\hat{R}$ for ϕ_2 (1.027) and the flattened density top might suggest that this interval was less informed by these data, likely reflecting limited detections.

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 *H. comalensis* or the *S. pecki*. 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. Ultimately, BEEtags were assessed because they could provide similar information as pChips, thus would be a more desirable tagging alternative.

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 *H. comalensis***.** 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 *H. comalensis* 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 edge 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.

We concluded our evaluation of BEEtags on beetles in December 2024 with the study lasting 50 days, coinciding with the conclusion of Trial three involving p-Chips. Out of the 10 *H. Comalensis* beetles initially tagged with BEEtags, only two beetles survived by the end of the

experiment. Similarly, for the 15 untagged H. Glabra beetles in the control chamber, only three beetles remained alive at the trial's conclusion. Survival concerns with BEEtags were observed within the first week, as only half of the tagged beetles remained alive (seven alive beetles, five observed mortalities, and three presumed deaths). Additionally, issues with tag retention was observed as two beetles in the control chamber were observed without their tags as early as the first week.

We hypothesizethat BEEtag size (1.2mm) was a potential factor influencing survival of BEEtagged beetles. This is likely due to greater hydrodynamic drag as the tag interfered with movement in flowing water. These issues suggest that reducing BEEtag size or rounding down edges could mitigate some of these effects. Currently, BEEtags can be downsized to ~0.7mm, making them closer in size to p-Chips (~0.5mm) while still maintaining readability. However, it remains uncertain how effective identification would be at a reduced size (~0.7mm) across large batches. To assess this, we conducted a validation test to determine the success rate of BEEtag identification at this reduced size.

Validation of BEEtags

The resizing of BEEtags was necessary to accommodate the small size of riffle beetles but may create inaccurate tag reading as these tags were reduced beyond their intended purpose. To ensure the accuracy of BEEtags, validating the accuracy of these resized tags was important to confirm their reliability in providing correct identification numbers. The tags we used for this validation test were numbered 824-1219, (series 200-299.pdf) and consisted of 100 tags total. Using the optimization procedure for reducing tags to <1mm (See above), tags were printed and then individually cut using a razorblade. To validate tag accuracy, we randomly selected tags (n = 40) from the total 100 tags using a random number generator in Microsoft Excel. Next, we took a photo of each tag using a standard phone camera, while a bright light (i.e., lamp) illuminated the tag. These images were then uploaded to the desktop version of MATLAB software (see above) for analysis. In MATLAB, I used the BEEtag software to identify each tag's ID number. Then, this number was compared to the original tag PDF template to confirm its accuracy, ensuring that both the ID number and QR code pattern matched (Figure 12). This process of image capture, tag identification, and verification was repeated for all 40 selected tags.

BEEtag Validation Results

Of the BEEtags chosen (n = 40), 36 tags were correctly identified by the software (90%) while 4 tags (10%) did not return an identification number (SD = 0.0474). One potential issue with BEEtags is the possibility of generating false positives (e.g., generation of an incorrect identification number). According to Crall et al. (2015), only five out of 11,166 codes tested resulted in incorrect values (i.e., success rate of 99.96%). This suggests that BEEtags have a high successful identification rate, which may remain true even when modified for specific applications. In this validation test, no false positives were observed. Instead, the tags that failed did so by not returning any identification number rather than an incorrect value. The performance of BEEtags depends heavily on visual information and can be substantially affected by image quality (i.e., resolution of phone camera) or uneven lighting (Crall et al. 2015), thus there could be possible explanations for unidentified tags at this size. We overcame the latter by ensuring each picture had similar lighting (i.e., illuminated by lamp), which likely helped when identifying them with computer software. A significant improvement could be to use photography equipment with better zoom, focus, or photo resolution to test if identification rate improves. While the current test did not identify any misclassified tags, it is still important to be aware of the potential for false positives in future applications.

Improving BEEtag shape

One potential issue with BEEtags may be their square shape protruding and creating drag in flowing water. To address this, we attempted to modify the tag shape to be more rounded. We used a handheld hole punch (Fiskars Brand Inc.) with a 0.15cm circular punch designed for paper. During this process, we found that for the tag to be recognized by the BEEtag software, the entire square code must remain fully intact. The software relies on detecting four right angles for tag recognition and identification. We initially tested this circular tag design by systematically cutting and validating tags (see above). However, many of these circular tags failed to be properly recognized. We observed that the punch tool would slightly distort the edges of the tag, impairing recognition. As a result, we reverted to using a razor blade to manually cut square tags, repeating the validation process with this original method. As the current waterproof paper used for BEEtags is noticeably thicker and more rigid than standard paper, this may

potentially prevent clean cuts. This technique may be improved upon by exploring printing tags on a more flexible material (i.e., silicone-like) to better accommodate round shapes.

Discussion

This study evaluated the feasibility of tagging extremely small aquatic invertebrates (*Stygobromus pecki* and *Heterelmis comalensis*) to enable individual identification and monitoring within captive refugia systems and experimental settings. The results demonstrate that while individual tagging of organisms of this size is technically possible, significant methodological and biological challenges remain. Both electronic micro-transponder tags (p-Chips) and image-based tags (BEEtags) could provide unique individual identification; however, each approach presented different logistical and biological limitations. The study highlights the difficulty of applying tagging technologies to organisms only a few millimeters in length, where tag weight, hydrodynamics, and attachment methods can influence mobility and survival. Despite these challenges, the work provides important preliminary insight into tagging approaches that could improve the ability to monitor individuals in both captive assurance programs and future biomonitoring efforts.

Passive monitoring housing: advantages and limitations

The passive monitoring chambers developed for this study provided a controlled system for evaluating tag detection and survival while maintaining flowing water conditions that more closely resemble spring habitats. The design allowed continuous scanning of p-Chips without repeated handling of animals, reducing disturbance and enabling time-stamped detections whenever individuals passed through the reader's detection field. Passive monitoring systems also have the advantage of generating large datasets on individual presence over time, which can support survival modeling and behavioral observations without intensive manual observation.

However, several limitations were also identified. The short detection range of the p-Chip system required individuals to pass within approximately 7 mm of the scanner to be detected, meaning that many live animals may have gone undetected if they did not move through the scanning field. Behavioral variability in beetle movement patterns further reduced detection frequency, particularly during the initial trial when chamber structure and substrate allowed animals to avoid the scanner area. Adjustments to the chamber design improved detection rates but did not eliminate imperfect detection. These findings emphasize that passive monitoring systems must be carefully designed to guide animal movement through detection zones and that statistical approaches capable of accounting for imperfect detections, such as the multistate survival modeling framework used in this study, are essential for interpreting the resulting data.

Physical impacts of tagging on beetles

The study identified several potential physical impacts associated with tagging extremely small beetles. The p-Chip tags, while small, are rigid. During trials, p-Chips occasionally caught on chamber materials or substrate surfaces, which could impede natural movement or increase the energetic cost of locomotion. Such mechanical interactions could contribute to reduced survival or altered behavior, particularly in environments with complex substrates similar to those found in natural spring habitats.

The BEEtag system presented a different set of physical considerations. Because the tags consisted of small pieces of waterproof paper and were larger than p-chips, they occasionally functioned as a "sail" when exposed to flowing water. In some cases, the increased drag created by the tag caused beetles to be pushed by the current or flipped onto their backs, potentially impairing their ability to move or right themselves. Even minor changes in hydrodynamics can be significant for organisms of this size, and tag design must therefore minimize drag and interference with natural swimming or crawling behavior.

Handling during the tagging process also represents a potential stressor. Tag attachment required immobilizing beetles under magnification, drying the elytra, and applying adhesive before positioning the tag. Although cold anesthesia was effective in slowing beetle movement and reducing handling time, the process of manipulating individuals and applying glue may still contribute to stress or mortality. Minimizing handling time and physical tag design will therefore be important considerations for future tagging approaches.

Use of surrogate species

The use of surrogate species in this study was an important step in minimizing risk to the endangered species. Experiments conducted with *Heterelmis glabra* and *Hyalella texana* allowed refinement of tagging techniques, chamber design, and monitoring protocols before applying them to the listed species. Because the surrogate species shares similar morphology and ecological characteristics, it provided a practical model for evaluating general feasibility and identifying potential methodological issues.

Nevertheless, the use of surrogate species cannot be considered a direct assessment of the impacts of tagging on the endangered species itself. Differences in physiology, behavior, or sensitivity to handling may influence survival outcomes. Consequently, while surrogate experiments provide valuable preliminary information, they should be viewed as a method-development step rather than definitive evidence of species-level impacts. Additional evaluation using *H. comalensis* and *S. pecki* would ultimately be required before implementing tagging methods at larger scales.

Evaluation of tagging technologies and future directions

After evaluating both tagging systems, we believe that the BEEtag approach has the greatest potential for future tagging efforts involving these species. The long-term availability of p-Chip technology is uncertain, and in our trials the presence of p-Chips appeared to reduce beetle survival or interfere with movement due to their rigidity and relative weight. In contrast, BEEtags are lightweight, inexpensive, and easily scalable, allowing tags to be produced at smaller sizes while still maintaining unique identification codes.

Several potential modifications to the BEEtag system could further improve its suitability for extremely small aquatic insects. One option is printing BEEtags directly onto waterproof adhesive sticker material, which would eliminate the need for glue during attachment and may reduce mortality associated with handling and adhesive application. However, retention of adhesive-backed tags in aquatic environments would need to be evaluated to determine whether this approach is feasible.

Another promising option is producing BEEtags using thin silicone materials. Silicone tags could maintain the binary identification pattern required for automated image recognition while providing increased flexibility and reducing the rigid edges that contribute to drag in flowing water. In addition, modifying tag geometry could reduce hydrodynamic effects. For example, producing circular tags rather than square tags and further reducing tag size may decrease drag and reduce the likelihood that tags act as sails in flowing water. Continued experimentation with tag material, size, and shape will likely be necessary to develop a tagging method that minimizes impacts on beetle mobility and survival.

Applications for refugia husbandry

If a reliable tagging method can be developed, individual identification would provide valuable tools for managing refugia populations. Tagging would allow husbandry staff to track individual survival, longevity, and reproductive output within captive populations. These data could improve colony management by enabling monitoring of individual health, identifying successful breeders, and evaluating how environmental conditions influence survival and reproduction.

Applications for biomonitoring

Beyond captive propagation, tagging technologies could support future biomonitoring and ecological research efforts. Individual tags would enable mark–recapture studies capable of estimating survival, movement patterns, and population dynamics for species that are otherwise extremely difficult to observe in the wild. Tagged individuals could also be used to evaluate the success of reintroduction or augmentation efforts by allowing researchers to track post-release survival and persistence in natural habitats.

In addition, tagging could facilitate experimental studies examining behavioral responses to environmental variables such as water flow, temperature, or energy availability. Because these variables can be manipulated under laboratory conditions, individually tagged organisms could provide insight into how environmental changes influence survival and behavior. Tagging wild individuals could validate laboratory observations. Such information would ultimately improve understanding of how spring-dependent species may respond to groundwater extraction, drought, or other environmental stressors affecting Edwards Aquifer ecosystems.

Overall, while substantial technical challenges remain, continued refinement of lightweight, minimally invasive tagging methods, particularly image-based systems such as BEEtags, has the potential to improve both husbandry practices and ecological monitoring for endangered spring invertebrates.

References

Authority, Edwards Aquifer. 2021. 2021 Work Plan. Edwards Aquifer Recovery Implementation Program, San Antonio, Texas

Barrajon, Enrique, and Laura Barrajon. 2020. Effect of right censoring bias on survival analysis. *arXiv preprint arXiv:2012.08649*.

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

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

Bowles, David E., Cheryl B. Barr, and Ruth Stanford. 2003. Habitat and phenology of the endangered riffle beetle *Heterelmis comalensis* and a coexisting species, *Microcylloepus pusillus* (Coleoptera: Elmidae) at Comal Springs, Texas, USA. Archiv für Hydrobiologie 156: 361-383.

Crall, J. D., N. Gravish, M. Mountcastle, and S. A. Combes. 2015. BEEtag: a low-cost, image-based tracking system for the study of animal behavior and locomotion. *PloS one* 10.9 (2015): e0136487.

Gelman, Andrew, and D. B. Rubin. 1992. Inference from iterative simulation using multiple sequences. Statistical Science 7: 457-472.

Gibbons, J. W., and K. M. Andrews. 2004. PIT tagging: simple technology at its best. BioScience 54:447-454.

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

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

Gruda, M. C., A. Pinto, A. Craelius, H. Davidowitz, W. M. Kopacka, J. Li, and W. Mandecki. 2010. A system for implanting laboratory mice with light-activated microtransponders. Journal of the American Association for Laboratory Animal Science 49:826–831.

Hewitt D. A., A. C. Janney, B. S. Hayes, R. S. Shively. 2010. Improving inferences from fisheries capture-recapture studies through remote detection of pit tags. Fisheries 35:217-231.

Hooten, M. B., and N. T. Hobbs. 2015. A guide to Bayesian model selection for ecologists. Ecological Monographs 85:3–28.

Horton, G. E., B. H. Letcher, and W. L. Kendall. 2011. A multistate capture–recapture modeling strategy to separate true survival from permanent emigration for a passive integrated transponder–tagged population of stream fish. Transactions of the American Fisheries Society 140:320–333.

Hougaard, P. 1999. Multi-state models: a review. Lifetime Data Analysis 5:239–264.

Huston, D. C., D. E. Bowles, J. N. Fries, and T. Brandt. 2015. Monitoring and marking techniques for the endangered Comal Springs riffle beetle, *Heterelmis comalensis* Bosse, Tuff, and Brown, 1988 (Coleoptera: Elmidae). Coleopterists Bulletin 69:793–798.

Huber, A. F., W. A. Fitzsimmons, and J. T. Westhoff. 2023. The smaller, the better? First evaluation of growth and mortality in crayfish internally tagged with p-Chips. Journal of Crustacean Biology 43:ruad071.

Kendall, W. L., J. D. Nichols, and J. E. Hines. 1997. Estimating temporary emigration using capture–recapture data with Pollock's robust design. Ecology 78:563–578.

Kendall, W. L., and R. Bjorkland. 2001. Using open robust design models to estimate temporary emigration from capture–recapture data. Biometrics 57:1113–1122.

Le-Rademacher, J. G., T. M. Therneau, and F. S. Ou. 2022. The utility of multistate models: a flexible framework for time-to-event data. Current Epidemiology Reports 9:183–189.

McCormick M. I., and S. Smith. 2004. Efficacy of passive integrated transponder tags to determine spawning-site visitations by a tropical fish. Coral Reefs 23:570-577.

Mollenhauer, R. and S. K. Brewer. 2017. Multinomial N-mixture models improve the applicability of electrofishing for developing population estimates of stream-dwelling Smallmouth Bass. North American Journal of Fisheries Management 37:211-224.

Moore, D. and S. K. Brewer. 2021. Evaluation of VIE, PIT, and p-Chip tagging methods in small bodied minnow species. North American Journal of Fisheries Management 41:1066-1078.

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

Pine, W. E., P. H. Pollock, J. E. Hightower, T. J. Kwak, and J. A. Rice. 2003. A review of tagging methods for estimating fish population size and components of mortality. Fisheries 28:10-23.

Pollock, K. H. 1982. A capture–recapture design robust to unequal probability of capture. Journal of Wildlife Management 46:752–757.

Price A. L., and J. T. Peterson. 2010. Estimation and modeling of electrofishing capture efficiency for fishes in wadeable warmwater streams. North American Journal of Fisheries Management 30:481-498.

Robinson, E. J. H., O. Feinerman, and N. R. Franks. 2014. How collective comparisons emerge without individual comparisons of the options. Proceedings of the Royal Society B: Biological Sciences 281:20140737.

Schaub, M., O. Gimenez, B. Schmidt, and R. Pradel. 2004. Estimating survival and temporary emigration in the multistate capture–recapture framework. Ecology 85:2107–2113.

Steffensmeier, Z., M. Wedgeworth, L. Yancey, N Santee, S. K. Brewer, and J. Perkin. 2022. Paradigm versus paradox on the prairie: Testing competing stream fish movement frameworks using a highly imperiled Great Plains minnow. Movement Ecology

Stroustrup, N., B. E. Anthony, Z. Nash, D. Gowda, J. Gomez, and others. 2016. The temporal scaling of *Caenorhabditis elegans* ageing. Nature 530:103–107.

Teixeira, A., and R. M. V. Cortes. 2007. PIT telemetry as a method to study the habitat requirements of fish populations: application to native and stocked Trout movements. Hydrobiologia 582:171-185.

Tenczar, P., C. C. Lutz, V. D. Rao, N. Goldenfeld, and G. E. Robinson. 2014. Automated monitoring reveals extreme interindividual variation and plasticity in honeybee foraging activity levels. Animal Behaviour 95:41–48.

Therneau, T. 2020. A package for survival analysis in R. R package version 3.2-7. Available: https://CRAN.R-project.org/package=survival. (March 2022).

Turkson, A. J. 2021. A closer look at the Kaplan–Meier and life table models in survival analysis. Open Access Library Journal 8:1–19.

U. S. Fish and Wildlife Service (USFWS). 2013. Biological Opinion for the Edwards Aquifer Recovery Implementation Program HCP (No. 21450-2010-F-0110; pp. 52–57). Ecological Services Field Office. Austin, TX.

Walters, C. J., B. T. van Poorten, and L. G. Coggins. 2012. Bioenergetics and population dynamics of Flannelmouth Sucker and Bluehead Sucker in Grand Canyon as evidenced by tag recapture observations. Transactions of the American Fisheries Society 141:158-173.

White, G. C., and K. P. Burnham. 1999. Program MARK: survival estimation from populations of marked animals. Bird Study 46(suppl. 1):S120–S139.

Zhang, Y., J. Li, H. Wang, and others. 2023. A hierarchical model for analyzing multisite individual-level disease surveillance data from multiple systems. Biometrics 79:1507–1519.

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 bushing and Sch 80 tube	5.08cm	4	Charlotte Pipe (Lowes)	$3.12
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

[1]Walter, Thomas, et al. "A new innovative real-time tracking method for flying insects applicable under natural conditions." BMC zoology 6 (2021): 1-11.

[2]Cowart, Dominique A., et al. "Environmental DNA (eDNA) applications for the conservation of imperiled crayfish (Decapoda: Astacidea) through monitoring of invasive species barriers and relocated populations." Journal of Crustacean Biology 38.3 (2018): 257-266.

[3]Crall, James D., et al. "BEEtag: a low-cost, image-based tracking system for the study of animal behavior and locomotion." PloS one 10.9 (2015): e0136487.

[4]Gui, Lian-You, et al. "Validation of harmonic radar tags to study movement of Chinese citrus fly." *The Canadian Entomologist* 143.4 (2011): 415-422.

[5]Crowe, Tasman P., Graeme Dobson, and Chan L. Lee. "A novel method for tagging and recapturing animals in complex habitats and its use in research into stock enhancement of *Trochus niloticus*." Aquaculture 194.3-4 (2001): 383-391.

[6]Blight, Olivier, et al. "Potential of RFID telemetry for monitoring ground-dwelling beetle movements: A Mediterranean dry grassland study." *Frontiers in Ecology and Evolution* 11 (2023): 1040931.

[7]Moffatt, Colin. "Using visible implant elastomer to tag insects across life stages: a preliminary investigation with blow flies (Diptera: Calliphoridae)." *The Canadian Entomologist* 145.4 (2013): 466-470.

[8]Piper, R. W. "A novel technique for the individual marking of smaller insects." *Entomologia experimentalis et applicata* 106.2 (2003): 155-157.

[9]Smith, Michael Thomas, Michael Livingstone, and Richard Comont. "A method for low-cost, low-impact insect tracking using retroreflective tags." *Methods in Ecology and Evolution* 12.11 (2021): 2184-2195.

[10]Lee, Inhee, et al. "mSAIL: milligram-scale multi-modal sensor platform for monarch butterfly migration tracking." *Proceedings of the 27th Annual International Conference on Mobile Computing and Networking*. 2021.

Figure 1. Image of treatment chamber where tagged beetles were housed during experiments evaluating tag retention and survival of tagged *H. comalensis*. 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 *H. comalensis*.

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

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

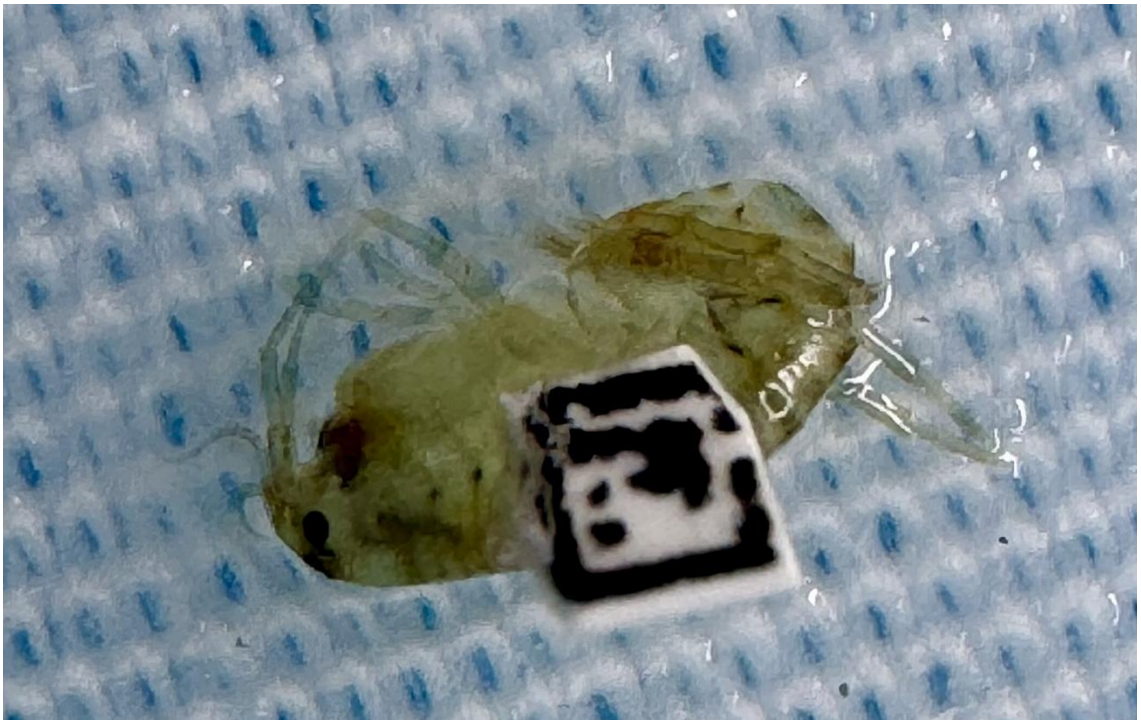

Figure 5. Example of an amphipod tagged with a 1.1-mm (1.3 mm with outside edge) BEEtag (QR codes).

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

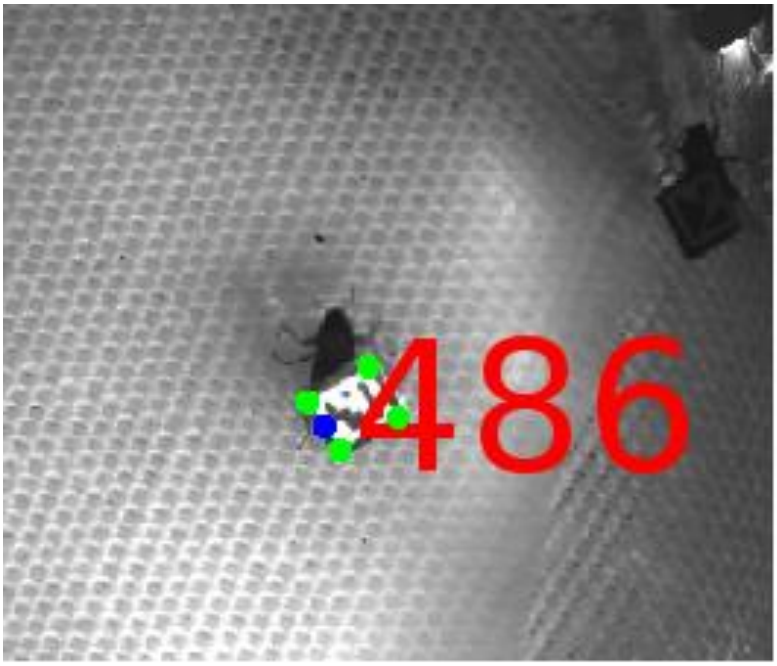

Figure 7. Image of BEEtag decoding performed using Matlab.

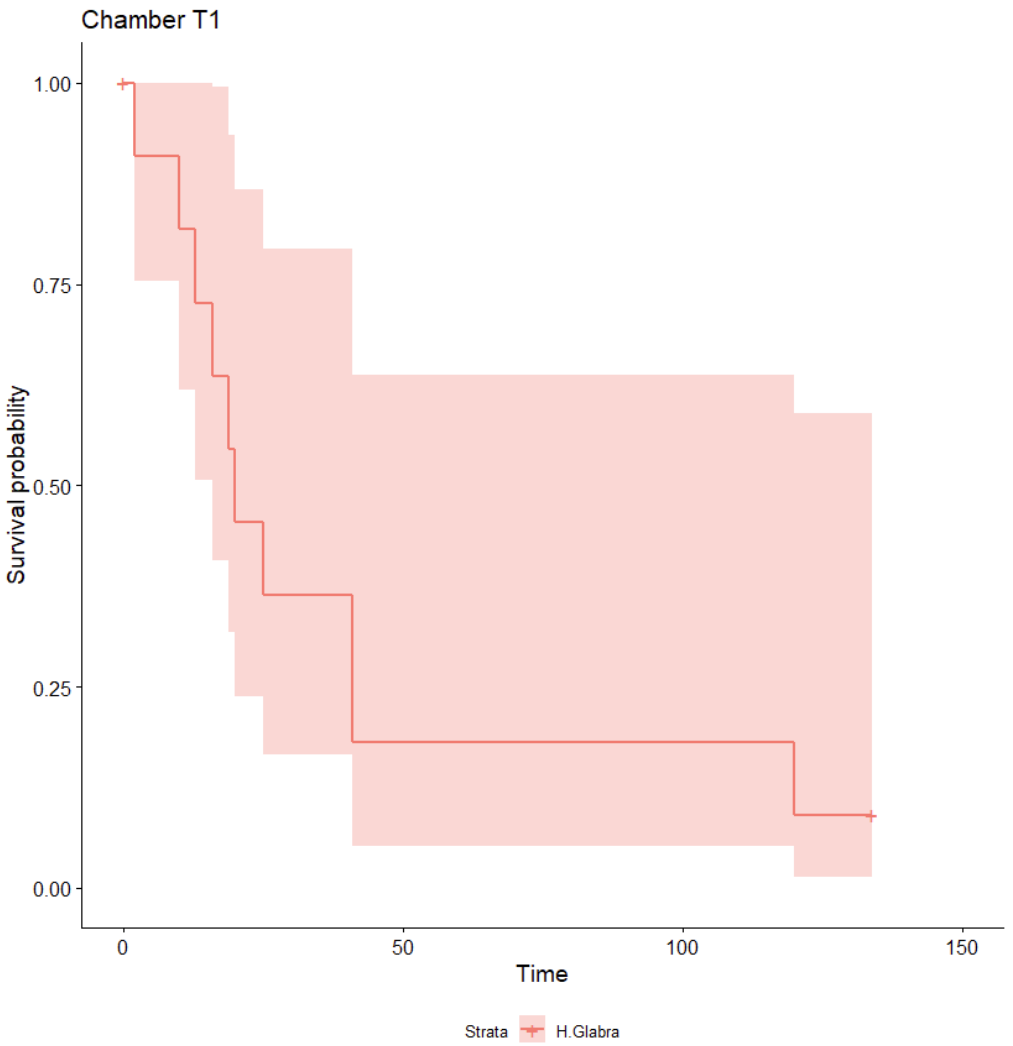

Figure 8. Kaplan-Meier plot for chamber T1. A plot of the Kaplan–Meier estimator is a series of declining horizontal steps which is assumed to approach the true survival function for that population, with a large enough sample size. The red line is the average survival, whereas the shaded area indicates the 95% confidence limits.

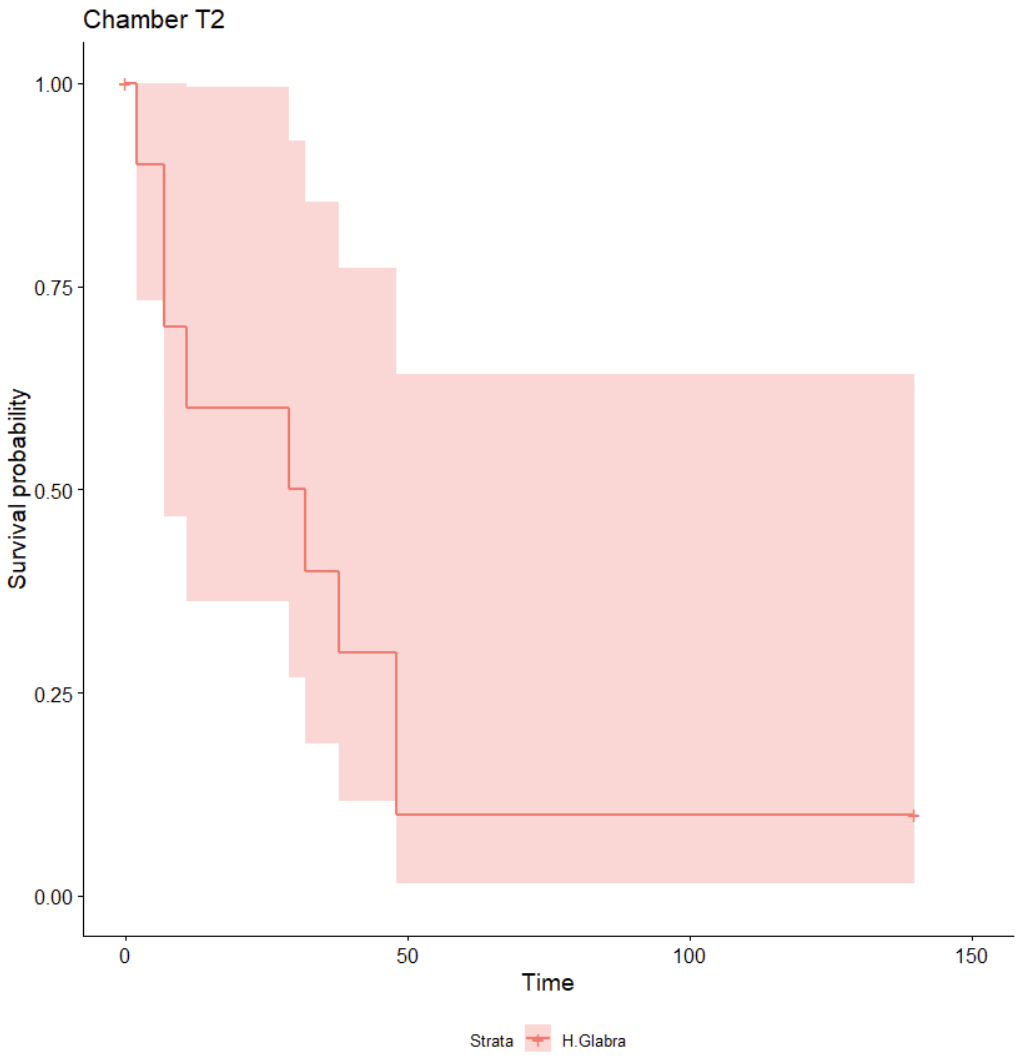

Figure 9. Kaplan-Meier plot for chamber T2. A plot of the Kaplan–Meier estimator is a series of declining horizontal steps which is assumed to approach the true survival function for that population, with a large enough sample size. The red line is the average survival, whereas the shaded area indicates the 95% confidence limits.

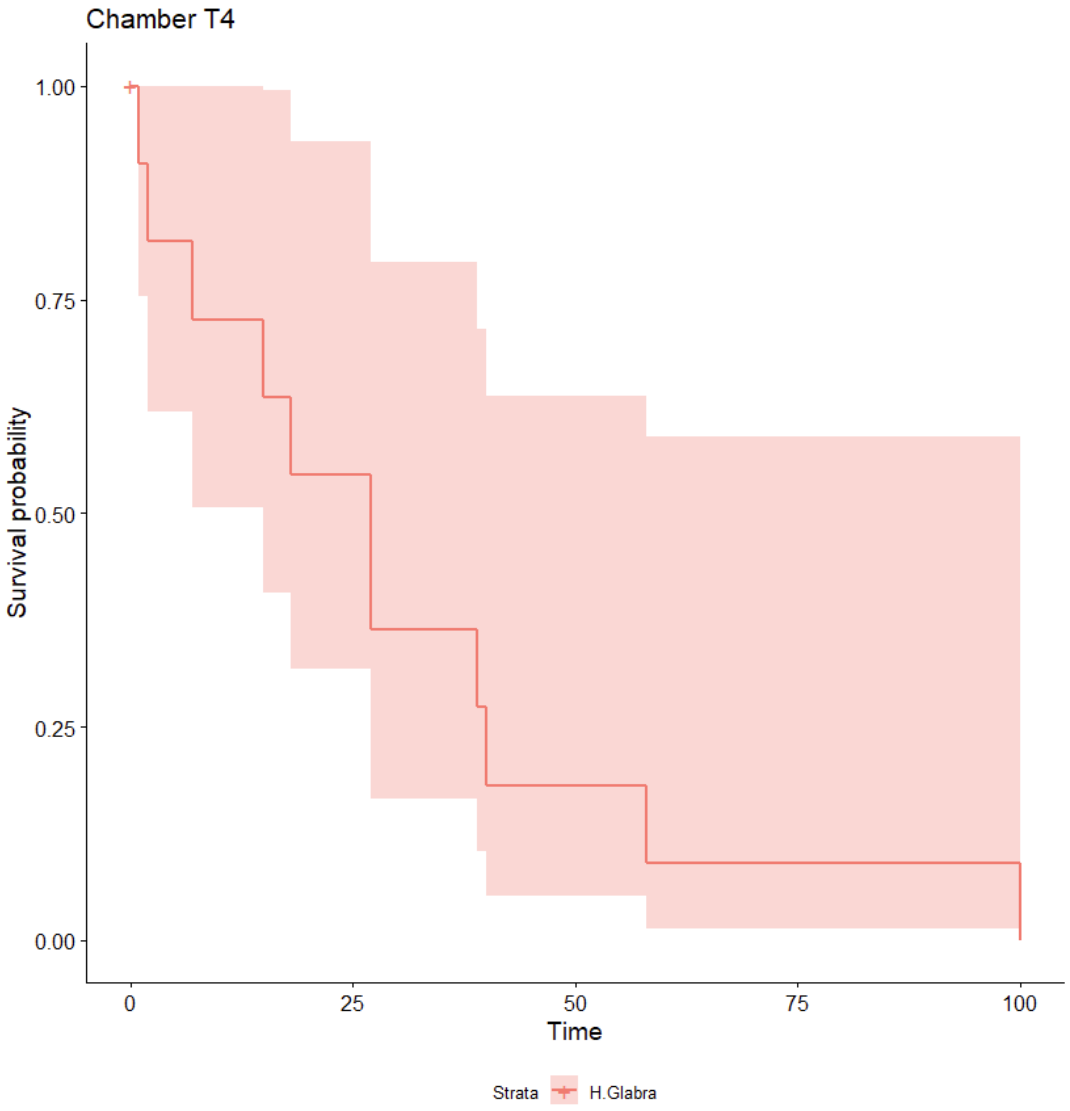

Figure 10. Kaplan-Meier plot for chamber T4. A plot of the Kaplan–Meier estimator is a series of declining horizontal steps which is assumed to approach the true survival function for that population, with a large enough sample size. The red line is the average survival, whereas the shaded area indicates the 95% confidence limits.

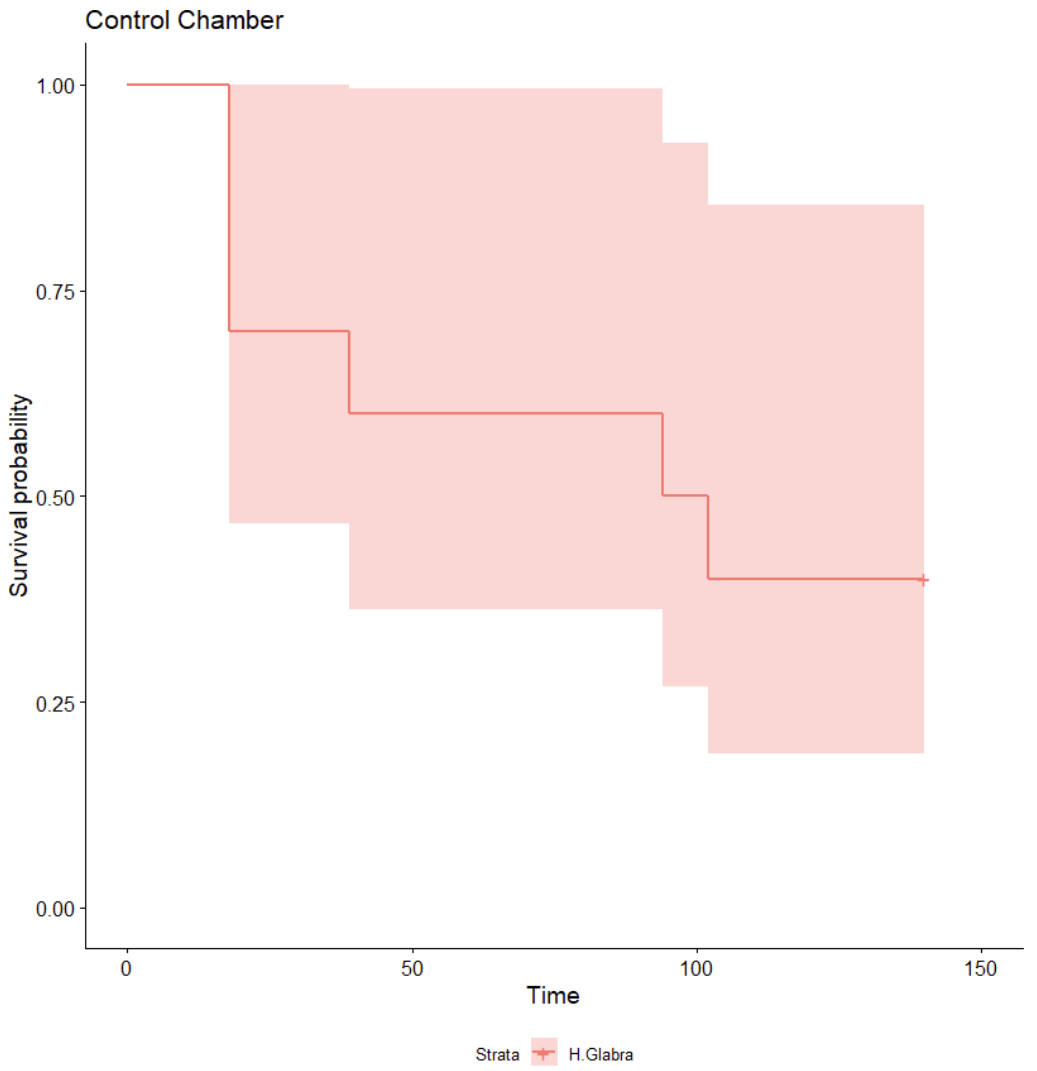

Figure 11. Kaplan-Meier plot for beetles held in the control chamber. A plot of the Kaplan–Meier estimator is a series of declining horizontal steps which is assumed to approach the true survival function for that population, with a large enough sample size. The red line is the average survival, whereas the shaded area indicates the 95% confidence limits.

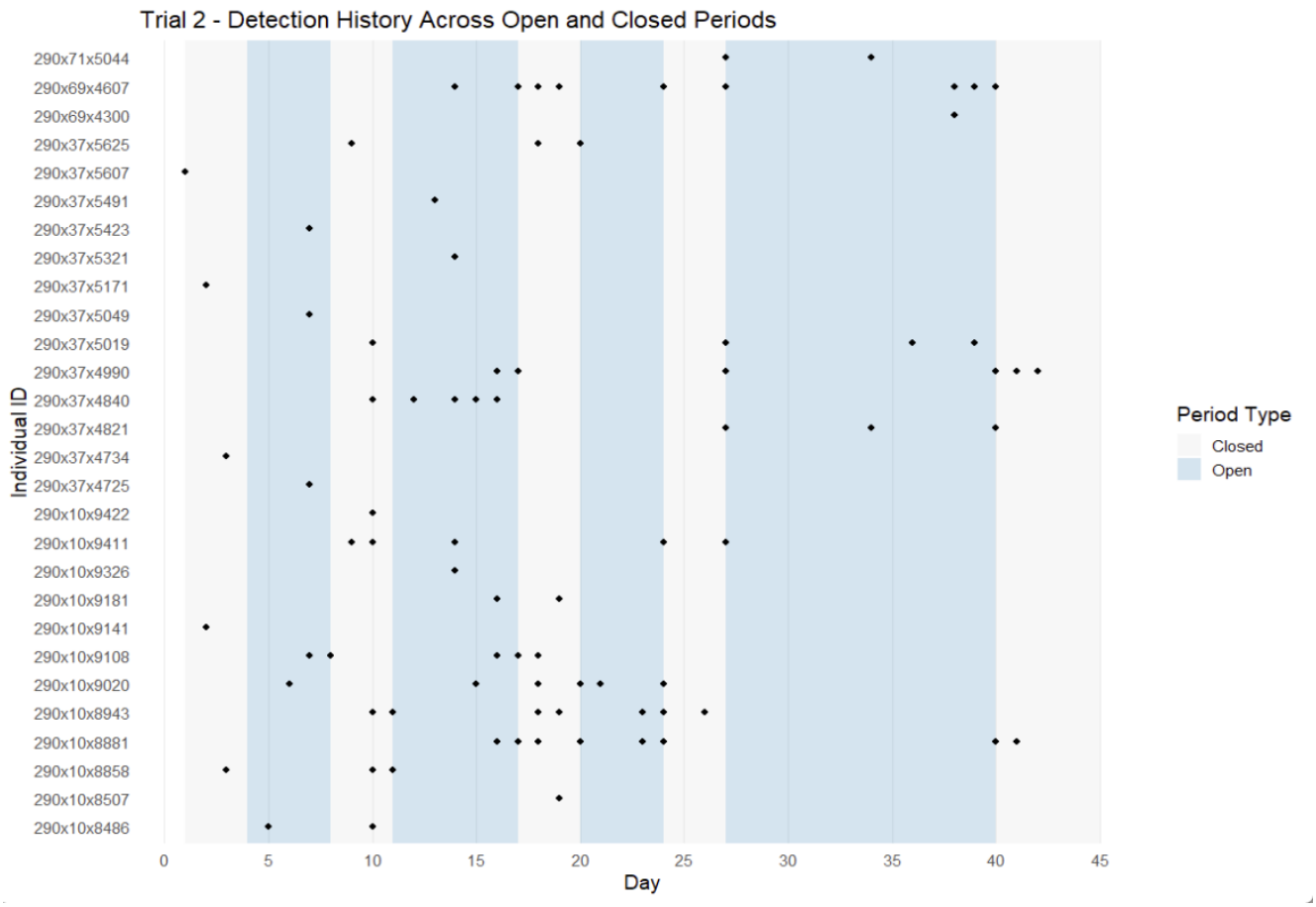

Figure 12. Detection scatterplot of all tagged beetles during Trial 2. Repeated detections are shown over time, with open(white) and closed periods (blue) indicated along the trial timeline to show the sampling structure of the analysis.

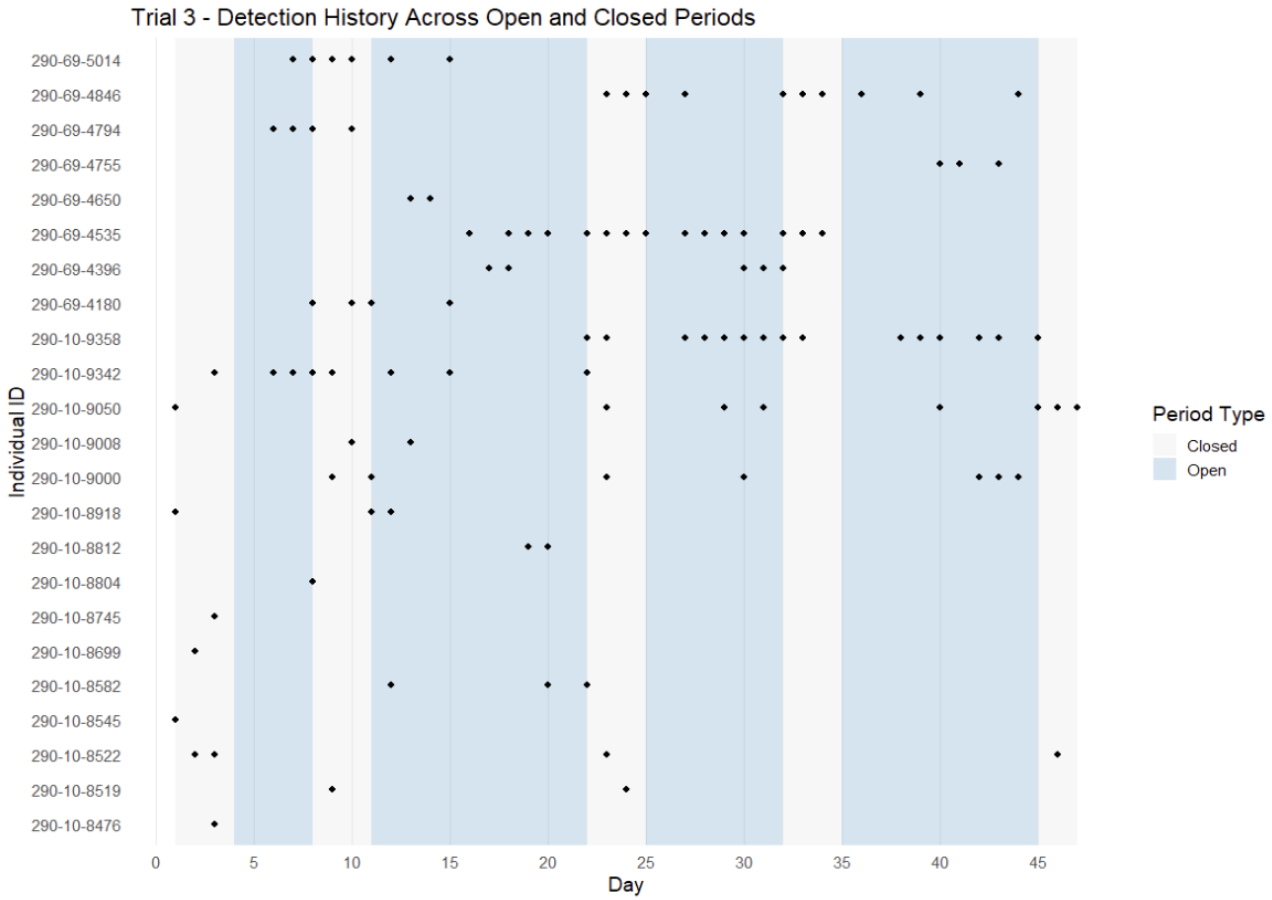

Figure 13. Detection scatterplot of all tagged beetles during Trial 3. Repeated detections are shown over time, with open(white) and closed periods (blue) indicated along the trial timeline to show the sampling structure of the analysis.

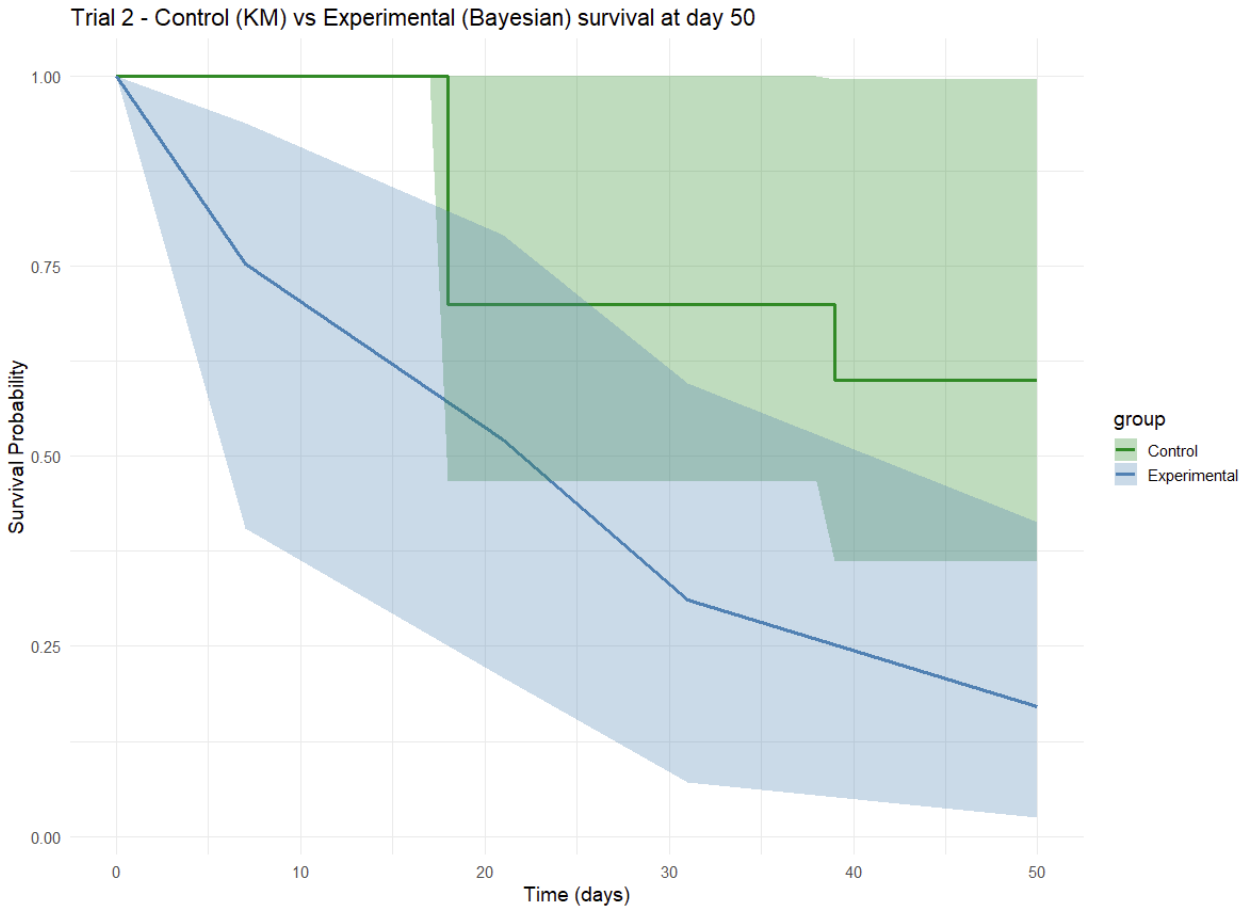

Figure 14. Comparison of survival curves for control and experimental groups for tagged beetles during Trial 2. Control survival was estimated using Kaplan-Meier analysis, whereas experimental survival was modeled with a Bayesian multistate framework. Shaded confidence (KM) and credible (Bayesian) intervals indicate uncertainty, with areas of overlap highlighting similarities in survival patterns across methods. Note: credibility and confidence intervals are not directly comparable but should provide improved comparison rather than just examining average values.

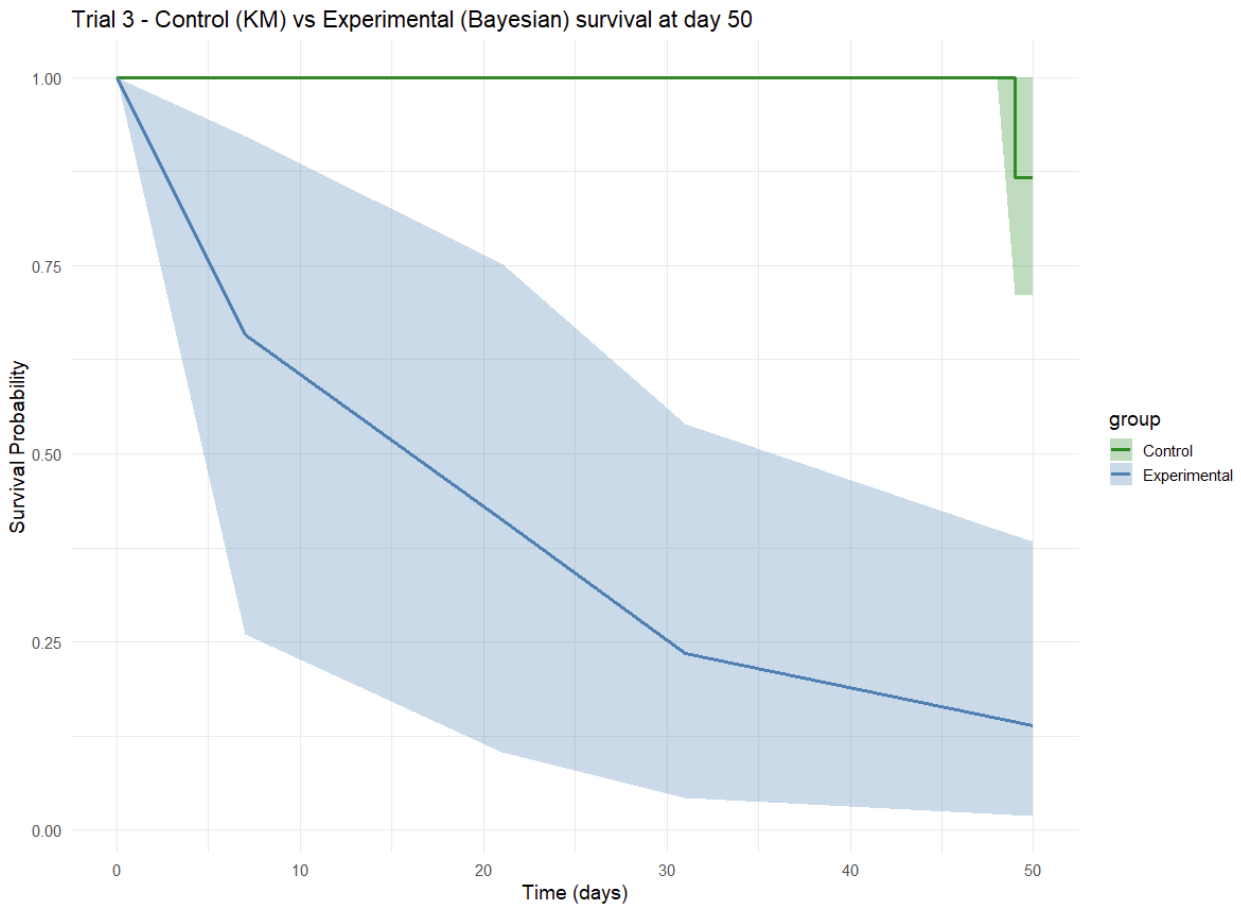

Figure 15. Comparison of survival curves for control and experimental groups for tagged beetles during Trial 3. Control survival was estimated using Kaplan Meier analysis, while experimental survival was modeled with a Bayesian multistate framework. Shaded confidence (KM) and credible (Bayesian) intervals show no overlap suggesting there is likely a stronger difference between groups. Note: credibility and confidence intervals are not directly comparable but should provide improved comparison rather than just examining average values.

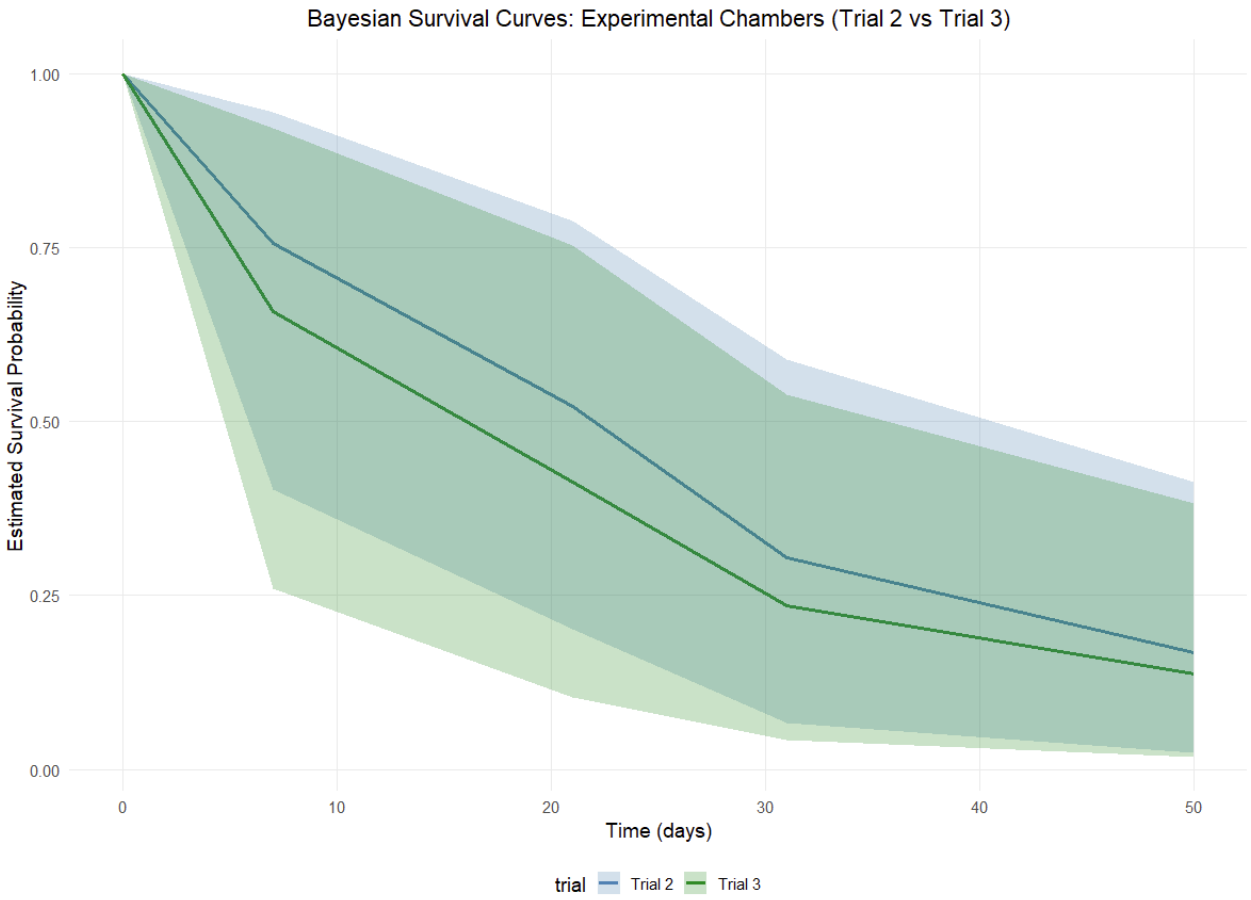

Figure 16. Comparison of experimental survival curves from Trials 2 and 3, both estimated using Bayesian multistate models in JAGS. The overlap of credible intervals indicate that survival estimates were reasonably similar across trials.

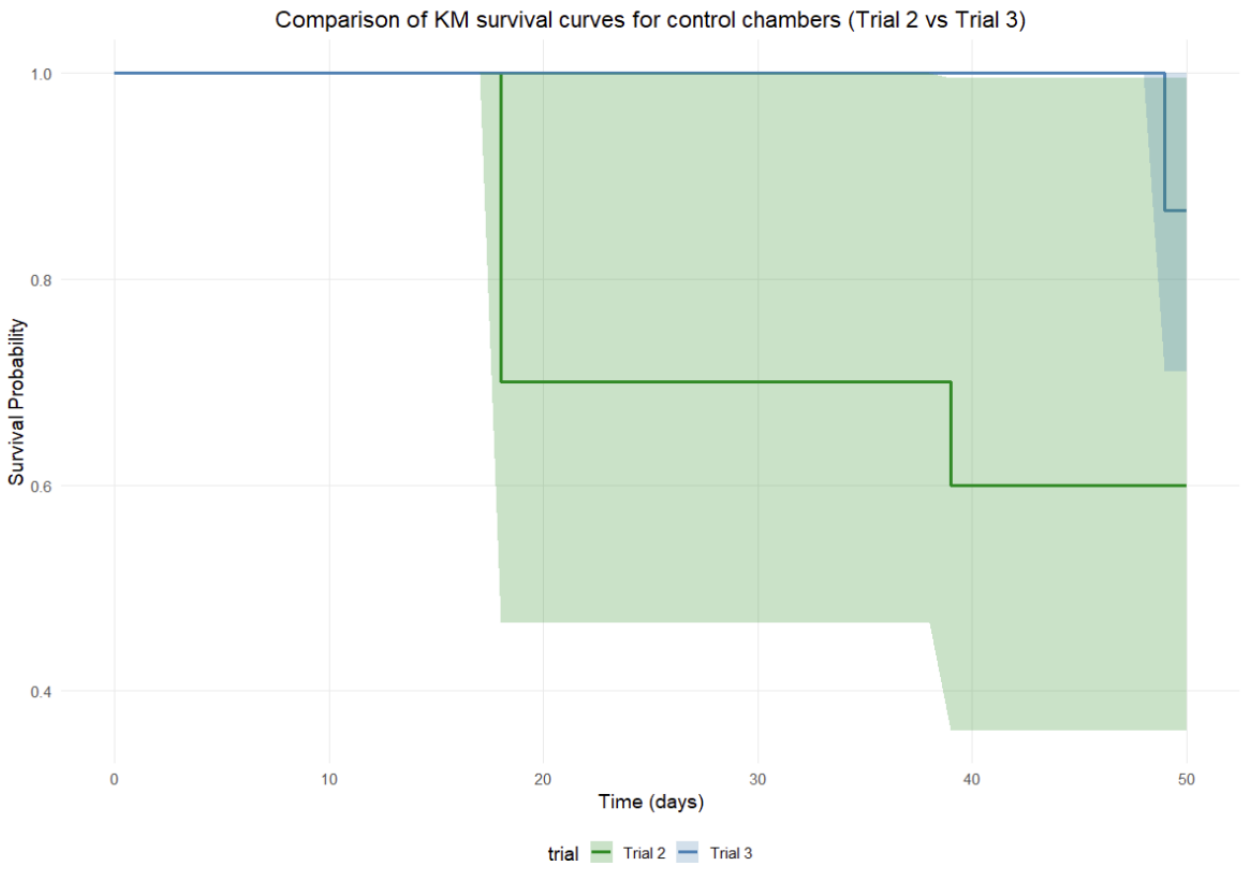

Figure 17. Comparison of Kaplan-Meier survival curves for untagged beetles between Trial 2 and 3. Confidence intervals show slight overlap, but survival in Trial 3 was markedly higher, with nearly all individuals surviving until the final days of the trial.

Figure 18. Comparison between the identification of BEEtag in MATLAB (left) and the original BEEtag image (right)

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.

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');
```

R code for Survival Analyses of untagged control beetles

Kaplan-Meier survival

```
library(survival)
library(ggplot2)
library(survminer)

#Plotting KM & Jags Curves
KM_Trial3=read.csv(file.choose())

fit <- survfit(Surv(Time, Death) ~ Trial, data = KM_Trial3)
logrank_result <- survdiff(Surv(Time, Death) ~ Trial, data = KM_Trial3)
logrank_result

km_fit <- survfit(Surv(Time, Death) ~ 1, data = KM_Trial2)  #Refit KM with updated data
km_summary <- summary(km_fit, times = 0:50) #trial 2

# Extract survival at exactly day 50
km_day50 <- summary(km_fit, times = 50)
```

```
# Print survival estimate and CI
cat("Estimated Control Survival at Day 50:\n")
cat("Survival =", round(km_day50$surv, 3), "\n")
cat("95% CI =", round(km_day50$lower, 3), "-", round(km_day50$upper, 3), "\n")

#data frame for plotting
km_trial2_df <- data.frame(
  time = km_summary$time,
  surv = km_summary$surv,
  lower = km_summary$lower,
  upper = km_summary$upper,
  group = "Control"
)

# Keep only the control group data
plot_df <- km_df
```

R code for Survival Analyses of tagged experimental beetles

Bayesian Multi-state analysis using JAGS

```
library(dplyr)
library(tidyr)
library(jagsUI)
data <- read.csv(file.choose(), stringsAsFactors = FALSE)
data <- data %>%
  mutate(
    Period_type = tolower(Period_type),
    seen_in_block = as.numeric(seen_in_block),
    count_str = as.numeric(count_str),
    length_days = as.numeric(length_days)
  )
```

```
df_full <- expand.grid(
  ID = unique(data$ID),
  Block = sort(unique(data$Block)),
  stringsAsFactors = FALSE
) %>%
  left_join(
    data %>% select(ID, Block, Period_type, seen_in_block, count_str, length_days),
    by = c("ID", "Block")
  ) %>%
  arrange(ID, Block)

df_clean <- df_full %>%
  group_by(ID, Block, Period_type) %>%
  summarise(
    seen_in_block = max(as.numeric(seen_in_block), na.rm = TRUE),
    count_str     = mean(as.numeric(count_str), na.rm = TRUE),
    .groups = "drop"
  )

df_clean <- df_clean %>%
  mutate(
    seen_in_block = ifelse(is.finite(seen_in_block), seen_in_block, NA),
    count_str     = ifelse(is.finite(count_str), count_str, NA)
  )
y_seen_df <- df_full %>%
  select(ID, Block, seen_in_block) %>%
  pivot_wider(names_from = Block, values_from = seen_in_block) %>%
  arrange(ID)

# (b) Count data (count_str)
y_count_df <- df_full %>%
```

```
  select(ID, Block, count_str) %>%
  pivot_wider(names_from = Block, values_from = count_str) %>%
  arrange(ID)

y_seen <- data.matrix(y_seen_df[,-1])
y_count <- data.matrix(y_count_df[,-1])
y_seen[is.na(y_seen)] <- 0
y_count[is.na(y_count)] <- 0

rownames(y_seen) <- rownames(y_count) <- y_seen_df$ID

period_info <- df_full %>%
  distinct(Block, Period_type, length_days) %>%
  arrange(Block)

period_vec <- period_info$Period_type
length_days <- period_info$length_days

# Create mask: 1 = closed (observation), 0 = open (unobserved)
period_vec <- df_full %>%
  distinct(Block, Period_type) %>%
  arrange(Block) %>%
  pull(Period_type)

y_mask <- ifelse(period_vec == "closed", 1L, 0L)
y_mask <- matrix(rep(y_mask, each = nrow(y_seen)), nrow = nrow(y_seen))

# Replace NAs with 0 (safe for numeric model input)
y_seen[is.na(y_seen)] <- 0
y_count[is.na(y_count)] <- 0
```

```
n_ind <- nrow(y_seen)
n_occasions <- ncol(y_seen)

# Closed = 1, open = 0
closed_blocks <- which(period_vec == "closed")

# Survival intervals occur between closed periods
surv_idx <- rep(NA, n_occasions - 1)
surv_idx[closed_blocks[-length(closed_blocks)]] <- seq_len(length(closed_blocks) - 1)

# Clean indices
surv_idx[is.na(surv_idx)] <- max(surv_idx, na.rm = TRUE)
safe_idx <- pmin(pmax(as.integer(surv_idx), 1L), max(surv_idx, na.rm = TRUE))
n_surv_intervals <- max(safe_idx)

z_init <- matrix(1L, nrow = n_ind, ncol = n_occasions)
jags.data <- list(
  y_seen = y_seen,              # presence/absence (1/0)
  y_count = y_count,            # count strength covariate
  y_mask = y_mask,              # closed (1) vs open (0)
  n_ind = n_ind,
  n_occasions = n_occasions,
  safe_idx = safe_idx,
  n_surv_intervals = n_surv_intervals,
  z_init = z_init
)
inits_function <- function() {
  list(
    logit_phi = rnorm(n_surv_intervals, 0, 0.5),   # survival interval parameters
    logit_p = rnorm(1, 0, 0.5),                    # detection intercept
    beta_count = rnorm(1, 0, 0.5)      # effect of count_str on detection)}
```

```
params <- c("phi", "p", "beta_count")
jags.out <- jags(
  data = jags.data,
  inits = inits_function,
  parameters.to.save = params,
  model.file = "jags_model_robust_simple",  # your JAGS model file
  n.chains = 3,
  n.iter = 10000,
  n.burnin = 5000,
  n.thin = 5
)
summary(jags.out)

cat("
model {

  # -----------------------------------------------
  # 1. Priors
  # -----------------------------------------------
  for (g in 1:n_surv_intervals) {
    logit_phi[g] ~ dnorm(0, 1)
    phi[g] <- ilogit(logit_phi[g])
}

  # weak transition priors
  for (t in 1:(n_occasions - 1)) {
    logit_gamma[t] ~ dnorm(0, 1)
    gamma[t] <- ilogit(logit_gamma[t])

    logit_delta[t] ~ dnorm(0, 1)
    delta[t] <- ilogit(logit_delta[t])
```

```
}

# Detection model priors (block-level binary detection)
beta0 ~ dnorm(0, 0.25)       # intercept on logit scale (mildly informative)
beta_count ~ dnorm(0, 0.25)  # effect of standardized count_str

# ------------------------------------------------
# 2. Survival indexing & transition probabilities
#    Uses safe_idx[t] where 0 => no survival estimated (phi_eff = 1),
#    and >0 => index into phi[1:n_surv_intervals].
# ------------------------------------------------
for (t in 1:(n_occasions - 1)) {
  is_surv[t] <- step(safe_idx[t] - 0.5)

  # safe index for phi (if safe_idx==0 we'll set phi_eff to 1)
  idx_t[t] <- max(1, safe_idx[t])

  # effective phi for this interval
  phi_eff[t] <- (1 - is_surv[t]) * 1.0 + is_surv[t] * phi[idx_t[t]]

  # clamp for numerical stability
  phi_clamp[t]   <- max(1e-6, min(0.999999, phi_eff[t]))
  gamma_clamp[t] <- max(1e-6, min(0.999999, gamma[t]))
  delta_clamp[t] <- max(1e-6, min(0.999999, delta[t]))

  # Transition matrix (states: 1 alive-undetected, 2 alive-detected, 3 dead)
  psi[1,1,t] <- phi_clamp[t] * (1 - gamma_clamp[t])
  psi[1,2,t] <- phi_clamp[t] * gamma_clamp[t]
  psi[1,3,t] <- 1 - phi_clamp[t]

  psi[2,1,t] <- phi_clamp[t] * delta_clamp[t]
```

```
  psi[2,2,t] <- phi_clamp[t] * (1 - delta_clamp[t])
  psi[2,3,t] <- 1 - phi_clamp[t]

  psi[3,1,t] <- 0
  psi[3,2,t] <- 0
  psi[3,3,t] <- 1
}

# ------------------------------------------------
# 3. Latent state process
# ------------------------------------------------
for (i in 1:n_ind) {
  z[i,1] <- z_init[i,1]   # initial states (1 or 2 as appropriate)

  for (t in 2:n_occasions) {
   z[i,t] ~ dcat(
    c(
      psi[z[i,t-1],1,t-1] + 1e-6,
      psi[z[i,t-1],2,t-1] * (1 - y_mask[i,t]) + 1e-6,
      psi[z[i,t-1],3,t-1] * (1 - y_mask[i,t]) + 1e-6
    ) / max(
      psi[z[i,t-1],1,t-1] +
      psi[z[i,t-1],2,t-1] * (1 - y_mask[i,t]) +
      psi[z[i,t-1],3,t-1] * (1 - y_mask[i,t]),
      1e-6
      )
    )
  }
}

# ------------------------------------------------
```

```
 # 4. Observation model (binary presence/absence at block-level)
 #    - y_seen[i,t] is 0/1 on closed blocks and should be NA on open blocks.
 #    - y_count[i,t] is a covariate (standardize in R) aligned to the same occs.
 # ------------------------------------------------
 for (i in 1:n_ind) {
  for (t in 1:n_occasions) {

    # linear predictor for detection (logit)
    logit(p_det[i,t]) <- beta0 + beta_count * y_count[i,t]

    # clamp detection probability
    p_det_clamp[i,t] <- max(1e-6, min(1 - 1e-6, p_det[i,t]))

    # alive indicator: 1 if state is 1 or 2, 0 if state is 3 (dead)
    alive[i,t] <- 1 - step(z[i,t] - 2.5)

    # probability of observing detection: alive * p_det, dead -> tiny eps
    p_obs[i,t] <- alive[i,t] * p_det_clamp[i,t] + (1 - alive[i,t]) * 1e-6
    p_obs_clamp[i,t] <- max(1e-12, min(1 - 1e-12, p_obs[i,t]))

    # Data node: binary detection. If y_seen[i,t] is NA in the data,
    # JAGS treats it as missing and ignores its likelihood.
    y_seen[i,t] ~ dbern(p_obs_clamp[i,t])
   }
  }
}
", file = "jags_model_robust_simple")
```

Graphing Bayesian survival and Kaplan-Meier Curves:

```
summary(jags.out)
```

```
posterior_phi <- jags.out$sims.list$phi  # matrix: iterations × 4 intervals
n_samples <- nrow(posterior_phi)

posterior_survival <- t(apply(posterior_phi, 1, cumprod))
phi_summary <- data.frame(
  interval = paste0("phi", 1:ncol(posterior_phi)),
  mean  = apply(posterior_survival, 2, mean),
  lower = apply(posterior_survival, 2, function(x) quantile(x, 0.025)),
  upper = apply(posterior_survival, 2, function(x) quantile(x, 0.975))
)

phi_summary$time <- c(7, 21, 31, 50)
bayes_df <- data.frame(
  time = c(0, phi_summary$time),
  surv = c(1, phi_summary$mean),
  lower = c(1, phi_summary$lower),
  upper = c(1, phi_summary$upper),
  group = "Experimental"
)

library(ggplot2)

plot_df <- rbind(km_df, bayes_df)
ggplot(plot_df, aes(x = time, y = surv, color = group, fill = group)) +
  geom_step(data = subset(plot_df, group == "Control"), size = 1.2) +  # KM as step
  geom_line(data = subset(plot_df, group == "Experimental"), size = 1.2) +  # Bayesian as line
  geom_ribbon(aes(ymin = lower, ymax = upper), alpha = 0.3, color = NA) +
  labs(
    x = "Time (days)",
    y = "Survival Probability",
    title = "Trial 3 - Control (KM) vs Experimental (Bayesian) survival at day 50"
```

```
) +
scale_color_manual(values = c("Control" = "forestgreen", "Experimental" = "steelblue")) +
scale_fill_manual(values = c("Control" = "forestgreen", "Experimental" = "steelblue")) +
theme_minimal(base_size = 14)
```

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.

Thermal Biological Assessment Devise Construction and Thermal Testing

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CONTENTS

Overview

The Thermal Biology Assessment Device (henceforth TBAD) is a novel apparatus designed to generate a linear, stable, and rapidly responsive thermal gradient in a straight trough of water, enabling simultaneous assessment of thermal preference, critical thermal minimum, (CTmin), and maximum (CTmax) for a variety of aquatic species. The TBAD employs counter-current heat exchange (CCHE) principles, using submerged conduit assemblies with heated and cooled fluids flowing in opposite directions to establish a predictable temperature gradient along the length of the trough. The whole trough is separated into two functional sections; the bottom half of the trough holds the CCHE conduits and drives the gradient, the upper half consists of a testing arena where organisms are held and observed during the thermal biology assessment. Most of our species of interest are local spring dependent and spring associated organisms and the ecological research collected with the TBAD on these species of interest could provide critical conservation or captive management improvement strategies. Spring dependent or spring obligate species are sensitive to changes in spring flows and water temperatures. Captive management strategies could be refined by keeping species at ideal temperatures, reducing stress and disease. These improved husbandry practices could also encourage reproduction. Determining preferred temperature ranges as well as minimum and maximum temperature tolerances allow for managers to predict how spring flows and fluctuations in water temperatures may impact species over time. For instance, species that are spring obligates or have a limited ability to migrate, such as *Heterelmis comalensis*, could be at significant risk of extirpation if spring flow was reduced or water temperatures increased. Alternatively, species that are mobile, like *Etheostoma fonticola*, may move out of degrading habitats into more suitable ones. Understanding thermal tolerances and thermal preferences could significantly improve long-term conservation efforts as well as salvage and restocking strategies. Refining minimum flows, identifying suitable alternative habitats, and reassess extirpation or extinction risks would improve wild management strategies. Refining species salvage and restocking conditions would benefit from thermal tolerance testing by identifying when and where species should be relocated or salvaged following habitat deterioration or reintroduced when the habitat recovers. Over the course of this past year, the Huffman Lab has been building the TBAD device, and construction has finally been completed. This update highlights the major parts of the device that have been completed over the past year.

TBAD Construction

Mobile Mounting Platform

The TBAD and all the associated instruments and components have been mounted on a rollable cart to improve transportability (Figure 1). The cart has a lower shelf for storage, a main counter that the trough rests on (3.28 m long and 0.75 m wide) and a small top shelf for additional storage and holds the flow in systems reservoir that gravity feeds into the trough. Under the top shelf, a board on PVC sliders was attached to the cart to house the camera, lights and the flow in system (Figure 2). The sliders allow the board to extend over the trough during a run and retract back under the top shelf when access to the inside of the trough is necessary. This mobility allows researchers to position instruments directly over the trough during a run to collect data with cameras, thermal dataloggers and introduce fresh water to the trough with the flow in system. Additionally, to keep the heater and chiller components at the same fluid level, we built an additional shelf attached to the hot end of the cart for the heater (Figure 3) and a detached cart for the chiller (Figure 4). The chiller cart rolls up to the cold end of the TBAD cart and uses the same castors.

The base of the cart rests on four heavy-duty castors and is equipped with 12 micro-adjustable support point levelers. These 12 support levelers will be capable of compensating for irregularities in floor surfaces and making it possible to bring the water in the Testing Arena of the trough to a uniform depth and keep it there through an extended study. The entire assembly is just wide enough to fit through a laboratory doorway, and since the castors make the whole device transportable, researchers can store the device or easily move it out of the way when not needed.

Figure 1. Full view of the cart and all the equipment attached that makes up the TBAD. White PVC frame over cart is the PVC frame for the shroud.

Figure 2. Sliding shelf with everything on it except for lights and cameras.

Figure 3. Heater shelf permanently attached to the cart.

Figure 4. Chiller cart.

Conduits and Gaskets

The conduits used for counter current heat exchange, which heat and cool the TBAD, have been cut, sanded and cleaned to perfectly match each other in length (Figure 5). Each of the two conduit subassemblies consists of eight conduits (four heated and four cooled) stacked in two conduits wide and four conduits tall configuration. The ends of the CCHE Conduit Assemblies have an additional silicone piece that seals the conduits off from each other and from the water in the trough (Figure 6). This was done by making casts of the ends of the conduit subassemblies with stainless-steel rods positioned in the center of the conduits. Once the casts were fully cured, we pull the silicone piece off the end of the conduits and are left with perfect fitting gaskets that fit over the end of the conduit assemblies with perfect inlet and outlet holes (created by the steel rods) connecting the square conduit to the rest of the circular plumbing loop. These inlet holes are just large enough that 1/4” OD plastic tubing can squeeze into the hole, pushing the silicone out around the tubing, making a tight fit around the inside of each conduit itself. The back side of the conduit gaskets are flat and are compressed against the outer bulkhead walls of the trough and seal the openings of the trough from leaks. The silicone gaskets also allowed us to try different possible hot and cold conduit configurations during dry testing (conduits not submerged in fluid) and compare them to each other to see which configuration had the best heat transfer along the length of the conduits (Figure 7).

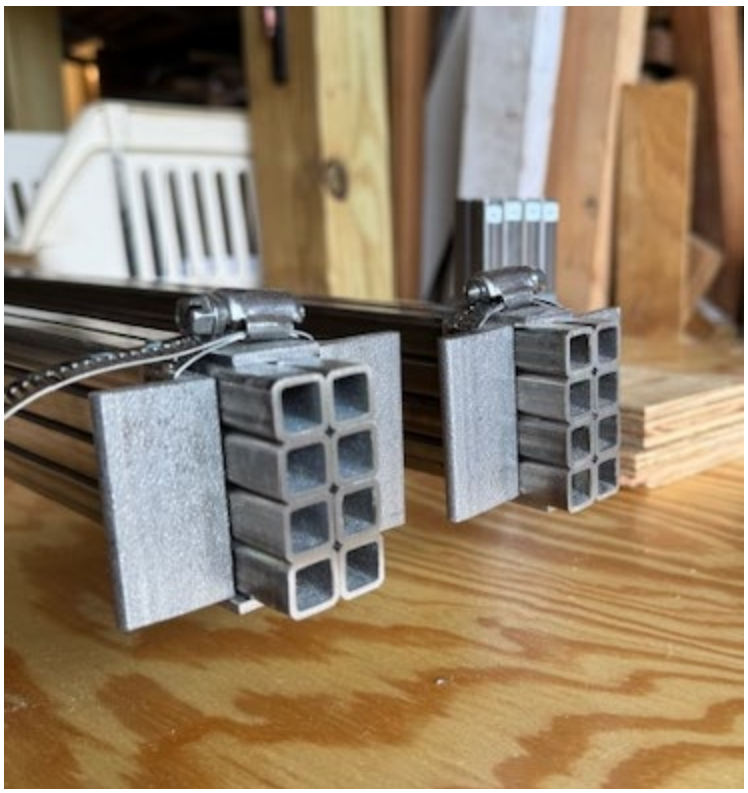

Figure 5. Cut Conduits used for CCHE design

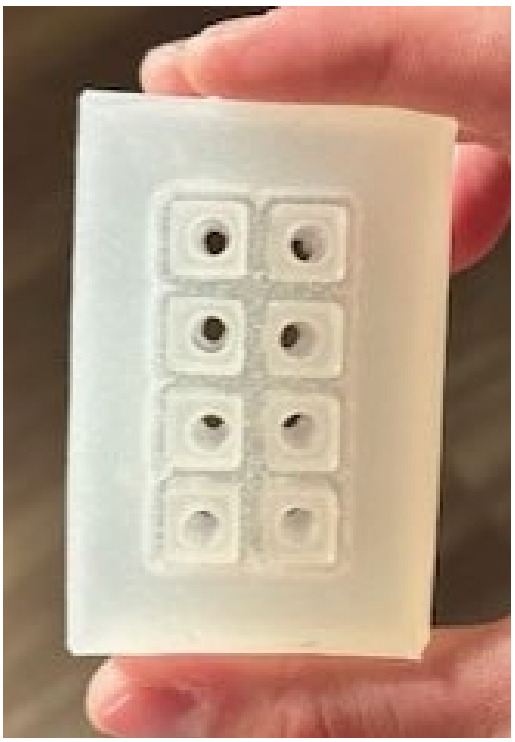

Figure 6. New gasket design that has inlet and outlet holes as part of the cast to eliminate the need for drilling while providing perfect holes for sealing and increased design durability.

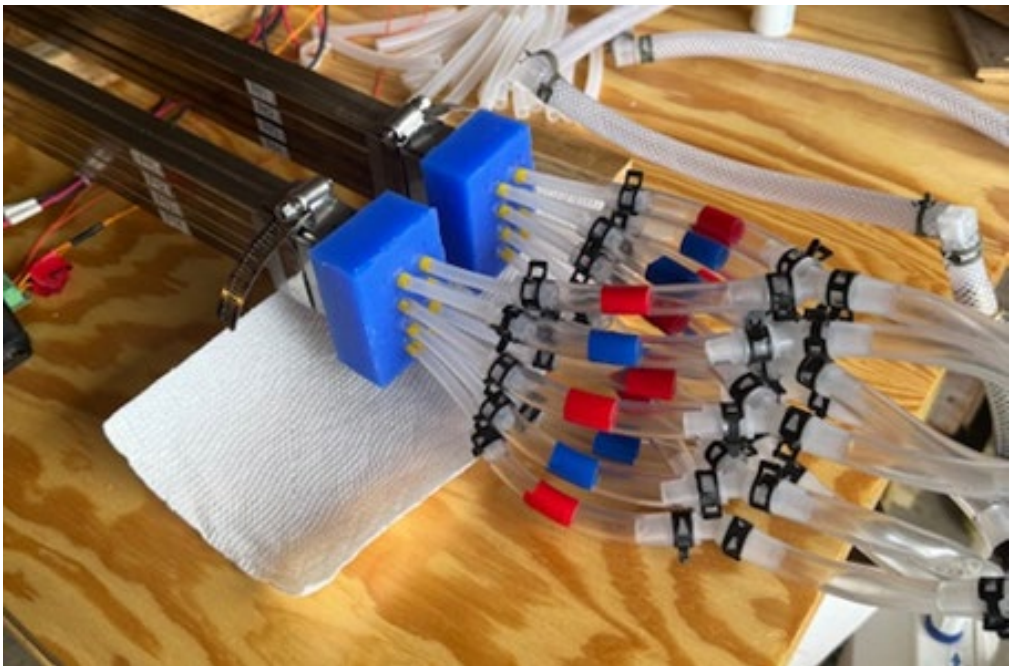

Figure 7. Waterproof gasket set up for initial dry testing with the hot and cold inlet and outlet hoses connected to each conduit. The paper towel below proved no leaks were present.

Counter Current Heat Exchange Flow Manifolds

While we waited for funding to secure the rest of the devices and units needed to complete the TBAD, we performed real-time simulations (Dry testing) to confirm our computational simulations performed on SolidWorks earlier this year. The goal of these simulations was to find out how the different hot and cold configurations are expected to source or sink heat between the conduits (Figure 7). The initial results of our real time simulation testing thermal images (Figure *8*) showed us that we had a difference in flow velocity going into each conduit, meaning there was an uneven amount of heated and cooled fluids going into each conduit due to the hose splits and minor differences in elevation between the inlet points of the heating and cooling loop (Figure 9). To remedy this, we decided to create CCHE flow manifolds with needle valves as a deterministic control (Figure 10) giving us the ability to evenly distribute the flow of water between the conduits. The CCHE flow manifolds allowing us to perform meaningful dry tests to understand how the conduits function outside of the water.

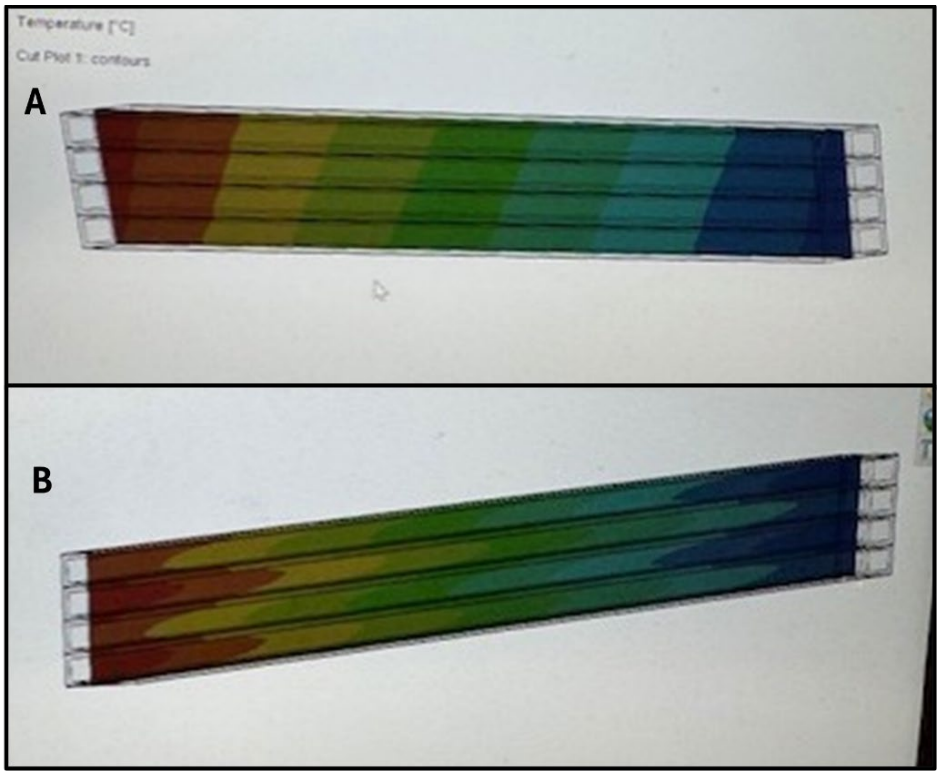

Figure 8. SolidWorks dry test simulation, A) Thermal readings of the transfer of heat between the stacked pairs of conduits, B) Thermal readings and conditions of the water inside the conduits.

Figure 9. Thermal readings of the outer surface of the conduits. The top cold conduit is much colder than the other cold conduit on the same side, showing a difference of flow.

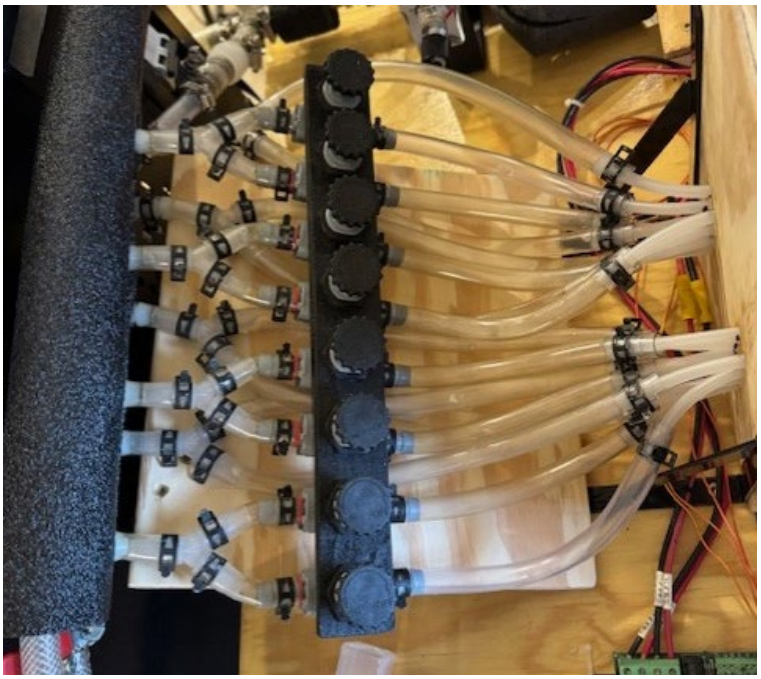

Figure 10. CCHE flow manifold with needle valves to control and regulate the flow rate and temperature distribution in the conduits.

Dry Testing

All the dry testing we conducted can be split into two major groups, flow tests and conduit configuration tests. The flow tests we performed have allowed us to replicate the same flow rate as the old TPAD device and find out which flow rate (working from high to low flow) allowed for the most transfer between the conduits. The conduit configuration tests allowed us to compare the different conduit configurations with set thermal conditions and flow that were chosen from the flow tests. The results of the dry testing have shown us that the low flow rates and the alternating hot and cold conduits configuration had the best thermal transfer out of all the configuration options available (Figure 11). This configuration allowed the most thermal transfer between conduits and the temperature of the water exiting the conduits was only a few degrees off from the entering conduit temperature on their respective sides, which is ideal to reduce the workload on the heater and chiller that are exchanging fluids at each end of the trough (Figure 12).

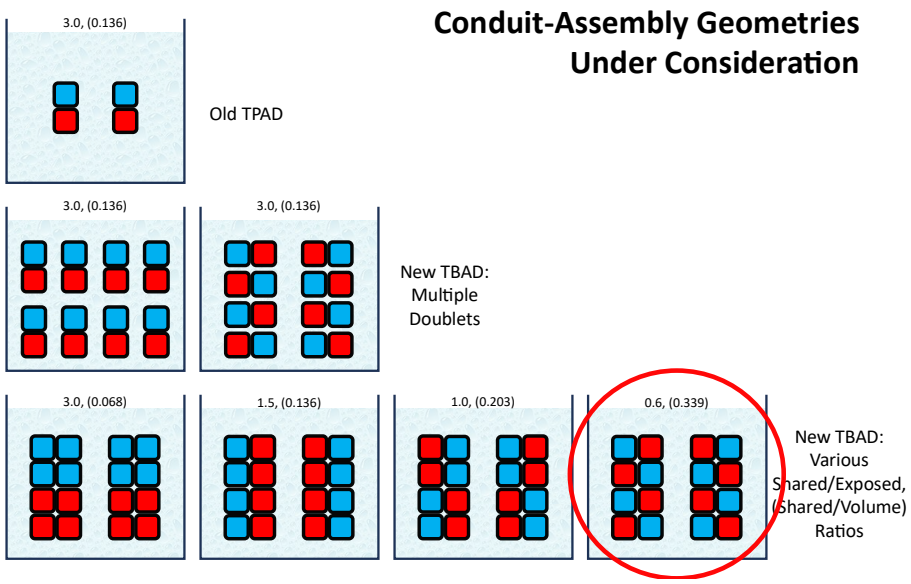

Figure 11. Different conduit configuration options for the TBAD. Configuration with the red circle around it had the best thermal transfer data out of all the options.

FR	Front C		FF	FC(FF)-FM(FF)	FC(FR)-FM(FR)	FR	Front M		FF	FM(FF)-FH(FF)	FM(FR)-FH(FR)	FR	Front H		FF
22.60	C	H	23.00	-6.10	4.70	27.30	C	H	29.10	-7.10	5.40	32.70	C	H	36.20
25.20	H	C	21.60	5.50	-7.60	32.80	H	C	27.10	6.10	-3.50	36.30	H	C	33.20
23.60	C	H	23.80	-8.10	4.30	27.90	C	H	31.90	-4.30	5.80	33.70	C	H	36.20
25.80	H	C	22.20	3.60	-3.40	29.20	H	C	25.80	4.90	-6.30	35.50	H	C	30.70

RR	Rear C		RF	RC(RF)- RM(RF)	RC(RR)-RM(RR)	RR	Rear M		RF	RM(RF)-RH(RF)	RM(RR)-RH(RR)	RR	Rear H		RF
22.50	C	H	31.80	-4.00	4.90	27.40	C	H	35.80	-2.00	6.00	33.40	C	H	37.80
26.70	H	C	21.80	6.10	-7.30	34.00	H	C	27.90	6.40	-4.00	38.00	H	C	34.30
24.90	C	H	22.80	-6.70	5.30	30.20	C	H	29.50	-6.80	6.00	36.20	C	H	36.30
29.20	H	C	21.00	4.90	-4.10	33.30	H	C	25.90	6.70	-4.90	38.20	H	C	32.60

Calc Avg Cold Temps: (IN)	Calc Avg Hot Temps: (OUT)
22.53	26.04

Avg Cold Temps: (OUT)	Avg Hot Temps: (IN)
33.35	36.81

Diff. of Left Temps:
3.51

Diff. of Right Temps:
3.46

Total Range:
14.29

Avg Diff across all Conduits Full length:
10.80

% of Gradient Range Used:
75.59

Figure 12. Data sheet showing some of the thermal information collected during dry testing. This is the alternating hot and cold conduits configuration that showed each conduit was on average transferring 75.6% of its thermal energy by the time it exited the conduit.

Heaters, Chillers and Plumbing Loops

To purchase the heater and chiller that drives the device, we met with a heater and chiller specialist from USA LAB to confirm that we were purchasing the heating and cooling devices needed for our unique design. After several consultations we decided on two units to support our needs. The cold end of the system is powered by a 3.5 KW USA Lab -30°C 30L Recirculating Chiller (Figure 3) and a LC-200 Pelteir-Thermoelectric liquid cooler (used in the old TPAD design). This chiller replaced the cold booster and one of the cold pelteirs on the cold end of the TBAD, increasing the cooling horsepower by over 4-fold. While the USA LAB chiller provides the necessary horsepower the TBAD needs, the dead band of the thermal control system on is too wide. The LC-200 Pelteir is used to tighten that deadband within a degree or range to help hold the set temperature steady while the TPAD operates. The USA Lab Recirculating Chiller also eliminates the need for the single speed pump on the plumbing loop since it has a positive displacement pump in the USA LAB chiller, further simplifying the plumbing on the cold end of the TBAD.

The hot end of the TBAD will be powered by a 3 KW USA Lab Recirculating Heater 200°C -20L ETL Certified (Figure 4) and a LC-061 Pelteir-Thermoelectric liquid heater (used in the old TPAD design) which also improves the heating performance by over 4-fold on the hot end. The LC-061 Pelteir performs the same task as the LC-200 Pelteir on the cold side which is to tighten the deadband of the set point. Like the USA Lab Chiller, this device will also replace the boost heater and the constant speed pump on the hot end of the TBAD.

Since we had not collected data with the USA Lab heater and chiller, we collected data on two different flow rates (1.2 LPM and 0.8 LPM) on the alternating hot and cold conduits configuration. This allows us to compare the data to when we had the same set up submerged and allows us to compare how the new heater and chiller are different from the old thermal devices which has effectively given us an extra 10 °C of thermal range out of the conduits during the dry configuration data collection. Before we could do this, we had to construct a new plumbing loop with an additional bypass loop to bleed off the flow we didn't need back into the units (since the pumps are not adjustable) (Figure 13). To make the bypass loop work, we calibrated L-valves on each side of the TBAD with flow gauges to have deterministic control of the flow coming out of the bypass loop that goes into the conduits. This design allows us to set the flow rate on both sides of the trough to have equal bath input and output on each side (Figure 14).

One challenge that we initially ran into was setting the flow gauges to balance the tanks in the system. Theoretically, exactly matching how much comes out of one and into the other thermal device would allow us to achieve a balanced transfer of fluids in and out of the tanks. In practice this is not the case, especially with the heating and cooling compressors in each of the units running. The changing viscosity of the water required us to create a procedure to regulate how much water is allowed into the system with the flow

gauges by watching the water level in the smaller heater reservoir. The procedure for balancing the tanks water levels with the heating and cooling condensers on and off has been added to the operation manual that will come with the device.

Figure 13. Bypass loops with the bleed off and in to feed back into the chiller, the heater side is identical.

Figure 14. Full view of the TBAD set up for the second trial of dry tests with the new heater and chiller.

Trough

The shell of the rectangular through is made of 1” PMMA acrylic plastic and has four sides and a bottom with an open top. The rectangular shape for the TBAD trough improves the dimensional stability of the trough, provides much more configurational flexibility, and simplifies system maintenance. The troughs acrylic panels were mended together using Weld-on-3 to which effectively chemically plastic welds all the pieces into one piece (Figure 15). The trough has the inside dimensions of 260 cm x 15.25 cm x 25.4 cm. The overall length of the trough was chosen to give roughly the same volume of water in each convection chamber as the TPAD trough. This was an important factor because as we increased the power and surface area of the conduits, we needed to keep the volume in the convection chambers relatively the same to allow us to use as much of the energy produced by the conduits in the convection chambers as possible. Now our increased number of conduits and thermal differences will drive the device more effectively, giving us more control over generating and manipulating the gradient. Another modification made to the trough design was drilling a hole for the Standpipe drain along the center of the back wall of the trough (Figure 16). The hole was fitted with stainless steel hardware that makes up the drain and was fixed in the hole with Plastic Bonder J-B weld.

Acrylic plastic is transparent and has a relatively low specific heat capacity of about 1,470 J/kg·K, so it will not provide much interference with attempts to change the temperature of the trough water. However, it has a thermal conductivity of about 0.2 W/(m·K) and an R-value close to zero, so is not a good insulator, and requires sheets of polyisocyanurate (R-value of 6) thermal insulation on the long front and back exposed surfaces during testing. The bottom panel of the trough is supported at all points by the mounting platform and rested on a sheet of 1-inch polyisocyanurate insulation, which thermally isolates the water in the trough from the platform.

Figure 15. Trough panels being supported by clamps and wooden struts while the mending process was occurring.

Figure 16. Left photo: The hole we drilled in an 8.75-foot-long sheet of acrylic that the overflow drain hardware will go through. Right photo: The stainless-steel fitting used as the overflow drain fixed in the hole with JB-weld.

Trough Bulkhead

The 2.6 m long stainless steel CCHE Conduit Assemblies are placed directly between the two acrylic end panels of the trough (bulkheads). Thus, thermal expansion of the Conduit Assemblies could apply destructive forces to the acrylic end panels during testing if they were both mended and fixed directly to the side and bottom panels of the trough. Therefore, only one bulkhead wall has been mended to the side and bottom panels, while the bulkhead wall on the other end was trimmed to fit tightly inside the other three panels on that end. On the inside edge of the open end of the trough, there is a 3/8th inch lip of acrylic that was mended to the inside walls where a lubricated silicone sealing gasket compresses against, creating a waterproof seal with the floating bulkhead wall (Figure 17). This allows the free bulkhead to move slightly back and forth with the thermal expansion or contraction of the Conduit Assemblies and allows us to seal the trough with

the same compression structure used to secure the conduits inside the bulkhead walls of the trough. Keeping all the movement and compression in a single plane.

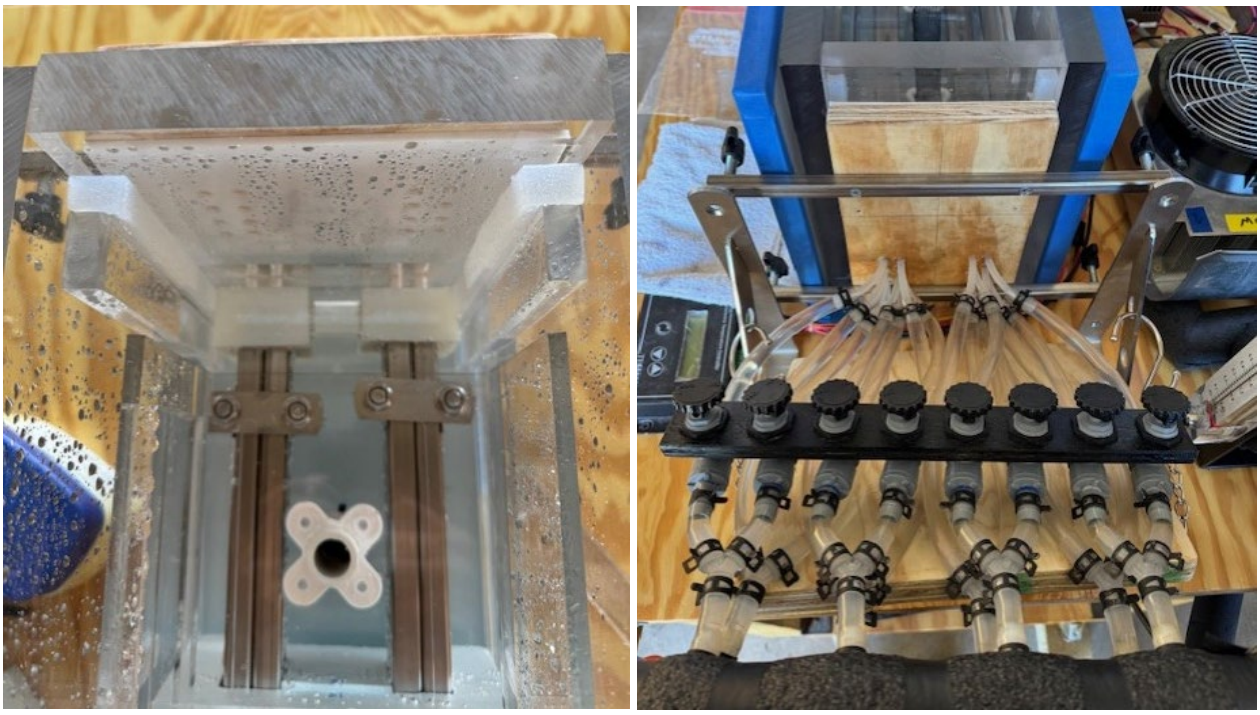

Figure 17. Left photo: The inside of the removable bulkhead wall with the gaskets squished against the acrylic strips mended to the outer walls of the trough. Right photo: The outside of the removable bulkhead with the compression brackets shown.

Blackout Shroud

A removable 1-inch PVC framework was built and mounted over the mounting platform (Figure 18). The framework supports a removable canvas shroud to isolate the Testing Arena from ambient room lighting and will allow the operator to control the illumination experience of test organisms independently of lighting conditions of the room (Figure 19). An awning hangs off the front side of the PVC frame allowing an observer to stand under the shroud without disrupting the shroud. The section of shroud that hangs down the awning on the front of the cart has a double layered vertical slit in the fabric where the edges overlapping, allowing researchers to access the trough and observe the organisms under the shroud with minimal exposure of light.

Figure 18. PVC frame held to the cart with pipe clamps.

Figure 19. Shroud on the PVC frame. Left photo is from the front showing the overlapping door panels. Right photo is from the back showing the wrap that goes down past the counter.

Arena Inserts

The arena floors will be specially designed to accommodate organism-specific morphometry and substrate modalities during testing, but the underlying structure will remain the same. The organism exclusion arena floor is an acrylic modular structure that perfectly fits within the walls of the TBAD. One section of the arena floor accounts for the area above three convection chambers (Figure 20), leading to 6 modular sections that make up the whole arena (Figure 21). The arena inserts are held together by clear H channel connectors to fill the gaps between sections (Figure 22). The modular design allows researchers to seal off the arena from the rest of the TBAD once it is assembled and can be disassembled after the run to be easily removed for cleaning. While most of the arena is completely sealed, there are a couple of strategic screened openings along the walls and floors of the arena inserts. Shown in Figure 23 along the back wall there are large, screened openings (three per modular section or one per convection chamber) and floor slits on both sides of hole that the bubble chimney comes through the floor (two floor slits per convection chamber). The screen on the back wall allows the water in the testing arena to flow through the back wall into our working space. The working space allows researchers to use a small section of the trough for input hoses and drains and keeps test species from interfering. The screen covering the floor slits keeps organisms from getting in the convection chambers below and allow for vertical circulation produced by the bubble chimney, which are glued to the bottom of the arena floor. The bubble chimney directs water and air bubbles through the arena floor from an air stone at the bottom of a small PVC pipe (in the bottom of each convection chamber), carrying oxygen and the thermal properties of the convection chamber below to the arena section above. The negative pressure pulls water from above the arena floor through the floor slits and back through the bubble chimney. This process circulates and refreshes the water in the testing arena and keeps the water in the area above the convection chamber hot or cold depending on its location in the trough. All the chimneys also have a cylindric screen cover that prevents test organisms from getting down the bubble chimney.

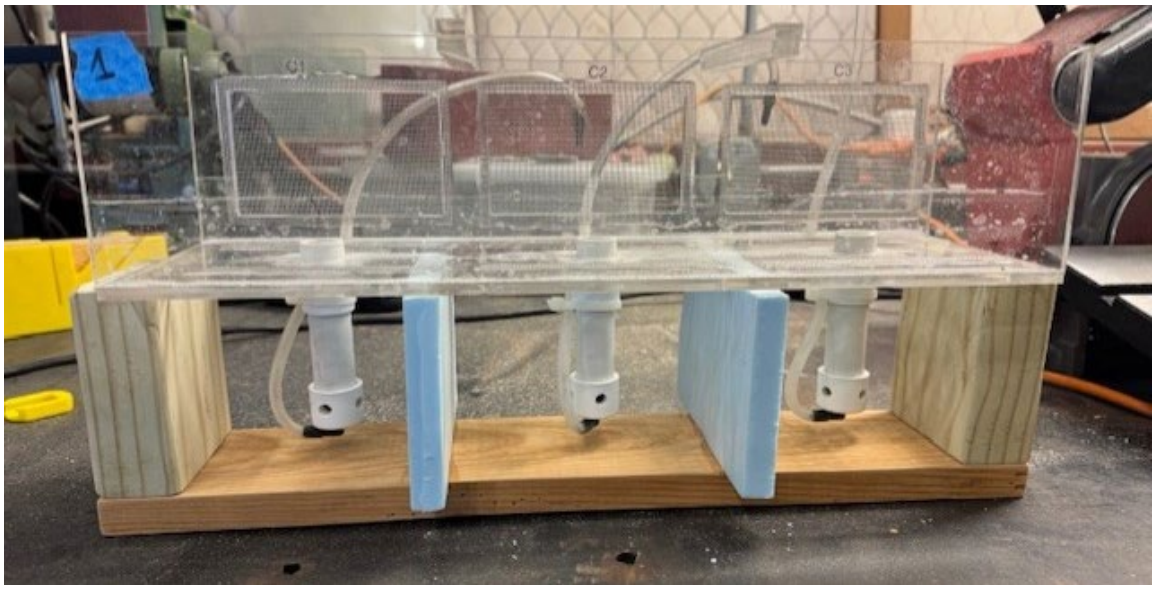

Figure 20. One section of the arena setup.

Figure 21. All 6 modular arena inserts held together with H connectors that span the whole length of the arena.

Figure 22. H channel connector used to connect modular arena inserts.

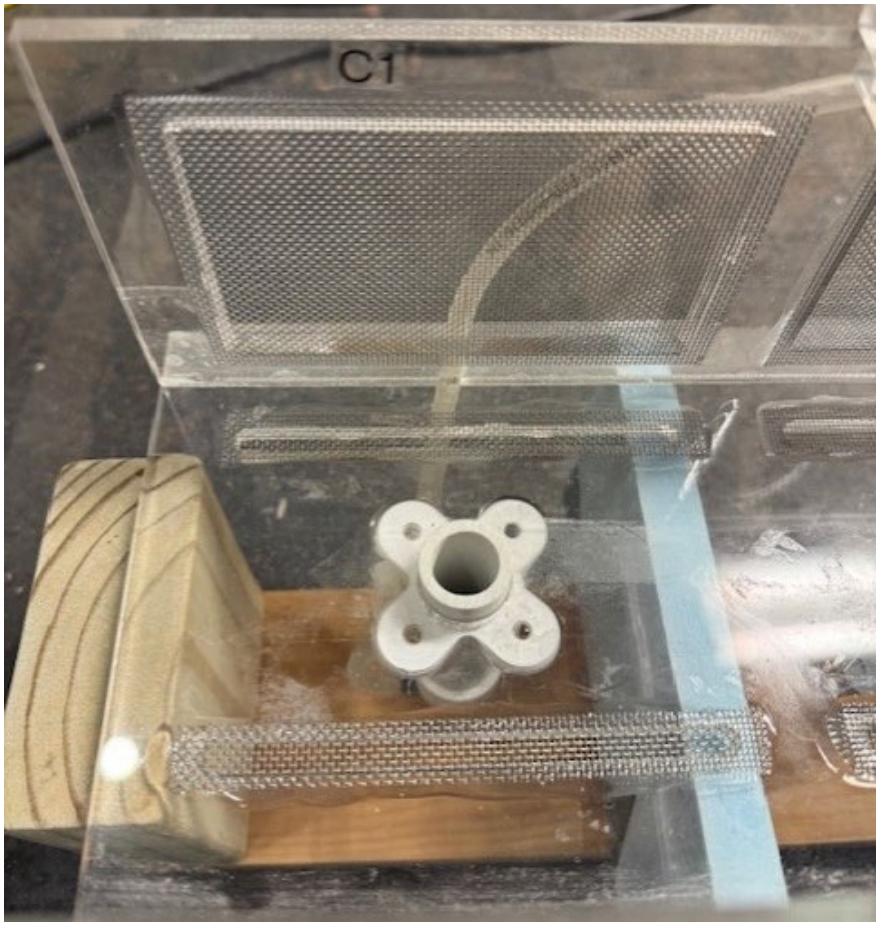

Figure 23. Arena insert with the openings screened closed.

Water Recycling and Overflow System

To ensure the clarity and freshness of the water in the Testing Arena during tests that require more than 12 hours of runtime, an water refreshment manifold (Figure 2) and an overflow standpipe drain has been added to the trough (Figure 16) to create a flow-through design. During a test, the water which specimens are exposed to can become stale over time and could collect unsafe levels of ammonia and CO_2. To ensure favorable water quality remains stable, fresh water is introduced from a flow-in reservoir that gravity feeds through a supply line (*Figure 24*) that connects to the water refreshment manifold that is attached to the sliding board that hangs over the trough. On the Flow in Reservoir there is a float valve that regulates the water depth in the reservoir and along the supply line there is a red handled ball valve that stops the flow to the water refreshment manifold. Along the water refreshment manifold there are 9 needle valves attached to the sliding board with square mounting brackets that control rate of flow into the tank. The overflow standpipe is located on the center of the back wall of the trough, as previously discussed in section 2.1.3. on the stainless-steel fittings fixed to the back wall is a barbed hose fitting with a .25” inside diameter plastic hose connected. There are a variety of different standpipe heights that fit inside the plastic hose and regulate the water height in the trough as the flow in system runs for different operational set ups.

To operate the flow in system, open the needle valves until the indicator on the dial meets the line on the mount below the dial. This is the setting for 36 drips per minute (6 drips per 10 seconds), which is enough inflow to exchange half of the water in 12 hours with the nine needle valves available and drain the water at a slow enough rate that the thermal gradient will be minimally disturbed. When testing is completed, turn the red handled ball valve to stop the flow to the water refreshment manifold. After the dripping stops, push the sliding shelf back all the way. When this feature is not needed for shorter tests, the inflow needle valve array can be shut, and the overflow drain can be plugged to run the same tests initially performed with the Donelon TPAD device.

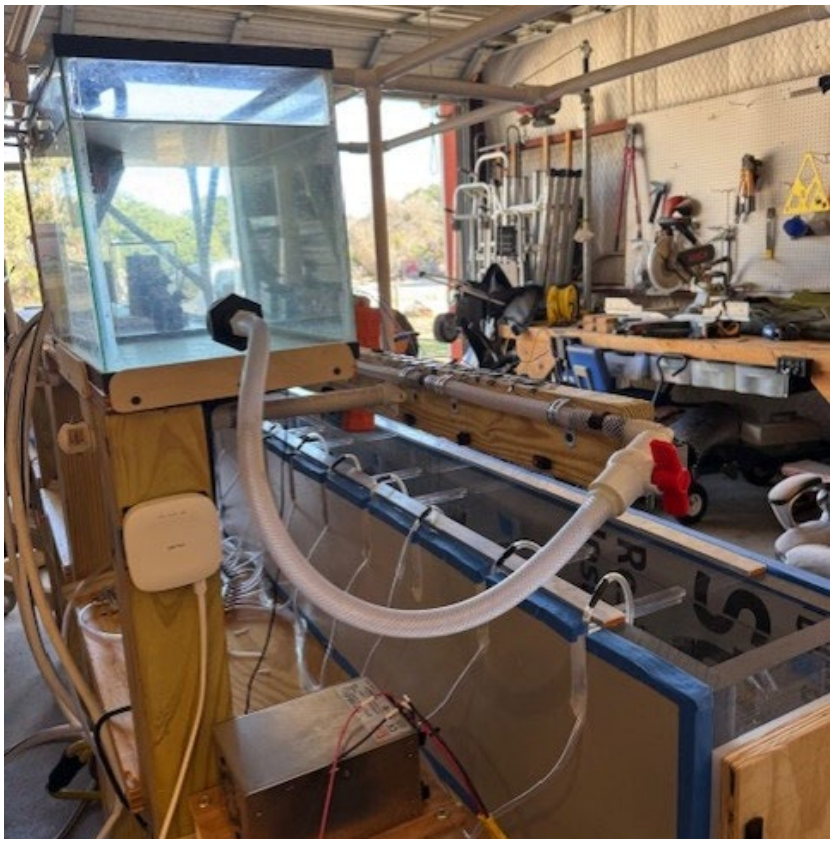

Figure 24. Reservoir and supply line leading to the water refreshment manifold on the sliding board that hangs over the trough.

Future Direction

We are still in the process of collecting data in the preliminary wet testing phase since the completion of building for the TBAD. We have already run the device under the same conditions as the dry tests while submerged in the trough to be able to account for the differences when the conduits are submerged. Now we are experimenting with other parameters, like how slow we can run the device before the gradient diminishes, how wide of a gradient is possible with this device, how quickly can we generate the gradient and move it while keeping the slope of the gradient stable and how far we can move the gradient along the trough and have it performed deterministically. These are all just a couple of examples of the extent of our research on the functioning of the device. Since a device has never been created with our exact parameters, there are many tests to run and data to collect. Figure 25 shows data from a run that generated a 16 °C gradient. Once the gradient stabilized with the 16 °C range, the gradient was moved by increasing the temperature set points on the heater and chiller by 5 °C moving the “ideal” center point (23 °C) six

chambers to the right. This is only one example of the types of tests we are running and the data we are collecting to better understand the device we have built. Once all the wet tests are completed in the next couple of weeks, we plan to start preliminary species testing, where we plan will use non-threatened invertebrates readily available in the San Marcos River to test how they react in the trough to the available gradient. Once we have a proper procedure for testing species and accounting for their species-specific requirements, the focus will be to test SMARC's species of interest and collect valuable information on their thermal biology. For instance, populations of *E. fonticola* have been found further downstream in the San Marcos River than previously recorded (Chappell et al. 2024). These populations are existing and reproducing at higher temperatures (29˚C; Chappell et al. 2024) than previously established as their thermal reproductive maximums (25˚C; Bonner 1996). Thermal testing to identify preferred and min/max thermal ranges for this species under reproductive and non-reproductive status would provide significant information on how upstream populations of *E.fonticola* could fair under low flow and/or high water temperatures when compared to the downstream population. We look forward to future collaborations with USFWS and the work we will do together.

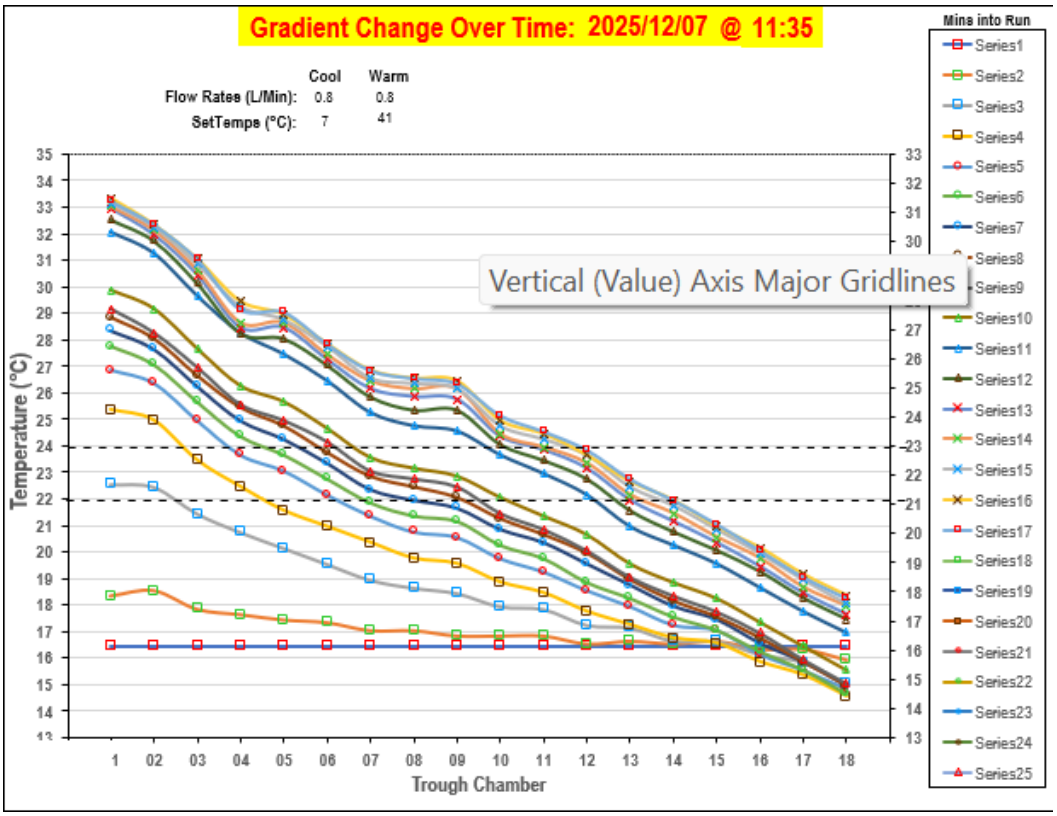

Figure 25. Wet test from December 12th, 2025, this run produced a 16 ℃ gradient, once stabilized the gradient was moved 6 cambers to the right as the trough heated up with changes to the parameters.

References:

Bonner, Timothy H. (1996). *Effects of Temperature on Egg Production and Early Life Stages of Fountain Darters,* Master's Thesis, Southwest Texas State University, https://digital.library.txst.edu/server/api/core/bitstreams/d5150991-4701-4146-bc41-3617fc0acb40/content

Lauren E. Chappell, Elibardo Leal, Joshua D. Tivin, Timothy H. Bonner "New Distributional Records for *Etheostoma fonticola* (Fountain Darter) in the San Marcos River, Texas," Southeastern Naturalist, 23(2), N6-N12, (4 June 2024)

Peck's Cave Amphipod (*Stygobromus pecki*) Offspring Exclusion: Improvements to Captive Propagation

2025 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by: Braden West, Dr. Katie Bockrath and Marisol Farias

Contents

Background

Successful captive propagation of Peck's cave amphipod (PCA) has historically been rare. PCA reproduces sexually and may only be receptive to mating for a short period following molting. Females release approximately 10 eggs per brood and may reproduce multiple times throughout their lifespan (Fries et al. 2004). Female PCA are known to exhibit cannibalistic behavior in captivity, including consumption of their own offspring, particularly when the female is larger than other amphipods (Nowlin, Schwartz, Worsham, and Gibson 2016). This behavior persists even when individuals are provided with food in excess. Recent observations suggest that gravid females may extract newly hatched offspring (neonates) from the marsupium using their extended pereopods. Such cannibalistic behavior in adult PCA presents a significant challenge to the development of a successful captive breeding program.

In captivity, PCA are housed in boxes containing Matala Filtration Media (Pentair Aquatic Eco-Systems) to mimic the interstitial spaces of spring openings. Individuals are maintained at low densities with abundant Matala to reduce the likelihood of cannibalism. Previous research has suggested incorporating additional substrate materials to provide refugia for newly hatched neonates (Kosnicki and Julius 2019), based on the assumption that neonates can escape adults within the Matala matrix. However, routine inventories rarely detect neonates, despite the presence of multiple gravid females and ample refuge substrate. This study evaluates two captive reproductive strategies for PCA: (1) manual removal of developed neonates from the female's brooding pouch and (2) passive separation following natural release of neonates from the brooding pouch.

Objectives

This research project had two objectives: Our first objective was to determine the efficacy of manually separation of PCA neonates and survival of the neonates and brooding female post separation. The second objective was to determine the efficacy of passive separation of PCA neonates from gravid female amphipods.

Methods

Pilot Study

Preliminary experimentation with manual separation began at Uvalde National Fish Hatchery in early 2024. Gravid females identified during quarterly inventories were housed separately and examined weekly under a microscope to monitor neonate development. Once neonates exhibited visible movement within the egg and measured approximately 0.95–1.0 mm, they were removed using blunt probes and housed separately from the mother to prevent predation. Excess matala and leaf litter were provided to ensure sufficient refuge habitat. Following separation, females were returned to the original inventory box. Neonates were assessed for survival every 30 days.

The manual removal process may require up to 10 minutes per female. Preliminary survival of the hand-hatched PCA is currently 100%, and growth of neonates has been observed. Because females were returned to their original boxes, post-separation survival of the females was not recorded.

In parallel with the manual removal pilot, passive exclusion chambers containing three designated sections (A, B, and C; Figure 1), stacked sequentially, were designed to evaluate amphipod movement and potential preferences related to substrate, position, and water flow. Triplicate chambers were used across 10 trials. A single PCA was placed in each system, with the starting section varied among trials. For example, in one trial a PCA began in section A of chamber one, section B of chamber two, and section C of chamber three. Flow and temperature were measured at the beginning and end of each trial. Amphipods remained in the chambers for 24 hours, after which movement was restricted using a knife-gate valve. Chambers were then disassembled, and amphipod position and condition were recorded.

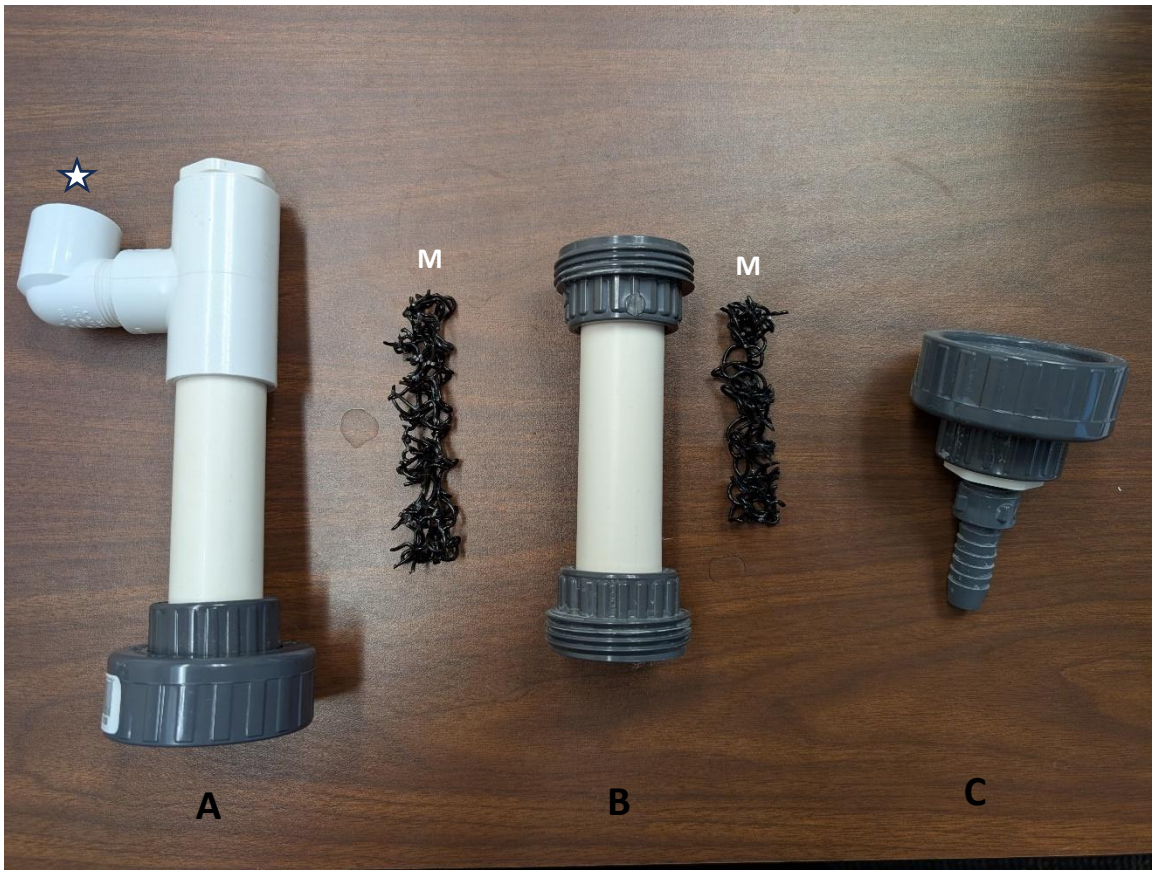

Figure 1. Disassembled passive exclusion housing. Housing is made of three sections, A, B and C. A houses the brooding female, B houses dropped neonates and eggs, C is the outflow cap. Matala substate (M) is placed in Sections A and B. Mesh on the top of Section B and the bottom of Section C separate the brooding female from dropped neonates and eggs. The star indicates the access port used to feed PCA while in the exclusion housing.

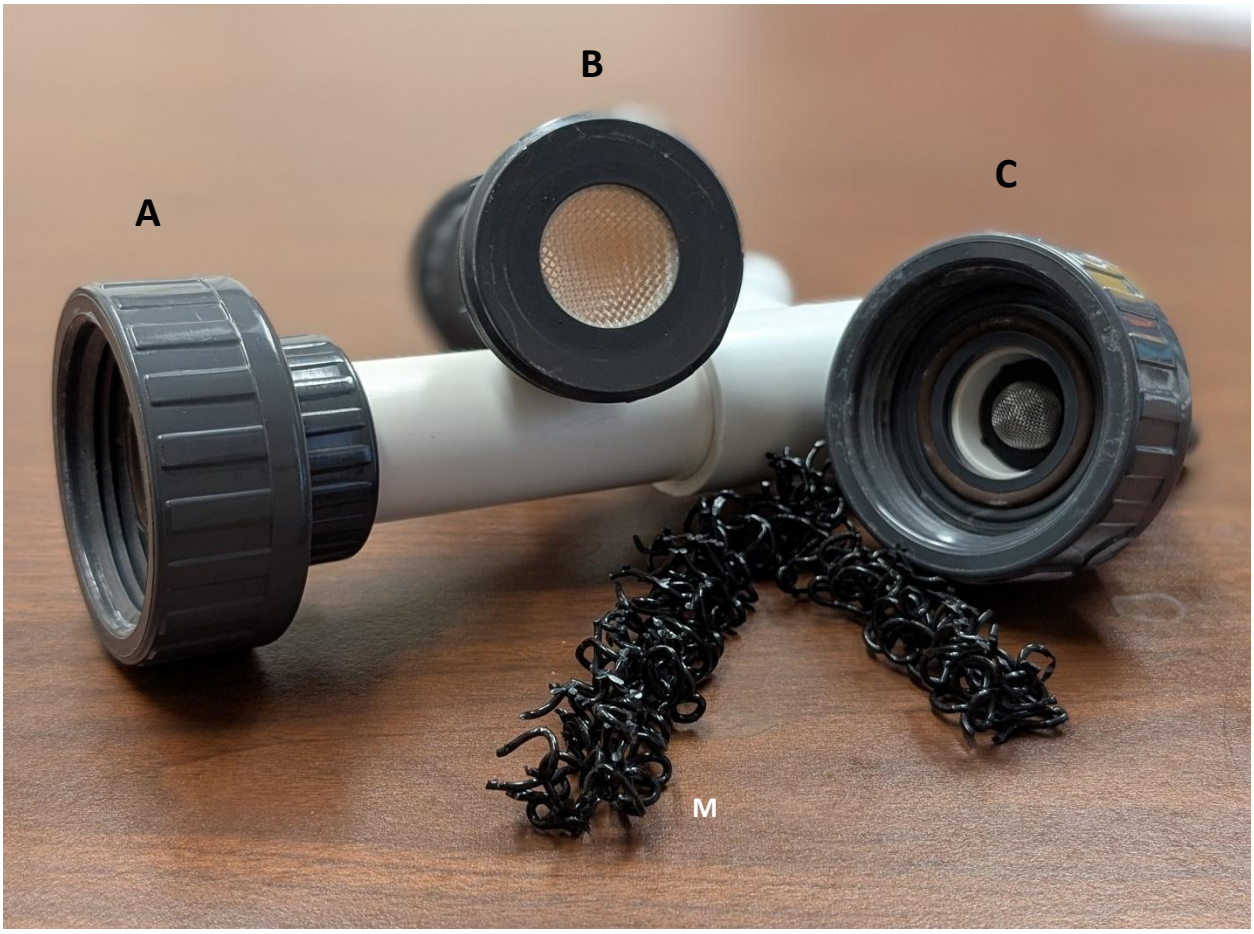

Figure 2. Alternative image of the passive exclusion housing highlighting the screens between sections (A, B and C). M designates Matala substrate.

In-situ passive separation trials

The three chamber passive exclusion housings were vertically oriented on a flowthrough system and supplied with Edwards Aquifer well water. Well water is maintained at 22˚C through countercurrent exchange by passing non-recirculated water through Titanium heat exchanger coils set up in a heated sump that sits below the passive exclusion housings (Figure 3).

Gravid PCA held in refugia were separated from group populations, placed into exclusion chambers (Chamber A), and monitored for up to four weeks. Amphipods that die within the first week of holding were replaced, if additional individuals are available. Chambers were monitored weekly by staff for flow, temperature, and mortality. PCA were fed through an access port in Chamber A (Figure 1) three times a week with 1ml ground fish flake mixed with well water.

Passive exclusion trials incorporated two chamber designs: the design established during spring pilot studies and the “brooding chamber” described in Kosnicki and Julius (2019) for the EAA. Each trial will include a minimum of three replicates, and ideally five, depending on the availability of gravid female PCA. Trials will run for four weeks and will be repeated no more than five times. When feasible, treatment trials for brooding chambers and passive exclusion chambers will be conducted simultaneously. If neither neonates nor the mother are detected during a monitoring check, the replicate chamber in the trial will be terminated and restarted with a new gravid PCA.

Figure 3. Passive exclusion set up. Ten vertically oriented passive exclusion tubes are shown with Chamber A at the top, followed by B and then C for each exclusion chamber. The black box with the yellow top was used to hold the physically isolated neonate. All chambers and the one box are on a flowthrough system. Countercurrent coils are in the green sump under the vertical housings and are used to maintain constant temperature.

Manual separation trials

Gravid PCA were separated from group populations at SMARC and maintained at reduced densities and monitored for neonate development. Gravid amphipods with underdeveloped neonates will be placed individually into the experimental containers designed at the beginning of this study and monitored weekly for neonate development. Neonates can be safely separated from the mother once they reach approximately 1 mm in length. Fully

developed neonates will be separated from gravid females as soon as practicable following transport.

Neonates were separated by immobilizing the female in a drop of water and gently coaxing the neonates from the marsupium using a blunt probe and a micro-hook dental tool. Both the female PCA and discharged neonates were then returned to separate, scaled-down habitat boxes that follow the general flow pattern and design of habitat boxes used by EARP. Amphipods maintained in these habitat boxes received the same feeding regimen as refugia-held individuals. The number of neonates present, the presence or absence of the adult female, and flow and temperature conditions were recorded weekly. Each trial will last four weeks following neonate separation and were conducted in quintuplicate, with no more than five repetitions. Any neonates produced during these trials were incorporated into SMARC refugia stocks. If neither neonates nor the adult female are detected during a monitoring check, the trial was terminated and restarted.

Results

Passive Exclusion

Thirty-two gravid PCA were monitored over the duration of this study. Gravid females remained gravid between 2-55 days with the majority being gravid between 8-35 days and an average of 19 days (Figure 3). Dropped eggs were observed in the separation chamber (Chambers B and **C**) but no neonates were observed in these chambers despite the adult PCA becoming non-gravid or observing a reduction in the number of neonates in the brooding pouch.

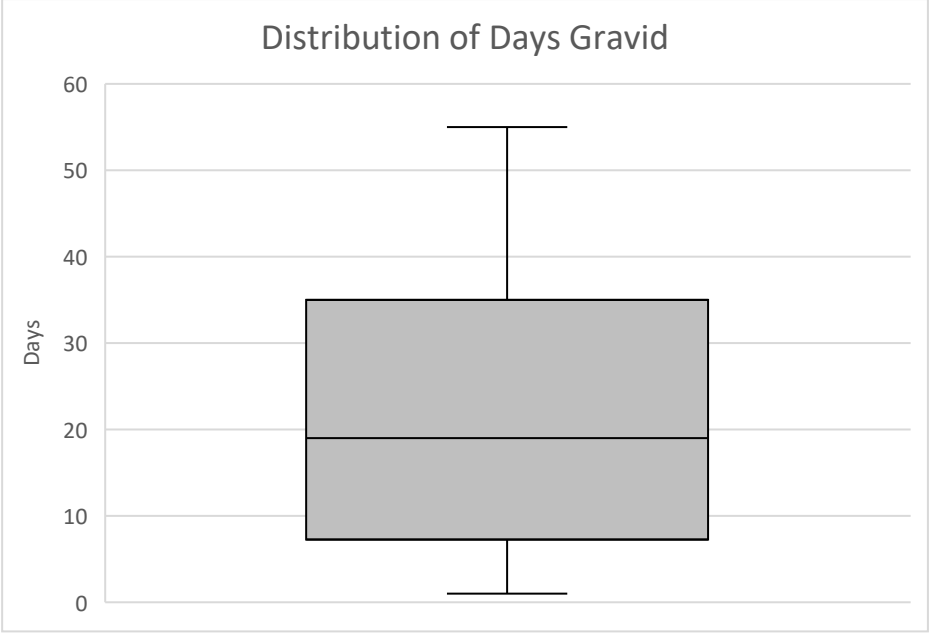

Figure 4. Box and Whisker plot showing the distribution of the number of days a PCA remained gravid. Days are on the Y-axis. Mean number of days is indicated by the line within the box. Frist quartile (25% of the data) is represented by the bottom line of the box. Third quartile (75% of the data) is represented by the top line of the box. The whiskers that extend from the box represent the extremes of the data (5% and 95%).

Manual Removal

One neonate was successfully removed from a gravid PCA with a single neonate in the brood pouch (Figure 4). Forceps were used to gently remove the neonate from a gravid PCA under red light. Manual removal took one minute and 15 seconds to complete. The neonate was active post removal. The neonate remains alive with growth observed at the writing of this report. The adult PCA died five weeks post removal of the neonate of unknown cause.

Figure 5. Manual removal of neonates from a gravid female. Panel A shows microdissection tools being used to brace the gravid female with a blunt end probe while a blunt microhook is used to sweep the neonates out of the brooding pouch. Panel B shows the freshly removed neonate and the adult female. Panel C shows the neonate one month post removal.

Discussion

Overall observations of gravid PCA showed they remain gravid as expected from previous research (Kosnicki and Julius 2019, Fries et al. 2004). Unfortunaly, no neonates were observed in the exclusion chamber. This could be due to the adult cannibalizing the eggs and/or neonates or being lost due to stress. The gravid PCA were fed frequently and in excess, which should have reduced the occurrence of cannibalization, but the gravid PCA may have preferred to eat the neonates over flaked fish food. Recently, the Edwards Aquifer Refugia Program has offered live feed, such as *Daphnia magna*, to captive held PCA in the Refugia population, which as shown some improvement in survival and neonate occurrence in the holding boxes. In future iterations of this effort, Daphnia will be offered as live food in addition to fish flake. Trials were also limited to 4 weeks (approximal 30 days). Longer trials of 60 days would fully encompass the full neonate brooding time and allow for neonates to be released and isolated in the lower chambers.

Manual separation of the neonate from the gravid PCA was successful for the one individual that was isolated in this study. Unfortunately, it was not an overwhelming success due to the death of the gravid PCA and the very low sample size. Manual separation is time consuming and requires very careful handling of both the gravid PCA and the neonates. There are many aspects of manual separation that can result in mortality if done poorly. For instance, if too little water is used while manually separating the neonates, both the neonates and the gravid PCA can overhead, desiccate or become very stressed. If too much water is used, the biologist will have a very difficult time separating the neonate from the gravid PCA, thus potentially causing physical damage to both neonate and gravid PCA.

Additional work in both manual and passive isolation is warranted. Physical isolation has resulted in successful isolation of a neonate and should continue to result in viable neonates. It is unclear what the full impact of physical isolation will be on the gravid PCA. Passive isolation has yet to produce a viable neonate, but the method could be promising if modifications to the duration and feeding strategy were made. Both passive and manual isolation efforts will continue as a husbandry practice improvement effort in 2026.

References

Fries, J.N., J.R. Gibson, and T.L. Arsuffi. 2004. Edwards Aquifer spring invertebrate survey and captive maintenance of two species. San Marcos National Fish Hatchery and Technology Center and Texas State University.

Kosnicki, E., and E.P. Julius. 2019. Life-history aspects of *Stygobromus pecki*. BIO-WEST, Inc. and San Marcos Aquatic Resources Center, Prepared for the Edwards Aquifer Authority. 24 pgs.

Nowlin, W.H., B.F. Schwartz, M.L.D. Worsham, and J. R. Gibson. 2016. Refugia research: development of husbandry and captive propagation techniques for invertebrates covered under the Edwards Aquifer habitat conservation plan. Texas State University and San Marcos Aquatic Resources Center prepared for the Edwards Aquifer Authority. 37 pp.

January 2025 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear and Dr. Katie Bockrath

With contributions from

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Task 1 Refugia Operations

Species Collection

The Edwards Aquifer Refugia Program (EARP) Team conducted four fountain darter collections in January. Sites in the Upper, Middle and Lower San Marcos River were sampled on January 6 and 7. A total of 274 San Marcos fountain darters were collected. 182 San Marcos fountain darters were brought to the San Marcos Aquatic Resources Center (SMARC) EARP quarantine while 91 San Marcos fountain darters were brought to the Uvalde National Fish Hatchery (UNFH) EARP quarantine. One San Marcos fountain darter was released. Sites in the Comal River (Spring Island, Old Channel and Landa Lake) were sampled on January 8 and 14. A total of 106 Comal Springs fountain darters were collected and placed in the SMARC EARP quarantine.

Husbandry

SMARC

Richelle Jackson and Shawn Moore constructed a new captive food propagation shelf in the SMARC quarantine to hold a blackworm tank and two artemia culture units. (Figure 1).

Braden West finished incorporating Comal Springs riffle beetle (CSRB), Comal Springs dryopid beetle (CSDB), and Peck's cave amphipod (PCA) from December 2024 collections into the Refugia. West incorporated 15 CSRB, 4 CSDB, and 10 PCA.

West worked closely with Kallan Padget to identify inconsistencies and recommendations for the controller box Standard Operating Protocol. West worked on one controller box for the SMARC.

Uvalde

General housekeeping duties were done throughout the month such as acid washing and bleaching tanks, rotating tank habitat, reorganizing and decluttering shelves. Two chillers were replaced in the main Refugia with new Raypak chillers. Ten Texas wild-rice plants were incorporated into the Refugia population from the December collection.

Dominique Alvear finished the PCA inventories along with the incorporation of 65 PCA into the Refugia from quarantine. Alvear administered various preventative treatments such as salt baths to tanks of fountain darters that were showing slight increases in mortalities.

Kallan Padget, Matthew Donelon and Noel Valenzuela-Charro began working on their first controller box to continue adding into the Refugia. (Figure 2)

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for January 2025. "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	182	91	0	0	30	39	255	294
Fountain darter: Comal	106	0	0	0	16	48	164	391
Comal Springs riffle beetle	0	NT	10	0	98	NA	456	36
Comal Springs dryopid beetle	0	NT	4	0	9	NA	40	30
Peck's cave amphipod	0	NT	15	65	25	41	100	168
Edwards Aquifer diving beetle	0	NT	0	0	0	0	0	0
Texas troglobitic water slater	0	NT	0	0	0	0	0	0
Texas blind salamander	0	NT	5	0	1	1	105	57
San Marcos salamander	0	NT	29	0	4	6	249	134
Comal Springs salamander	0	NT	0	0	2	9	80	64
Texas wild-rice	0	NT	15	10	0	9	191	132

Task 2 Research

Developing tools to determine thermal tolerances and preferences of current and future covered HCP species

Dr. Katie Bockrath worked on setting up a cooperative agreement with Texas State University to fund the improvements to the thermal tolerance testing prototype.

San Marcos Salamander Photo Mark and Recapture

Dr. Katie Bockrath worked with SCA to renew Marina Draeger's internship to continue working on processing photos that were collected during the previous p-Chip mark and recapture effort.

Peck's Cave Amphipod Offspring Exclusion

Dr. Katie Bockrath worked with SCA to advertise for an intern to work on the PCA exclusion project. Braden West designed exclusion housings and the flowthrough husbandry set up for brooding females during this study.

Genetic Assessment of fountain darters

No significant updates to report.

Genetic Assessment of Texas blind salamanders

No significant updates to report.

Genetic Assessment of San Marcos salamanders

No significant updates to report.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

Dr. Katie Bockrath submitted the final reports for research conducted in 2024 and the 2024 EARP annual report.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss Husbandry needs, Collection plans, and Research updates.

Figure 1. The new captive food propagation setup in the SMARC quarantine.

Figure 2. (from left to right). Matthew Donelon, Kallen Padget and Noel Valenzuela-Charro following the Controller Box SOP to wire a controller box. Noel enthusiastically identified the wire they had been looking for within the box.

February 2025 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear and Dr. Katie Bockrath

With contributions from

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Task 1 Refugia Operations

Species Collection

On February 6, Edwards Aquifer Refugia Program (EARP) staff collected eighty Peck's cave amphipods (PCA) from the Spring Island area of the Comal River. Eighty PCAs were retained for incorporation into refugia. Thirty-nine PCAs were transported to the quarantine building at the SMARC. Forty-one were transported to the quarantine building at the Uvalde National Fish Hatchery (UNFH). Six San Marcos salamanders were also collected and taken to the quarantine building at the UNFH.

On February 25, EARP staff collected PCA from the Spring Island area of the Comal River (Figure 1). 123 PCA were collected and 117 were retained for incorporation into the Refugia and transported to the quarantine building at the SMARC.

Figure 1. Shawn Moore (Left) and Richelle Jackson (Right) colleting Peck's cave amphipods from the Spring Island area of the Comal River, New Braunfels, TX.

On February 24, Shawn Moore and Richelle Jackson set Texas blind salamander (TBS) traps at Primer's Fissure and Johnson's Well in the Purgatory Creek Natural Area, San Marcos, TX. On February 26, four TBSs were captured. One TBS was retained for incorporation into refugia and

transported to the quarantine building at the SMARC. Three TBSs were released into their respective collection locations.

On February 26, Dr. Katie Bockrath, Randy Gibson (SMARC) and Justin Crow (SMARC) repaired a tear in the Diversion net, reset floats to hold the net up, and inspected the ropes and ties holding the net in place (Figure 2).

Figure 2. Randy Jackson and Justin Crow swimming to the Diversion Springs Net for inspection and repairs.

Husbandry

SMARC

Moore and Jackson worked with Kallan Padget to identify improvements to the SMARC's *Daphnia* culture system. Moore and Jackson re-designed the system by providing a constant water supply and food, greatly increasing productivity.

Moore and Jackson met with Padget to discuss plans for future captive animal diet improvements. Padget recommended the program move toward complete food independence by reducing the amount of pre-prepared diets and increasing the proportion of live feed.

West began construction on a single-tank partially recirculating system in the SMARC refugia.

Uvalde

Dominique Alvear, Noel Valenzuela-Charro, Tanner Donelon and Padget worked on troubleshooting issues with existing controller boxes. Relays within the boxes were replaced to get the tanks back up and running.

Valenzuela-Charro and Donelon organized the field gear storage area and got everything separated by collection site for ease of use in the future.

Alvear conducted quarterly invertebrate box inventories starting with the Comal Springs riffle and dryopid beetle boxes.

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for February2025. "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	0	NT	2	0	0	0	240	296
Fountain darter: Comal	0	NT	175	0	0	19	175	372
Comal Springs riffle beetle	0	NT	0	0	0	16	412	32
Comal Springs dryopid beetle	0	NT	0	0	0	25	40	0
Peck's cave amphipod	39	39	0	0	0	0	100	168
Edwards Aquifer diving beetle	0	0	0	0	0	0	0	0
Texas troglobitic water slater	0	0	0	0	0	0	0	0
Texas blind salamander	4	NT	3	0	1	0	105	57
San Marcos salamander	0	6	0	0	1	6	244	128
Comal Springs salamander	0	NT	0	0	1	7	80	57
Texas wild rice	0	NT	0	0	0	2	191	130

Task 2 Research

Developing tools to determine thermal tolerances and preferences of current and future covered HCP species

Dr. Katie Bockrath worked on setting up a cooperative agreement with Texas State University to fund the improvements to the thermal tolerance testing prototype.

San Marcos Salamander Photo Mark and Recapture

Dr. Katie Bockrath worked with SCA to renew Marina Draeger's internship to continue working on processing photos that were collected during the previous p-Chip mark and recapture effort.

Peck's Cave Amphipod Offspring Exclusion

Dr. Katie Bockrath worked with SCA to advertise for an intern to work on the PCA exclusion project. Braden West designed exclusion housing and the flowthrough husbandry set up for brooding females during this study.

Tagging Aquatic Invertebrates

Braden West and Dr. Bockrath temporarily took down the flowthrough system that housed the beetles during the tagging trials. The system was descaled and cleaned.

Dr. Bockrath discussed potential dates for Dr. Shannon Brewer and Brian De La Torre to visit the SMARC to conduct one final trial using QR codes.

Genetic Assessment of fountain darters

No significant updates to report.

Genetic Assessment of Texas blind salamanders

No significant updates to report.

Genetic Assessment of San Marcos salamanders

No significant updates to report.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

EARP Staff contributed to the monthly report. Dr. Bockrath finalized edits for the 2024 Comal Springs riffle beetle genetic assessment report.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss Husbandry needs, Collection plans, and Research updates.

March 2025 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear, Kallan Padget and Dr. Katie Bockrath

With contributions from

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Task 1 Refugia Operations

Species Collection

Richelle Jackson and Shawn Moore checked Texas blind salamander traps set in the Purgatory Creek Natural Area, San Marcos, TX on March 3, 5, 7, and 10. One TBS was captured and released.

On March 26th, Jackson and Moore replaced the drift net on Diversion Spring in Spring Lake, San Marcos, TX. Two San Marcos salamanders were caught and released.

Husbandry

SMARC

Staff were alerted to a drip from the ceiling in the refugia building. After inspection, approximatly10-15% of the insulation batts in the three highest bays in the ceiling were saturated with water and in danger of falling (Figure 1a). Jackson and Moore quickly moved refugia animals out of the building and into temporary holding facilities in quarantine. Braden West worked with Juan Martinez (SMARC), Dr. Katie Bockrath, Dr. Scott Walker (UNFH), and Mike Montagne (SMARC) to assess the extent of required repairs and to purchase the necessary materials to repair the ceiling. West, Martinez, and Marin DeBolt (Student Conservation Association) removed the damaged insulation through the end of March (Figure 1b). West and Martinez repaired the dehumidifier and purchased a second dehumidifier to reduce condensation build up in the remaining insulation.

Figure 1. Left(a) initial discovery of waterlogged insulation. The orange arrow points to the centermost (ceiling peak) batt that first separated from the ceiling. Right (b) removal of wet insulation.

UNFH

Dominique Alvear, Tanner Donelon, Noel Valenzuela-Charro and Kallan Padget continued working to maintain existing controller boxes and chillers. One chiller was replaced, and two controller boxes had extension cords replaced. Valenzuela-Charro and Donelon began moving rock and potting materials to the quarantine rice area for rice collections next month. Padget began working on renovating the plumbing for another tank and gathering parts for building controller boxes. Alvear continued acid washing tanks to remove excess calcium carbonate. All employees worked together to ensure optimal animal health and assist in moving animals between tanks.

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for March 2025. "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	0	0	116	75	45	10	311	293
Fountain darter: Comal	0	0	69	0	42	59	142	313
Comal Springs riffle beetle	0	0	0	0	NA	0	412	32
Comal Springs dryopid beetle	0	0	0	0	NA	0	40	0
Peck's cave amphipod	0	0	60	30	NA	0	160	168
Edwards Aquifer diving beetle	0	0	0	0	0	0	0	0
Texas troglobitic water slater	0	0	0	0	0	0	0	0
Texas blind salamander	1	0	0	0	3	0	96	57
San Marcos salamander	0	0	0	2	3	6	248	115
Comal Springs salamander	0	0	0	0	0	2	77	55
Texas wild rice	0	0	0	0	26	0	165	130

Task 2 Research

Developing tools to determine thermal tolerances and preferences of current and future covered HCP species

Dr. Katie Bockrath worked with Texas State University and GrantSolutions to set up a cooperative agreement with Texas State University to fund the improvements to the thermal tolerance testing prototype.

San Marcos Salamander Photo Mark and Recapture

Dr. Katie Bockrath worked with SCA to extend Marina Draeger's internship to continue working on processing photos that were collected during the previous p-Chip mark and recapture effort. EARP staff met with Desiree Moore (former EARP) to discuss writing the manuscript for the mark and recapture study.

Peck's Cave Amphipod Offspring Exclusion

Braden West and Dr. Katie Bockrath interviewed SCA intern candidates to assist in the husbandry and maintenance of PCA during the exclusion trials.
Braden West continued to build the flowthrough system to house the exclusion trials and finalized the active exclusion housing. West continued to optimize the passive exclusion housings.

Tagging Aquatic Invertebrates

Dr. Bockrath set up the flowthrough system that houses the beetles during the tagging trials. Brian De La Torre and Dr. Shannon Brewer tagged Fx Comal Springs riffle beetles and placed them in control boxes and experimental tubes (Figure 2). Half of the beetles in each box and tube were tagged and the other half were not tagged to further investigate the impacts of tagging on survival. Dr. Bockrath monitored the system, ensuring temperatures and flows were within ideal range. Dr. Bockrath checked the beetles in the control boxes, flipped tubes, and reported movement data weekly.

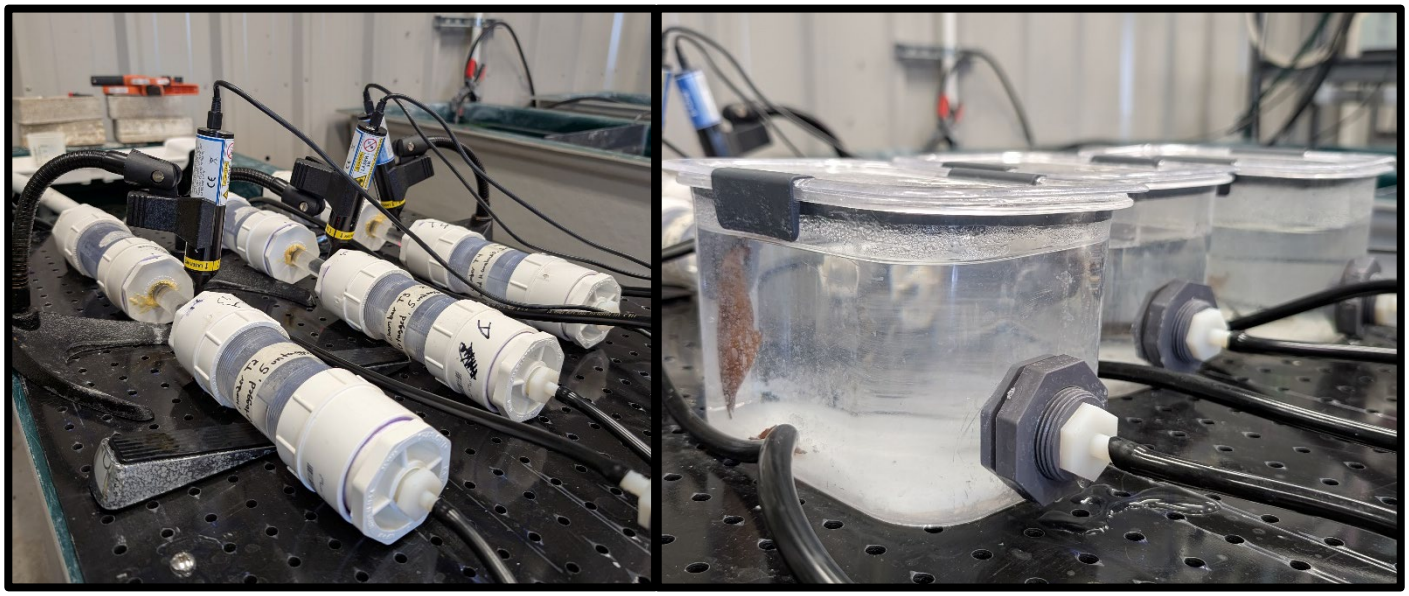

Figure 2. Tagged invertebrate experimental tubes (Left) and control housing (Right).

Genetic Assessment of fountain darters

Dominiqe Alvear and Shawn Moore began consolidating all the wild stock mortalities from the 2024 Seasonal Collections.

Genetic Assessment of Texas blind salamanders

Dominiqe Alvear began consolidating all the wild stock and Fx Texas blind salamander mortalities at Uvalde National Fish Hatchery.

Genetic Assessment of San Marcos salamanders

Dominiqe Alvear began consolidating all the wild stock and Fx San Marcos salamander mortalities at Uvalde National Fish Hatchery.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

EARP Staff contributed to the monthly report. Dr. Bockrath finalized edits for the 2024 Comal Springs riffle beetle genetic assessment report.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss Husbandry needs, Collection plans, and Research updates.

Dr. Bockrath and West met with Dr. Chad Furl and Kristy Kollaus during the quarterly EARP meeting.

April 2025 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear, Kallan Padget and Dr. Katie Bockrath

With contributions from

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Task 1 Refugia Operations

Staffing

Shawn Moore and Richelle Jackson (both SMARC) resigned from their positions in the U.S. Fish and Wildlife Service on April 17. Dr. Bockrath increased her role in the day-to-day animal husbandry operations and trained other SMARC biologists to step in while being short staffed.

Species Collection

Braden West met Christa Kunkel (BIO-WEST Inc.) to transfer fountain darters from the BIO-WEST led biomonitoring efforts in the San Marcos and Comal Rivers. USFWS received a total of 348 San Marcos River fountain darters and 677 Comal River fountain darters. Dominique Alvear (UNFH) transferred 219 San Marcos River fountain darters to the Uvalde National Fish Hatchery on April 24. SMARC retained the remaining 129 San Marcos fountain darters. All 677 Comal River fountain darters remained at the SMARC.

Jackson, Moore, Alvear, and Kallan Padget (UNFH) collected San Marcos salamanders from the Eastern Spillway (Middle San Marcos) of the San Marcos River on April 16. Thirteen salamanders were caught and retained for refugia at the UNFH.

Jackson, Moore, Alvear, and Padget collected San Marcos salamanders from the Hotel area of Spring Lake (Upper San Marcos) on April 17. Fourty-two salamanders were caught and retained for refugia at the UNFH.

West and Alvear coordinated with Dr. Matt Pintar (BIO-WEST Inc.) on April 11 to collect Comal Springs riffle beetle (CSRB) and Comal Springs dryopid beetle (CSDB). Forty-eight CSDB and eighty-four CSRB were caught and retained for refugia at the UNFH.

Jackson, Moore, and Alvear collected Peck's cave amphipods (PCA) from the Spring Island area of the Comal River on April 15. Thirty-eight PCA were caught and retained for refugia at the UNFH.

Jackson, Moore, Alvear, and Matthew (Tanner) Donelon (UNFH) collected Texas wild-rice (TWR) from the Eastern Spillway of the San Marcos River on April 8. Sixty plants were collected, with forty retained for refugia at the UNFH and twenty retained for refugia at the SMARC.

Jackson, Moore, Donelon, and Alvear collected TWR from the San Marcos River near San Marcos City Park on April 9. Thirty plants were collected, with twenty retained for refugia at the UNFH and ten retained for refugia at the SMARC.

Jackson, Moore, and West continued monitoring the drift net over Diversion Spring in Spring Lake during the month of April. Twenty-five San Marcos salamanders were captured, and eleven

were retained for refugia at the SMARC.

West, Padget, and Donelon, along with Victor Castillo III (Texas State University, Edwards Aquifer Research and Data Center) scouted Rattlesnake Cave and Rattlesnake Well to gauge the viability of collecting Texas blind salamanders in 2025. No TBS were observed, but the cave and well were both found to be accessible and held sufficient water to set traps.

Husbandry

SMARC

Construction on the waterlogged ceiling in the EARP refugia building was completed at the end of April. While many staff contributed to the efforts, Juan Martinez (SMARC) and Marin DeBolt (Student Conservation Association) were fundamental to successfully removing and replacing the insulation (Figure 1).

Animals that were held in temporary housing during the ceiling repair and were returned to their original tanks in the SMARC refugia after the repair project was completed.

West continued the construction of RE-01 at the SMARC EARP refugia.

Multiple staff members at the SMARC stepped up in the month of April to assist in the daily operations of the EARP (Figure 2). Dr. Katie Bockrath led cross-training efforts to increase the efficiency of daily tasks such as taking temperatures, collecting and logging mortalities, feeding, and captive food culture maintenance.

UNFH

Construction of the new plumbing system on RE00 was finished this month. The system is currently being disinfected and prepped to receive fish by the end of May. Plumbing for RE01 is currently being designed and installed and will be finished and holding fish by the end of May as well.

Donelon and Padget continued working on controller boxes last month. Currently four boxes are being prepped for installation. One is almost complete. There are also two boxes already mounted near their respective tanks and ready to be tied into a walchem box.

Valenzuela, Padget, Alvear and Donelon conducted successful husbandry last month, expanding animals brought into the facility for quarantine and maintaining existing refugia. Annual fish health monitoring was done this month with 15 Comal fountain darters and San Marcos fountain darters being sacrificed.

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for April 2025. "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	129	219	0	0	6	36	235	317
Fountain darter: Comal	677	0	0	0	11	85	136	226
Comal Springs riffle beetle	NT	84	0	0	93	0	319	32
Comal Springs dryopid beetle	NT	48	0	0	0	0	40	0
Peck's cave amphipod	NT	38	0	0	43	0	117	168
Edwards Aquifer diving beetle	NT	0	0	0	0	0	0	0
Texas troglobitic water slater	NT	0	0	0	0	0	0	0
Texas blind salamander	NT	0	2	0	1	1	97	56
San Marcos salamander	11	55	0	0	1	14	247	101
Comal Springs salamander	NT	0	0	0	0	4	77	51
Texas wild rice	30	60	0	0	0	0	165	130

Task 2 Research

Developing tools to determine thermal tolerances and preferences of current and future covered HCP species

Dr. Katie Bockrath worked with Texas State University and GrantSolutions to set up a cooperative agreement with Texas State University to fund the improvements to the thermal tolerance testing prototype. Enzo Silvagni volunteered every Friday morning to learn more about the EAHCP species and refugia husbandry practices to better prepare for future thermal tolerance testing trials.

San Marcos Salamander Photo Mark and Recapture

Marina Draeger's internship to continue working on processing photos that were collected during the previous p-Chip mark and recapture effort was successfully renewed. Marina will begin working on the photos in May.

Peck's Cave Amphipod Offspring Exclusion

No new activities to report.

Tagging Aquatic Invertebrates

Dr. Bockrath monitored the flowthrough system that house tagged and untagged beetles during the tagging trials. Dr. Bockrath ensuring temperatures and flows were within ideal range and checked the beetles in the control boxes for mortalities, flipped tubes, and reported movement data weekly.

Genetic Assessment of fountain darters

Dominiqe Alvear and Shawn Moore began consolidating all the wild stock mortalities from the 2024 Seasonal Collections. Alvear transferred UNFH mortalities to SMARC for DNA extraction.

Genetic Assessment of Texas blind salamanders

Dominiqe Alvear began consolidating all the wild stock and Fx Texas blind salamander mortalities at UNFH and transferred them to SMARC for DNA extraction.

Genetic Assessment of San Marcos salamanders

Dominiqe Alvear consolidated all the wild stock and Fx San Marcos salamander mortalities at UNFH and transferred them to SMARC for DNA extraction.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

EARP Staff contributed to the monthly report. Dr. Bockrath worked on and submitted the 2025 workplan and budget amendments as well as the 2026 work plan.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss Husbandry needs, Collection plans, and Research updates.

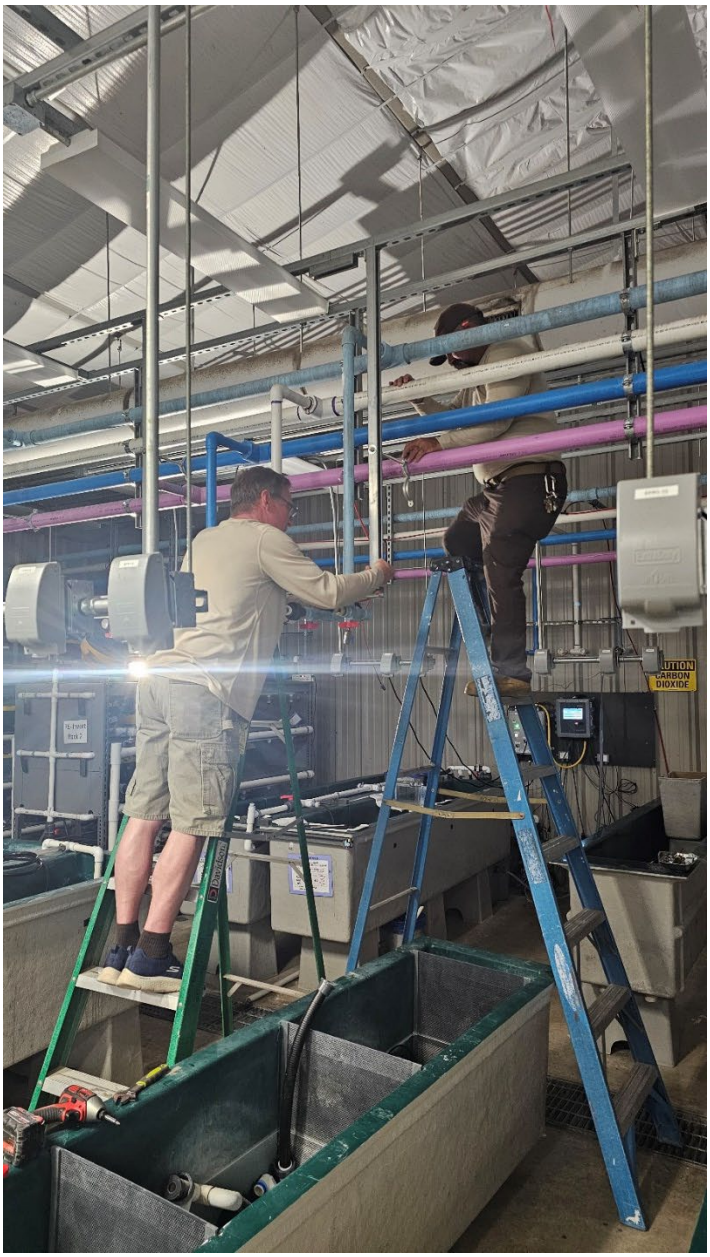

Figure 1. Mike Montagne (Acting Center Director, SMARC, foreground) and Juan Martinez (Facility Operations Specialist, SMARC, background) reinstall plumbing supports in the SMARC refugia.

Figure 2. Dr. Katie Bockrath (foreground), training Randy Gibson (Supervisory Biologist, SMARC, background) on daily temperature readings taken in the EARP refugia.

May 2025 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear, Kallan Padget and Dr. Katie Bockrath

With contributions from

Matthew (Tanner) Donelon

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Task 1 Refugia Operations

Staffing

Species Collection

Dominique Alvear and Braden West collected 40 Texas wild-rice tillers on May 19 from the City Park area of the San Marcos River in San Marcos, Texas. The tillers were brought to the UNFH and placed in their rice quarantine tanks.

Alvear and Kallan Padget collected fountain darters from the Spring Island area of the Comal River in New Braunfels, Texas on May 22. Three hundred ten fountain darters were transferred to the SMARC for incorporation into the refugia quarantine.

Alvear and Padget collected Comal River fountain darters from the Spring Island area of the Comal River on May 23. Three hundred eighty-one fountain darters were transferred to the SMARC for incorporation into the refugia quarantine.

Dr. Katie Bockrath, Randy Gibson (Supervisory Biologist, SMARC), and West collected Comal River fountain darters from the Spring Island area of the Comal River on May 24. One thousand six hundred four Comal River fountain darters were transferred to the SMARC for incorporation into the refugia.

Alvear and West collaborated with Dr. Matt Pintar (BIO-WEST, Inc.) to collected Comal Springs riffle beetle (CSRB) and Comal Springs dryopid beetle (CSDB) from the Spring Island area of the Comal River on May 12. Eighty-three CSRB and forty-five CSDB were transferred to the UNFH for incorporation into the refugia.

Dr. Bockrath, West, Gibson, and other SMARC staff checked the Diversion net regularly during the month of May. Seven San Marcos salamanders were captured and transferred to the SMARC for incorporation into the refugia.

West set Texas blind salamander (TBS) traps in Primer's fissure and Johnson's well on May 7. West and other SMARC staff checked the traps on Mondays, Wednesdays, and Fridays until May 21. Six TBS were captured and three were transferred to the SMARC for incorporation into the refugia.

Husbandry

SMARC

San Marcos Aquatic Resources Center staff set up seven additional trough tanks in the SMARC research pad (hereafter the pad) to accommodate the large influx of Comal River fountain darters (Figure 1). West, Dr. Bockrath, Somerley Swarm (Freshwater Mussel Biologist, SMARC), Gibson, Mike Montagne (acting Center Director, SMARC), Juan Martinez (Facility Operations Specialist, SMARC), Marin DeBolt, Marina Draeger, Garrison Engstrom (all Student Conservation Association, SMARC), as well as several volunteers worked to implement the recirculating systems. West, Martinez, and DeBolt moved four circular tanks into the pad to start four new cultures of *Daphnia* (Figure 2).

Figure 1: Seven new systems were constructed in the SMARC research pad to expand capacity for Comal River fountain darters collected in May.

*Figure 2: **Four additional Daphnia cultures were started to bolster live food culture capabilities for the EARP at the SMARC.***

UNFH

Uvalde National Fish Hatchery staff performed a refuge census this month. Padget, Donelon and Alvear recounted San Marcos fountain darters and San Marcos salamanders. Alvear incorporated Pecks cave amphipods, Comal Springs riffle beetles and Comal Springs dryopid beetles into the refuge last month. San Marcos salamanders were incorporated into the refuge last month by Padget and the entire staff finished working on controller boxes for refuge tanks 12 and 13.

On May 30th a gas supersaturation event occurred in the quarantine building. It is unclear what caused gas to become supersaturated in the waterlines supplying the quarantine building. This event led to mass mortality of the refuge populations of Comal fountain darters and Comal Springs salamanders. Staff are installing a bypass valve that will prevent supersaturation events from occurring in the future.

Animal Health

West transferred 60 fountain darters from the San Marcos River and 60 fountain darters from the Comal River to the Southwestern Fish Health Unit in Dexter, NM for parasite enumeration and health inspection in compliance with the SMARC Biosecurity plan.

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for May 2025. "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	0	99	0	8	18	326	276
Fountain darter: Comal	2295	0	387	0	1	226	522	0
Comal Springs riffle beetle	NT	83	0	44	NA	0	319	76
Comal Springs dryopid beetle	NT	45	0	35	NA	0	40	35
Peck's cave amphipod	NT	0	0	24	NA	0	117	192
Edwards Aquifer diving beetle	-	-	-	-	-	-	-	-
Texas troglobitic water slater	-	-	-	-	-	-	-	-
Texas blind salamander	3	0	0	0	3	1	94	55
San Marcos salamander	7	0	0	48	1	3	259	146
Comal Springs salamander	NT	0	0	0	0	51	76	0
Texas wild rice	NT	40	30	55	0	9	195	176

Task 2 Research

Developing tools to determine thermal tolerances and preferences of current and future covered HCP species

The TBAD (Thermal Biology Assessment Device) has been making many improvements over the past couple of months. The conduits used for counter current heat exchange, which heat and cool the TBAD have been modified to perfectly match each other in length. To seal these conduits, Enzo Silvagni (Texas State University) made casts of the ends of these conduits with silicone rubber to create a waterproofing gasket for the trough bulkhead. This method seals each conduit individually from each other, allowing dry tests to be performed on the conduits (Figure 3).

The mobile cart to house the TBAD is built and is now being used to hold the components to perform dry tests. The dry tests are to confirm computational simulations performed earlier this year to look at heat transfer in the conduits. The simulation showed different hot and cold configurations expected to source or sink heat in the trough. The results of our real time simulation testing thermal images have shown a difference in flow velocity going into each conduit, skewing the amount of heat or cold going into the conduits individually due to the hose splits and minor differences in elevation between the inlet points of the heating and cooling loop (Figure 4). To remedy this, Needle valves were used as a deterministic control instead of focusing on matching the geometry of the inlet and outlet hoses to mitigate this issue.

Lastly, simulations are being run to create a formula for the function of this device. The goal is to simulate the conditions of a wet test to provide crucial information on how this device will function, making operation, maintenance and performance more predictable and user friendly.

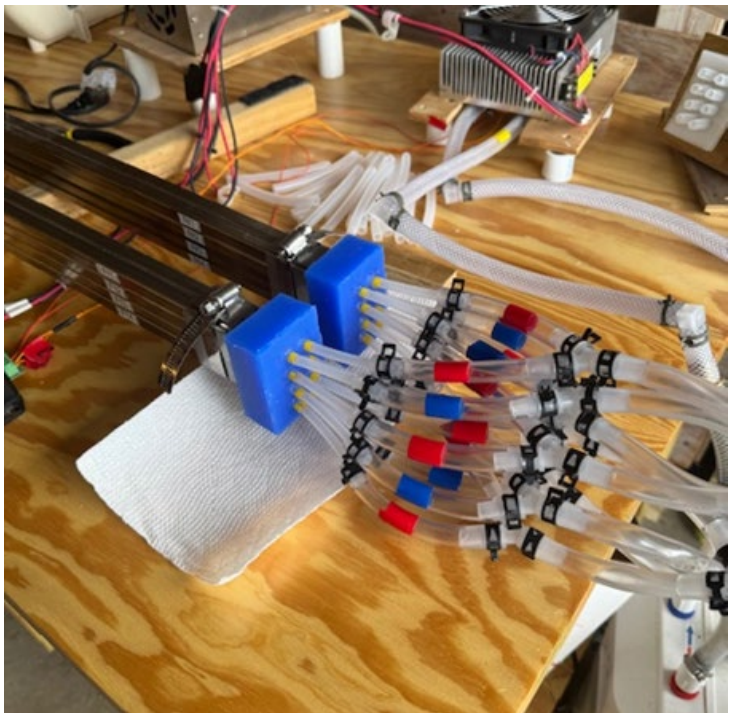

Figure 4.Waterproof gasket in action with the hot and cold inlet and outlet hoses connected to each conduit. The paper towel below proved no leaks were present.

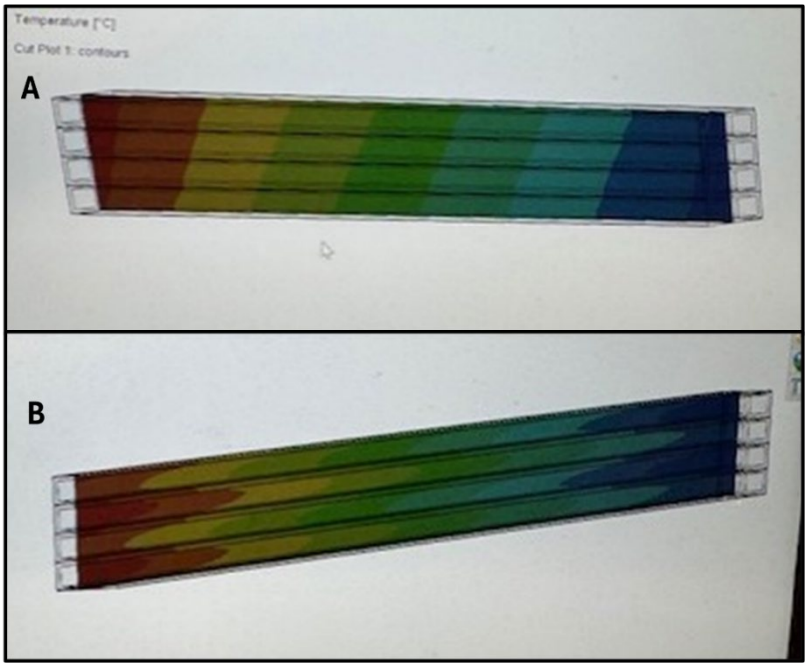

Figure 3: SolidWorks dry test simulation, A) Thermal readings of the transfer of heat between the stacked pairs of conduits, B) Thermal readings and conditions of the water inside the conduits.

San Marcos Salamander Photo Mark and Recapture
No new updates to report.

Peck's Cave Amphipod Offspring Exclusion
Marisol Farias (Student Conservation Association) started with SMARC. She has been training in animal husbandry and system construction in preparation for starting PCA exclusions.

Tagging Aquatic Invertebrates
To make space in quarantine for incoming Comal fountain darters, Dr. Bockrath took down the system that held the experimental tubes and control boxes and converted the system to hold fish. There are only a few adult CSRB and *Heterelmis glabra* in the Refugia, which is insufficient to conduct another trial. It is unlikely another trial will occur this year. Dr. Bockrath will work with Dr. Shannon Brewer and Brian De La Torre on completing the report.

Genetic Assessment of San Marcos Salamanders
Dr. Chris Nice (Texas State University) has taken on this project. Dr. Bockrath is in the process of setting up a cooperative agreement with Texas State University.

Task 4 Species Reintroduction
No work has been completed for this task area.

Task 5 Reporting
EARP Staff contributed to the monthly report.

Task 6 Meetings and Presentations
EARP staff met weekly to discuss Husbandry needs, Collection plans, and Research updates.

June 2025 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear, Kallan Padget and Dr. Katie Bockrath

With contributions from

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Task 1 Refugia Operations

Staffing

Marisol Farias (Student Conservation Association intern, SMARC) started at the SMARC on June 2. Marisol began work on the *Peck's Cave Amphipod Offspring Exclusion* project. Farias will fulfill a 12-month internship with the Edwards Aquifer Refugia Program.

Species Collection

On June 17, Matthew (Tanner) Donelon (UNFH), Farias, and Braden West (SMARC) collected Peck's cave amphipod (PCA) from the Spring Island area of the Comal River, New Braunfels, TX. A total of 42 PCA were captured. Seventeen were transferred to the UNFH and twenty-five to the SMARC for incorporation into the refugia.

On June 18, Dominique Alvear (UNFH), Farias, and West collected PCA from the Spring Island area of the Comal River, New Braunfels, TX. A total of 67 PCA were captured. Forty-two were transferred to the UNFH and twenty-five to the SMARC for incorporation into the refugia.

On June 24, Dr. Katie Bockrath (SMARC) received 32 San Marcos salamanders (SMS) captured at Diversion Spring by Pete Diaz, Somerley Swarm, Justin Crow, and Randy Gibson (All SMARC) during biomonitoring activities. All SMS were transferred to the SMARC for incorporation into the refugia.

On June 25, Alvear, Farias, and West met Dr. Matt Pintar (BIO-WEST, Inc.) at the Spring Island area of the Comal River, New Braunfels, TX. Farias and West collected 161 PCA and Alvear collected 12 Comal Springs riffle beetle and 18 Comal Springs dryopid beetle. All organisms were transferred to the UNFH for incorporation into the refugia.

Various SMARC staff checked the net over Diversion Spring on Mondays and Thursdays throughout the month of June. A total of 19 SMS were captured and transferred to the SMARC for incorporation into the refugia.

Husbandry

SMARC

West began training Farias on the various aspects of daily animal care and system maintenance to boost cross-training capabilities. Farias also received training on PCA field collections and inventory procedures. West plumbed heater/chiller units to the seven new recirculating systems housing Comal Springs fountain darters in the SMARC research pad to assist in temperature modulation within the systems.

UNFH

Kallan Padget (UNFH) and Alvear plumbed the new bypass valve at UNFH and began wiring the electronic shut off switch for the main line. Staff are waiting for the total gas pressure probe to arrive in order to complete the project. Padget and Alvear also began plumbing a new collection head box for supplying water to the quarantine area. Both projects are part of a collective effort to make water delivery more efficient and avoid any future gasification events. West traveled to the UNFH to deliver several components necessary to complete the bypass project at the UNFH quarantine building. In the refuge Alvear and Donelon incorporated 20 more rice plants and staff continued a collective effort to improve rice husbandry. Donelon and Noel Valenzuela (Student Conservation Association intern, UNFH) continue to build refuge systems. Currently 4 are finished with 5 remaining.

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for June 2025. "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	0	0	0	70	23	256	253
Fountain darter: Comal	NT	0	2201	0	18	0	2705	0
Comal Springs riffle beetle	NT	12	0	30	NA	0	319	106
Comal Springs dryopid beetle	NT	18	0	33	NA	0	40	68
Peck's cave amphipod	50	161	0	64	NA	0	117	188
Edwards Aquifer diving beetle	-	0	-	-	-	0	-	-
Texas troglobitic water slater	-	0	-	-	-	0	-	-
Texas blind salamander	NT	0	0	0	0	0	94	55
San Marcos salamander	51	0	16	0	0	9	238	121
Comal Springs salamander	0	0	0	0	0	0	76	0
Texas wild rice	0	0	0	20	0	4	195	172

Task 2 Research

Developing tools to determine thermal tolerances and preferences of current and future covered HCP species

The needle valves were successful at improving the distribution of water flow through the manifolds (Figure 3). This design evenly distributes the flow of water through the conduits, allowing for initial flow tests and dry tests to be completed. The dry test show the alternating hot and cold conduits configuration has the best thermal transfer out of all the configurations available (Figure 4). The temperature of the water exiting the conduits was only a few degrees off from the entering conduit temperature on their respective sides, which meets the goal of reducing the workload on the heater and chiller (Figure 5) and increase thermal stability.

Now that preliminary dry test data are complete, Enzo Silvagni (Texas State University) is prepared to start wet testing. To prepare for this, Enzo met with a heater and chiller specialist to confirm the heating and cooling devices we need for our unique design. Enzo purchased a second 12 channel thermocouple datalogger to provide instant thermal reading above all 18 of the convection chambers. Enzo is also working on the final measurements for the plexiglass trough frame which is the last item needed to start wet testing.

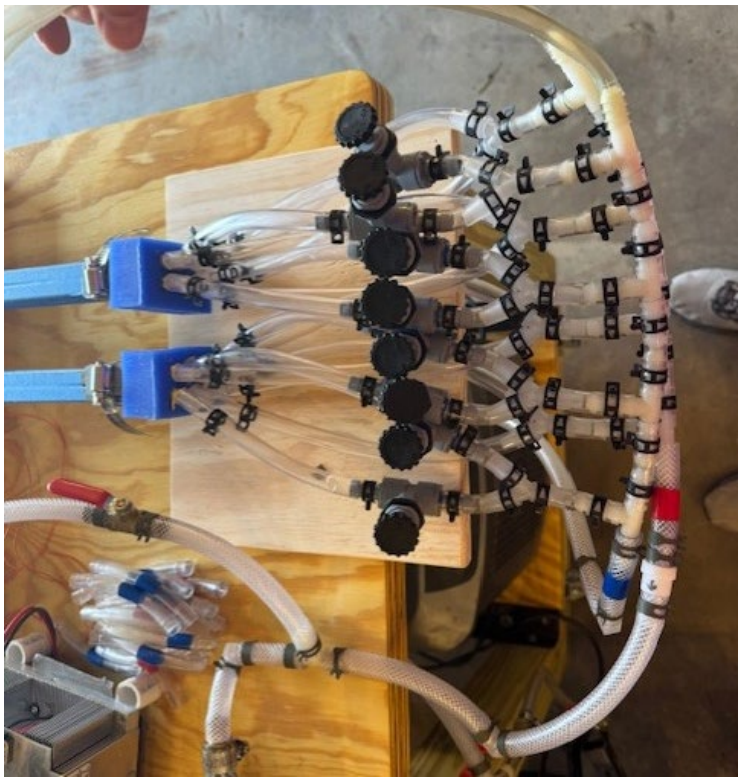

Figure 1. New Manifold with needle valves to control and regulate the flow rate and temperature distribution in the conduits.

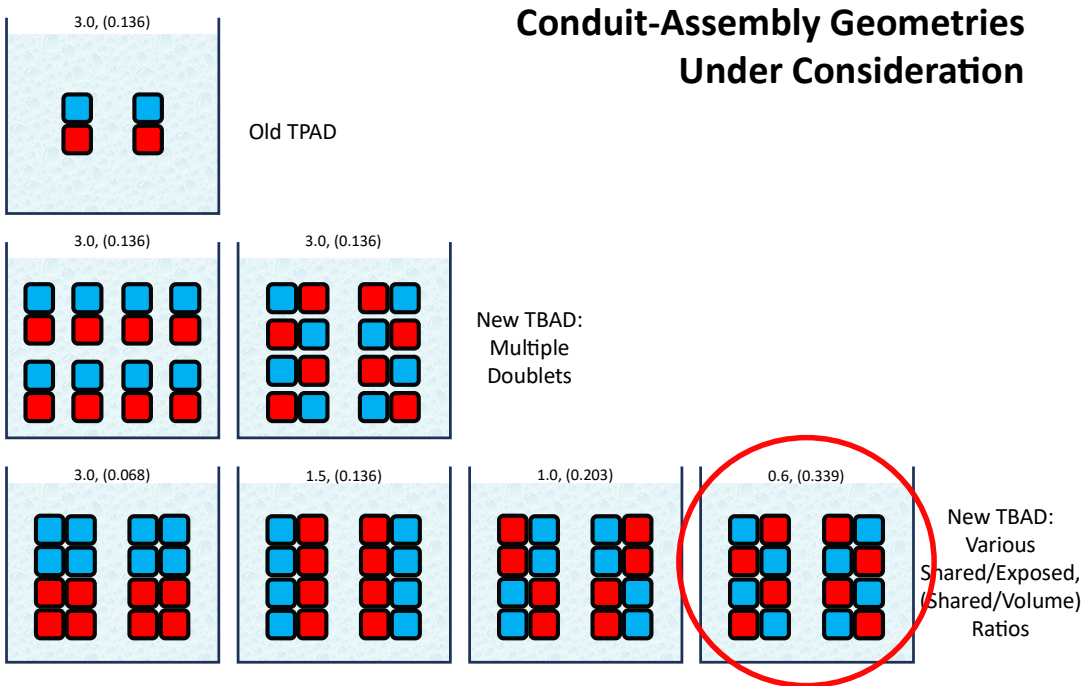

Figure 2. Different conduit configuration options for the TBAD. Configuration with the red circle around it had the best thermal transfer data out of all the options.

FR	Front C		FF	FC(FF)-FM(FF)	FC(FR)-FM(FR)	FR	Front M		FF	FM(FF)-FH(FF)	FM(FR)-FH(FR)	FR	Front H		FF
22.60	C	H	23.00	-6.10	4.70	27.30	C	H	29.10	-7.10	5.40	32.70	C	H	36.20
25.20	H	C	21.60	5.50	-7.60	32.80	H	C	27.10	6.10	-3.50	36.30	H	C	33.20
23.60	C	H	23.80	-8.10	4.30	27.90	C	H	31.90	-4.30	5.80	33.70	C	H	36.20
25.80	H	C	22.20	3.60	-3.40	29.20	H	C	25.80	4.90	-6.30	35.50	H	C	30.70

RR	Rear C		RF	RC(RF)- RM(RF)	RC(RR)-RM(RR)	RR	Rear M		RF	RM(RF)-RH(RF)	RM(RR)-RH(RR)	RR	Rear H		RF
22.50	C	H	31.80	-4.00	4.90	27.40	C	H	35.80	-2.00	6.00	33.40	C	H	37.80
26.70	H	C	21.80	6.10	-7.30	34.00	H	C	27.90	6.40	-4.00	38.00	H	C	34.30
24.90	C	H	22.80	-6.70	5.30	30.20	C	H	29.50	-6.80	6.00	36.20	C	H	36.30
29.20	H	C	21.00	4.90	-4.10	33.30	H	C	25.90	6.70	-4.90	38.20	H	C	32.60

Calc Avg Cold Temps: (IN)	Calc Avg Hot Temps: (OUT)
22.53	26.04

Avg Cold Temps: (OUT)	Avg Hot Temps: (IN)
33.35	36.81

Diff. of Left Temps:
3.51

Diff. of Right Temps:
3.46

Total Range:
14.29

Avg Diff across all Conduits Full length:
10.80

% of Gradient Range Used:
75.59

Figure 3. Data sheet showing some of the thermal information collected during dry testing. This is the alternating hot and cold conduits configuration that showed each conduit was on average transferring 75.6% of its thermal energy by the time it exited the conduit.

San Marcos Salamander Photo Mark and Recapture

No new updates to report.

Peck's Cave Amphipod Offspring Exclusion

Marisol Farias (Student Conservation Association) started with SMARC. She has been training in animal husbandry and system construction in preparation for starting PCA exclusions.

Tagging Aquatic Invertebrates

No new updates to report.

Genetic Assessment of San Marcos Salamanders

Dr. Chris Nice (Texas State University) has taken on this project. Dr. Bockrath is in the process of setting up a cooperative agreement with Texas State University.

Task 4 Species Reintroduction

No work has been completed for this task area.

Task 5 Reporting

EARP Staff contributed to the monthly report.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss Husbandry needs, Collection plans, and Research updates.

July 2025 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear, Kallan Padget and Dr. Katie Bockrath

With contributions from

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Task 1 Refugia Operations

Staffing

The SMARC is in the process of hiring a temporary (6 months) Biological Technician. The position was advertised on USAJOBS for one week. The advertisement closed on July 28 and we are waiting on a candidate list to start interviews.

Species Collection

Braden West traveled to Corsicana, TX on July 21 to meet Dr. Ryan Shartau (formerly University of Texas at Tyler). Dr. Shartau donated 10 Comal Springs salamanders to the SMARC. The salamanders were captured from the Spring Island area of the Comal River in 2023 and were previously used as a control group in Gas Bubble Disease research. The salamanders will undergo normal quarantine at the SMARC and be incorporated into the refugia population.

West set traps for Texas blind salamanders (TBS) in the Purgatory Creek Natural Area on July 23. West, Marisol Farias (Student Conservation Association, SMARC), and various other SMARC staff checked the traps on Mondays, Wednesdays, and Fridays until the end of July. West captured three TBS in July; all were retained for refugia at the SMARC.

Husbandry

SMARC

Kallan Padget travelled to SMARC on July 31 to assist West with constructing new recirculating systems in the refuge. With assistance from Farias and Padget, West assembled two pallet racks and began mounting and structuring all essential parts to construct the systems.

UNFH

Padget, Dominique Alvear, Matthew (Tanner) Donelon and Noel Valenzuela-Charro (Student Conservation Association, UNFH) worked on the quarantine bypass sump project this month. Staff completed the wiring of the bypass valve and have plumbed in the sump to deliver water to the quarantine area and are adding in extra degassing measures to ensure appropriate water quality of the well water. Once this portion of the project is concluded, staff will be ready to transfer Comal fountain darters from SMARC to the UNFH EARP quarantine.

UNFH staff focus on Texas wild-rice (TWR) this month. Padget and Alvear introduced new husbandry techniques, with the assistance of Chris Hatchcock (Botanist, SMARC), to create more uniform flow pattern, increase the light the plants receive and create an even density of plants in each of the refuge tanks. The new pump setup was designed to encourage water movement over each plant to simulate flow in the river.

Donelon and Valenzuela-Charro have been busy performing day to day husbandry and caring for darters and salamanders. Staff are finishing work on plumbing two more refuge tanks in the refugia area in preparation for fall collections.

Fish Health

The SMARC completed the annual Southwestern Fish Health Unit (SFHU) annual fish health inspection on July 22. This annual inspection maintains SMARC hatchery rating. West provided 60 Comal River fountain darters and 40 San Marcos River fountain darters for parasite enumeration and disease analysis. West also assisted SFHU staff by collecting skin swabs from all species of salamander held in refugia.

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for July 2025. "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	0	21	57	235	196
Fountain darter: Comal	NT	NT	0	0	214	0	2491	0
Comal Springs riffle beetle	NT	NT	0	0	NA	NA	523	106
Comal Springs dryopid beetle	NT	NT	0	0	NA	NA	40	68
Peck's cave amphipod	NT	NT	0	0	NA	NA	117	188
Edwards Aquifer diving beetle	-	-	-	-	-	-	-	-
Texas troglobitic water slater	-	-	-	-	-	-	-	-
Texas blind salamander	3	0	0	0	2	0	92	55

San Marcos salamander	NT	NT	0	0	7	7	231	114
Comal Springs salamander	10	NT	0	1	0	0	76	1
Texas wild rice	NT	NT	0	36	0	0	195	157

Task 2 Research

Developing tools to determine thermal tolerances and preferences of current and future covered HCP species

Enzo Silvagni and Dr. David Huffman (both Texas State University) prepared the trough and started wet tests. To prepare the trough, Silvagni and Dr. Huffman finalized the dimensions of the plexiglass trough (105" x 11" x 8"). The overall length of the trough (which was the biggest change to the original plan) was modified from the original prototype to provide the same volume of water in each convection chamber along the trough, increase the number of convection chambers and maintained power efficiencies along the trough. These modifications will drive the device more effectively by a magnitude of 4x compared to the prototype, allowing for more control over generating and manipulating the gradient. The conduits have been cut to the new length and have been cleaned and prepped for wet testing.

Silvagni and Dr. Huffman are working with Texas State University purchasing account manager to secure the heater and chiller needed to perform the wet tests. They expect the heater/chiller to arrive in August. All other materials needed for wet testing have been secured. After wet testing is complete, the data generated will be used to confirm the dry test models and the TBAD unit will be ready for on specimen testing and procedure development.

San Marcos Salamander Photo Mark and Recapture

Marina Draeger (Student Conservation Association) worked on cropping and reorienting headshot photos of San Marcos Salamanders that were collected during the 2023-2024 mark and recapture effort.

Peck's Cave Amphipod Offspring Exclusion

Marisol Farias (Student Conservation Association) inventoried the Peck's cave amphipods at SMARC and found 3 gravid females. These females were separated into individual housing and observed each week to monitor embryo development. Passive exclusion housing designs were finalized, and the brooding females will be placed in these housings in August. Farias will continue to monitor the Refugia population of PCA for brooding females and will start manual exclusion trials with the next set of brooding females.

Tagging Aquatic Invertebrates

No new updates to report.

Genetic Assessment of San Marcos Salamanders

Dr. Chris Nice (Texas State University) has taken on this project. Dr. Bockrath is in the process of setting up a cooperative agreement with Texas State University.

Task 4 Species Reintroduction

No work has been completed for this task area.

Task 5 Reporting

EARP Staff contributed to the monthly report.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss Husbandry needs, Collection plans, and Research updates.

Dr. Bockrath and Dr. Scott Walker (Acting Center Director, UNFH/SMARC) attended a discussion about Texas wild-rice current and future conservation efforts and HCP successes.

August 2025 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear, Kallan Padget and Dr. Katie Bockrath

With contributions from

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Task 1 Refugia Operations

Species Collection

Per the work plan collection schedule, staff did not schedule or conduct any collections in August. Collections will resume in September.

Husbandry

SMARC

West incorporated 57 San Marcos Salamanders, bringing the San Marcos refuge total to 278 animals. West continues to maintain Comal fountain darter from the May collection and has begun assisting with the transfer of some of these darters to Uvalde in an effort to re-establish a refuge population at both facilities. West and Padget worked on updating inventory numbers for the SMARC invertebrates. Many of the invertebrates at SMARC were either reaching a year in captivity or were over a year in captivity. Comal Springs riffle beetles have a lifespan of 1 year and it is around this time drops in refuge populations are observed. Although some of the drops were significant, staff are not surprised at these drops due to the age of the animals. Staff will make fall collections a priority to recover those numbers. West and Padget continue redesigning some of SMARC's tank systems to work as recirculating aquaculture systems. There is an observable increase in fountain darter longevity in the new systems. These new recirculating systems will continue to help West and other staff at SMARC optimize animal health and system functions.

UNFH

Invertebrate inventories were also a priority at Uvalde this month. Alvear incorporated 92 Pecks Cave Amphipods and 16 beetles; 8 Comal Springs riffle beetles and 8 dryopid beetles. Padget finished building RE00 and RE01 this month and transferred San Marcos Darters into the improved systems. The use of Low Head Oxygenators, filtration media and UV treatment has improved water quality conditions in many of the tanks and is improving the health of refuge animals. This month Padget, Donelon and Alvear finished the oxygenator in quarantine to reduce the impact of gasification events and improve water quality by introducing more dissolved oxygen into the water. So far this has been a success and staff have begun the process of re-establishing a refuge population of Comal fountain darters. Donelon and Padget continue to work with rice 4 days a week. Staff have disinfected and cleaned all tanks and reworked the way the rice tanks are set up. Plant health continues to improve under these new husbandry conditions.

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Species	SMARC Collections	UNFH Collections	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	0	0	5	51	230	145
Fountain darter: Comal	NT	NT	0	0	118	0	2373	0
Comal Springs riffle beetle	NT	NT	0	8	435	0	88	114
Comal Springs dryopid beetle	NT	NT	0	8	26	0	14	76
Peck's cave amphipod	NT	NT	0	92	70	0	47	284
Edwards Aquifer diving beetle	-	-	-	-	-	-	-	-
Texas troglobitic water slater	-	-	-	-	-	-	-	-
Texas blind salamander	NT	NT	0	0	2	0	90	55
San Marcos salamander	NT	NT	57	0	4	2	278	112
Comal Springs salamander	NT	NT	0	0	0	0	76	1
Texas wild rice	NT	NT	0	0	0	14	195	176

Task 2 Research

Developing tools to determine thermal tolerances and preferences of current and future covered HCP species

Dr. Huffman and Enzo Silva determined the layout and size of the blackout shroud on the trough (Figure 1). The blackout shroud will allow light exposure to be controlled during temperature experiments. Another design modification was on the removeable bulkhead wall seal (Figure 2). The sealing gasket was moved on the inside of the trough walls instead of the outside edge (like originally planned) allowing the bulkhead wall to be sealed using the same structure used to hold the inside of the trough onto the conduit gaskets caps. This will keep the movement and compression in a single plane.

Enzo received the plexiglass and heater/ chiller on September 22nd, 2025. Upon receiving the units, a heater/chiller shelf and a chiller cart was built to keep the coolant at the same level as the water line (Figure 3) to ensure there is no chance of overflow out of the open baths when the system is primed.

An organism exclusion arena floor as added to the TBAD with an attached air system (Figure 4). Figure 4 is a smaller model only accounting for the area above 3 convection chambers (final mode will be 5x as long). This allows for over half the arena floor assembly to be one piece that can be install and remove easily without having any cracks or holes where small organisms may escape the arena, while still working with larger organisms as well. The arena will still have species specific additions to them to accommodate for each study species specific needs but will use the same bare bones arena insert.

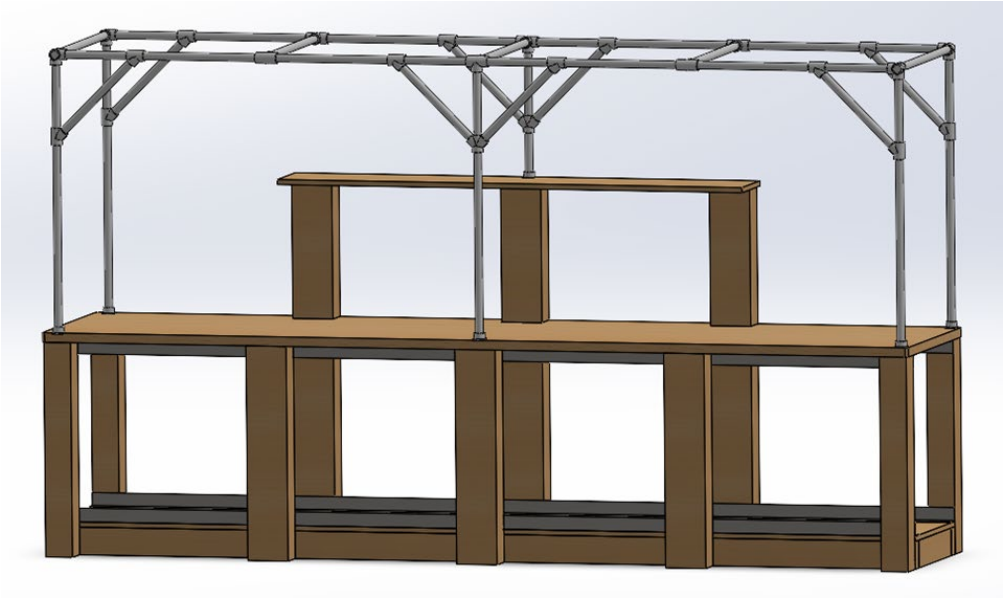

Figure 1. Cartoon mockup of the TBAD stand showing the PVC frame for the blackout shroud.

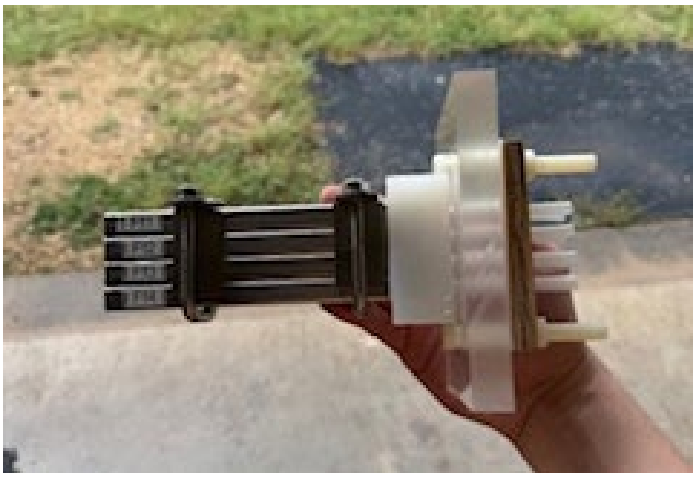

Figure 2. Removable bulkhead with attached countercurrent channels.

Figure 3. Full view of the TBAD set up with the addition of the new heater and chiller and removable bulkheads.

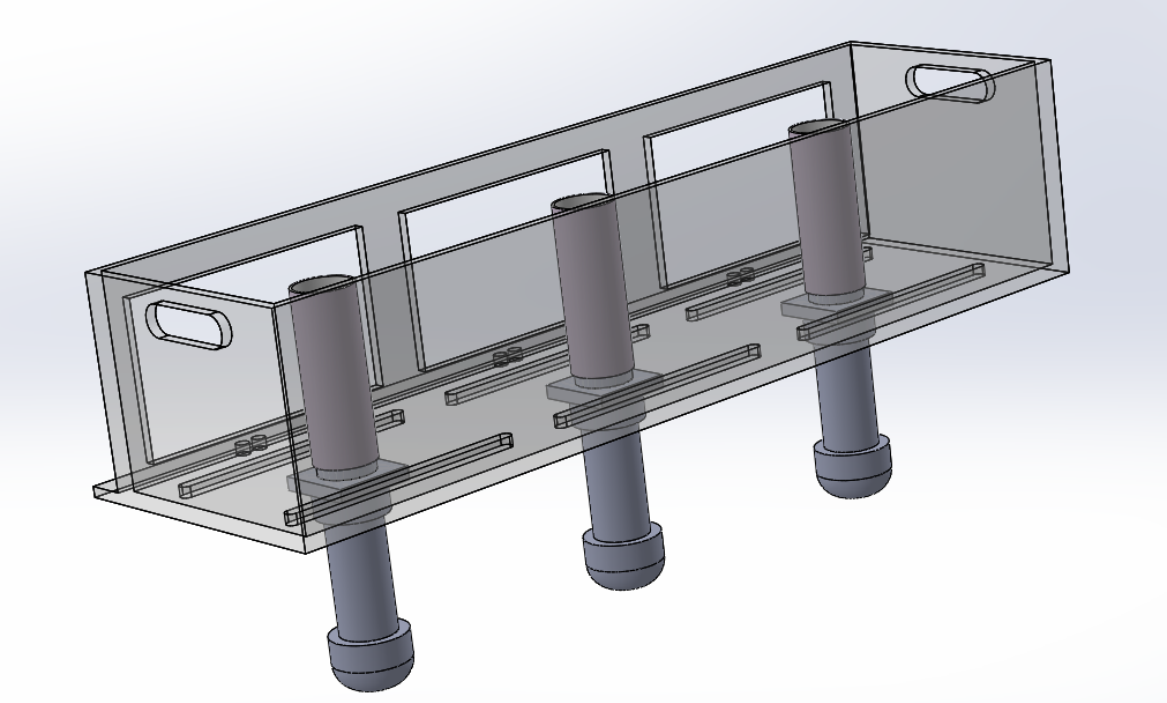

Figure 4. Digital mockup of the organismal exclusion arena floor. All inserts are clear plexiglass, windows and floor slits are screened, lower chimneys are made of PVC, and light purple (in the figure) is fine nylon screen exclusion chimneys.

San Marcos Salamander Photo Mark and Recapture

Photos of the tagged San Marcos salamanders were compared to photos of recapture events with known tagged salamander recaptures. Marina Draeger (Student Conservation Association) cropped these photos to uniform size and ran them through Wild.ID. Wild.ID generated a list of detections with confidence scores (range between 0-1). Confidence scores ranged from 1% to over 80%. Matches in the Wild.ID database were visually confirmed to be the same salamander. These matches were then categorized into matches between tagged/tagged salamanders, tagged/nontagged salamanders and non-tagged/non-tagged salamanders. The p-chip ID for tagged/tagged salamander matches were compared, and unfortunately, they did not match. Upon further investigation, there is a was a miss-match of photo ID and salamander tag ID when the photos were being cropped. The photos are of the same salamander, but in the database, they have the incorrect p-chip assigned to them. Dr. Bockrath and Dreager are QA/QC checking the photos and redoing the Wild.ID analysis.

Peck's Cave Amphipod Offspring Exclusion

Marisol Farias (Student Conservation Association) constructed additional passive exclusion setups using the design finalized in July. Passive setups were tested using *Hyalella azteca* amphipods, a smaller, surface-dwelling amphipod. Two brooding female PCA were placed in

separate passive exclusion setups in August, one with five neonates, and one with eight. Setups are monitored biweekly for neonate dispersal. Farias worked on drafting a plan for anesthesia of PCA using temperature to limit stress levels during manual exclusion trials.

Tagging Aquatic Invertebrates

No new updates to report.

Genetic Assessment of San Marcos Salamanders

This project has stalled as Texas State University refused to honor the pre-approved 17.5% indirect rate under the CESU agreement between Texas State University and the U.S. Fish and Wildlife Service. Texas State University is insisting on a 50.5% indirect rate, removing approximately $10,000 from the project budget to cover the increased indirect rate. A cooperative agreement between Texas State and USFWS has not yet been awarded for this project.

Task 4 Species Reintroduction

No work has been completed for this task area.

Task 5 Reporting

EARP Staff contributed to the monthly report.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss Husbandry needs, Collection plans, and Research updates.

September 2025 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear, Kallan Padget and Dr. Katie Bockrath

With contributions from

Matthew (Tanner) Donelon

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Task 1 Refugia Operations

Species Collection

Edwards Aquifer Refugia Program staff collected San Marcos salamanders, Pecks Ccve amphipods and Comal Springs riffle beetle this month. Braden West and Kallan Padget collected 41 Comal Springs riffle beetles Amphipods in the Spring Island Area of the Comal River, New Braunfels, Texas, with the help of Dr. Matthew Pintar of BIOWEST. Padget, Marisol Farias, West, Noel Valenzuela-Charro (Student Conservation Association) and Dominique Alvear all contributed to the collection of 197 Pecks Cave Amphipods in the same area of the Comal River. Later in the month, Padget, Valenzuela-Charro, Dr. Katie Bockrath, Farias and Marina Draeger (Student Conservation Association) all worked together to collect and transport 89 San Marcos Salamanders to Uvalde.

Husbandry

SMARC

The focus this month at SMARC has continued to be maintenance of Comal fountain darters. Uvalde did receive 604 Comal darters from SMARC, however, West still has roughly 1700 darters to care for daily. West has successfully increased his daphnia and blackworm cultures to supply adequate amounts of live feed for these darters and continues to provide them a diversified diet. West has also continued building new recirculating systems at SMARC that will follow a similar design plan as the systems at Uvalde. The long-term plan is to utilize the same strategies at SMARC and Uvalde to improve water quality and animal longevity. West has put a significan amount of time into working on Texas wild-rice this month as well as he prepares the refuge to receive new plants during the fall and winter months. Staff continue annual tank cleaning, sorting plants by size and consolidation of rice numbers in preparation.

Figure 1. Braden West (Center), Marisol Farias (Left) and Delaney LeStourgeon (Right) cleaning Tank 3 in the SMARC Greenhouse and grooming Texas wild-rice.

UNFH

The focus this last month at Uvalde has been fountain darter husbandry. Padget continues working with Tanner Donelon and Valenzuela-Charro on improving daily husbandry techniques. The primary focus is increasing daily water exchange to reduce bacterial load and improve oxygen levels in tanks. All darters are now in tanks that are equipped with LHO's (Low Head Oxygenators), biofiltration and UV treatment. This helps control bacterial load and increases oxygen levels. Staff also changed feeding routines and are moving towards a diversified diet of daphnia, artemia and frozen feed mixed together for feeding. In the coming months we will continue to share updates on how these changes contribute to the improvement of the refuge population of fountain darters. Uvalde received 604 Comal darters transferred from SMARC. The new system design for the quarantine has been tested, and water is adequate to maintaining animals again. In November, the transferred Comal darter population will be incorporated into the Uvalde refugia.

Staff continue working on the new refuge systems at Uvalde. We are beginning to see how beneficial these new systems are in improving longevity of our animals and are working diligently to update the rest of the systems to this new design. Central parts to the new design include the Low Head Oxygenation and biofiltration elements that optimize oxygen delivery and reduce bacterial load. A new outflow sump design that allows staff to efficiently perform more effective water exchanges and UV treatment that sterilizes water as it is recirculated within the system.

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for September 2025. "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	SMARC Transfer to UNFH	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	0	0	3	0	15	227	130
Fountain darter: Comal	NT	NT	0	0	76	604	0	1693	0
Comal Springs riffle beetle	41	NT	0	0	0	0	0	88	114
Comal Springs dryopid beetle	NT	NT	0	0	0	0	0	14	76
Peck's cave amphipod	197	NT	0	0	0	0	0	47	284
Edwards Aquifer diving beetle	-	-	-	-	-	-	-	-	-

Texas troglobitic water slater	-	-	-	-	-	-	-	-	-
Texas blind salamander	NT	NT	4	0	1	0	0	94	55
San Marcos salamander	NT	89	0	0	14	0	1	264	111
Comal Springs salamander	NT	NT	0	0	0	0	0	76	1
Texas wild rice	NT	NT	0	0	4	0	19	191	157

Task 2 Research

Developing tools to determine thermal tolerances and preferences of current and future covered HCP species

Enzo Silvagni and Dr. David Huffman (Texas State University) completed the dry on the TBAD (Figure 1). Since receiving the new heater and chiller, they collected data on two different flow rates (1.2 LPM and 0.8 LPM) on the alternating hot and cold conduits configuration. The improvements on this model compared to the prototype has resulted in an extra 10 °C of thermal range out of the conduits. One challenge that they faced was setting the flow gauges to balance the water level in the tanks in the system. Theoretically, exactly matching the amount of water coming out of one thermal devise and into the other thermal device would result in a balanced transfer of fluids in and out of the tanks. In practice this is not the case, especially with the heating and cooling condensers running. The changing viscosity of the water has required them to create a procedure to regulate how much water is allowed into the system with the flow gauges by watching the water level in the smaller heater tank. The procedure for balancing the tanks water levels with the heating and cooling condensers on and off has been added to the operation manual that will come with the device.

Also, we have created the display model of the arena insert (Figure 2) and created a working model of the bubble chimney that will circulate the water in the trough (Figure 3). This all has led to the final model of the arena insert that will be laser cut on October 8th. The final model is modular and held together by clear H channel connectors to close the gaps between the modular sections (Figure 4). Once the pieces are cut, everything just needs to be bonded together and given a couple days to dry, and the preliminary wet tests will commence.

Lastly, while waiting for access to the laser cutter, Silvagni and Dr. Huffman started work on the shroud that will control light availability during a run. While the shroud is not necessary for the wet tests, it is something that will be critical for light sensitive species testing when we get to that point. Once wet tests are done, preliminary species testing can start. The goal is to start testing organisms at SMARC in November.

Figure 2. Set up for dry test with new heater and chiller.

Figure 3. Display model of arena insert setup.

Figure 4. Bubble chimney that circulates water from the convection chamber below into the arena above.

Figure 5. H channel connector used to connect modular arena inserts.

San Marcos Salamander Photo Mark and Recapture

Marina Dreager (Student Conservation Association) cropped and standardized the orientation of photos of San Marcos salamander taken during the 2023-2024 mark and recapture effort. These photos were run through Wild.ID and produced matches. When confirming salamander p-Chip tag ID, we discovered there was an error in photo transcription resulting in an error in matched salamander tag ID. After some quality checks, Dr. Katie Bockrath took a subset of the photos and correctly labeled them and reran them through Wild.ID. Photos of tagged salamanders were

correctly matched to photos of the same salamanders recaptured at a later date (Example seen in Figure 1). Wild.ID generates a quality score and we had hoped that quality score would correlate to matches, allow us to use a minimum quality score to quickly confirm matches, but the quality score is heavily impacted by photo quality and differences in photo attributes (See Figure 1). Visual confirmation of matches is still required.

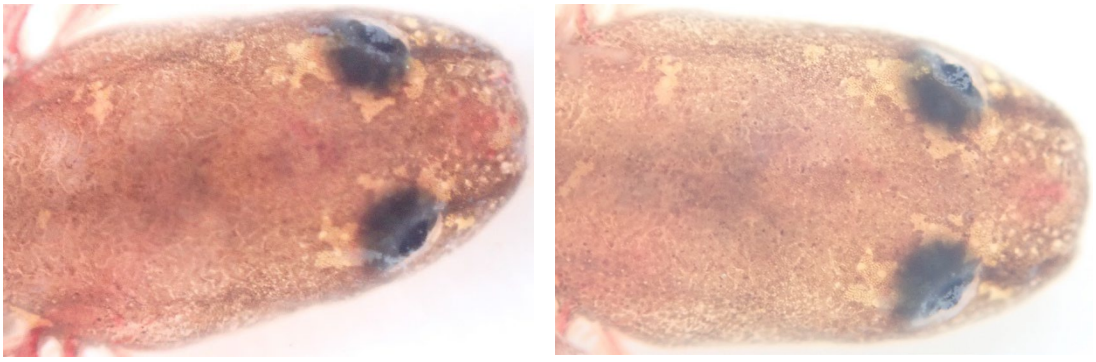

Figure 6. Photo of a tagged San Marcos salamander that successfully matched to a photo of the same tagged salamander from a later collection date. The salamander on the left was collected on June 20, 2023, and the salamander on the right was collected on March 13, 2024. Both photos are of the same salamander confirmed by the p-Chip ID. The quality score for this match is 22%.

Peck's Cave Amphipod Offspring Exclusion

Marisol Farias (Student Conservation Association) finished constructing the passive exclusion tubes, set the flow to 15 L/sec and tested the set up using *Hyalella*. Farias checked the tubes after a week and the *Hyalella* were still alive and present in the setup, showing the mesh size will keep Peck's cave amphipod neonates from being ejected from the tube and they will not be smashed against the mesh in the tube. Farias identified 5 brooding females and placed one in the top chamber of each passive exclusion tube. Farias will check the top and bottom chamber of each tube throughout October to check for mortalities and the presence of eggs and/or neonates in the bottom chamber.

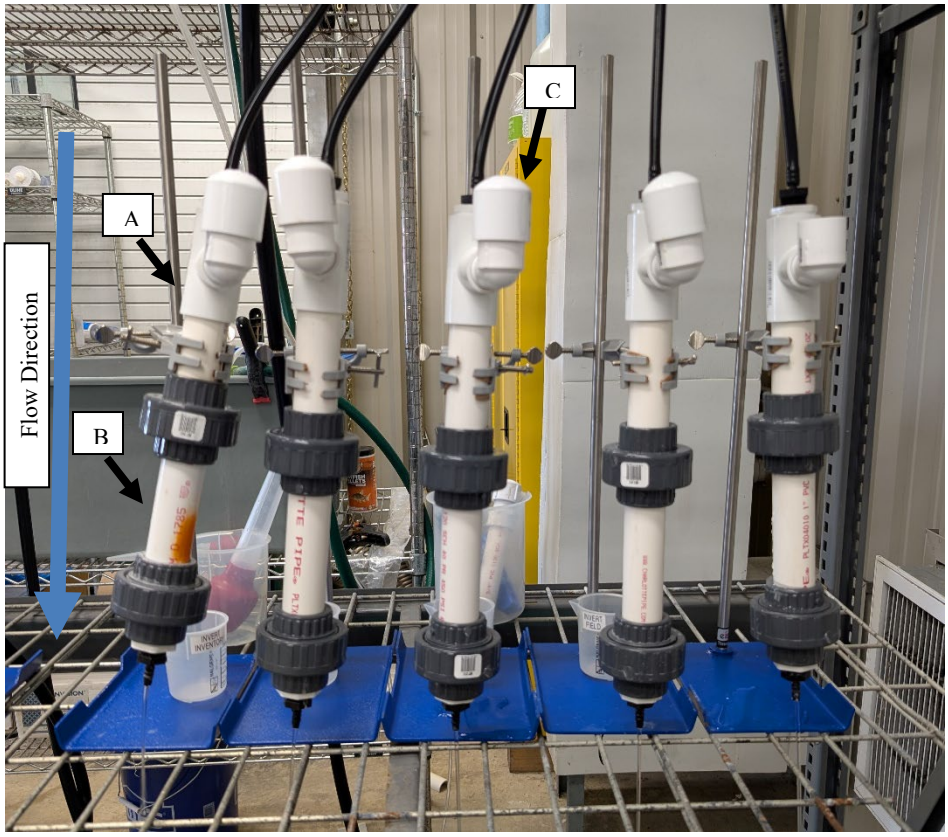

Figure 7. Picture of 5 vertical flowthrough passive exclusion tubes. Each tube is separated into a top (A) and bottom (B) chamber, with M1000 mesh. A brooding female PCA is placed in the top chamber and neonates can move through the mesh into the bottom chamber where the female is excluded by the mesh. Food is placed in each tube through the access port to the top chamber (C).

Tagging Aquatic Invertebrates

Brian De La Torr (Auburn University) has developed and is testing models to assess tagging efficacy on both Comal Springs riffle beetles and the surrogate species, *Heterelmis glabra*. Results are forth coming.

Genetic Assessment of San Marcos Salamanders

This project is currently on hold as we attempt to negotiate with Texas State University on the overhead rate.

Task 4 Species Reintroduction

No work has been completed for this task area.

Task 5 Reporting

EARP Staff contributed to the monthly report.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss Husbandry needs, Collection plans, and Research updates.

Dr. Bockrath attended the EAHCP Science Committee Meeting on September 9, 2025.

Dr. Katie Bockrath and Braden West met with Dr. Chad Furl and Kristy Smith for the EARP/EAA quarterly meeting on September 23, 2025.

October 2025 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear, Kallan Padget and Dr. Katie Bockrath

With contributions from

Matthew (Tanner) Donelon

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Task 1 Refugia Operations

Species Collection

Staff focused on collecting Pecks Cave Amphipods and San Marcos Salamanders this month. Our coordinated effort with Dr. Matthew Pintar of BIOWEST also continued this month to collect Comal Springs riffle beetles and Comal Spring dryopid beetles. EARP staff conducted three collections of Pecks cave amphipods at Spring Island. All three collections were very productive yielding over 100 PCA each collection. EARP staff conducted three San Marcos salamander collections this month as well. The first at Eastern Spillway yielded 15 salamanders. The other two collections were conducted at Spring Lake with 49 salamanders being collected across the two collection days.

Husbandry

SMARC

This month at SMARC Braden West has been focused on Comal fountain darter husbandry. He worked with Kallan Padget to developed new procedures to improve husbandry of darters collected during the salvage event in May. These darters are held in basic partial recirculating flow through tanks, thus do not have the superior water quality provided by the UV sterilizers, CO_2 injectors, and overall water quality monitors/equipment that are on the main refugia systems. As a result, the number of mortalities in these tanks are greater than that of the main refugia. The goal is to reduce mortality significantly for the remainder of their time in the refuge. Farias successfully incorporated 85 PCA into the refuge this month and continues to be very resourceful in assisting with daily husbandry when needed. Due to the increase in collections for the fall West is working on systems much less than before and is spending more time in the field now.

Last month an inventory of the Comal fountain darters was done, and it was found that refuge numbers were lower than anticipated from the logged collection numbers and mortality counts. These discrepancies are likely a result of miscounts during collection and unlogged mortalities following the salvage event in May, and mortalities recorded in the incorrect location on the inventory tracking sheets. Following the salvage event, staff focused on husbandry of the large number of fish brought on station and did not have adequate time to accurately record mortalities as they occurred. Most mortalities were retained but there are likely some that were not. Staff were able to log the retained mortalities four months later in September. Approximately 180 unlogged mortalities were recorded, and incorrectly logged mortalities were corrected. The remaining discrepancy in numbers is likely due to a miscount that occurred in the field during the salvage collection of the fish. Although a significant number of fish were collected it was not as many as had been originally anticipated. SMARC transferred 604 Comal fountain darters to UNFH in September, further reducing the number of fish on hand at SMARC. As of this report, there are 1,061 Comal fountain darters at SMARC which is 252 fewer fish than expected.

UNFH

At UNFH Padget, Alvear, Donelon and Valenzuela-Charro continue to make significant progress on systems work and husbandry developments. All staff are rotating rice husbandry to clean tanks every day now. There have been significant improvements in the health of the plants since staff have begun using this procedure. Padget, Alvear and Valenzuela-Charro continue updating refuge systems. Staff are doing their best to update as many systems as possible before San Marcos fountain darters begin entering the refuge this winter. Significant improvement in health and condition of the fish has been observed when utilizing these new systems. Staff are also working on expanding live food culturing to introduce more dietary options to animals daily feeding routine. Padget has observed a noticeable change in condition of fish since introducing more live feed into their diet and will continue to utilize more live food options. Staff are also trying new filter systems for invertebrates to improve water quality and exchange rate.

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for October 2025. "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	SMARC Transfer to UNFH	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	0	0	0	0	1	0	8	226	123
Fountain darter: Comal	0	0	0	0	56	0	9	1061	0
Comal Springs riffle beetle	0	56	0	0	0	0	0	88	114
Comal Springs dryopid beetle	0	6	0	0	0	0	0	14	76
Peck's cave amphipod	217	140	85	0	0	0	111	132	173
Edwards Aquifer diving beetle	-	-	-	-	-	-	-	-	-
Texas troglobitic water slater	-	-	-	-	-	-	-	-	-
Texas blind salamander	0	0	0	0	0	0	0	94	55
San Marcos salamander	9	64	0	81	20	0	2	250	199
Comal Springs salamander	0	0	0	0	1	0	0	75	1

Texas wild rice	0	0	0	0	29	0	13	162	144

Task 2 Research

Developing tools to determine thermal tolerances and preferences of current and future covered HCP species

Major progress has been made in the development of the TBAD. The troughs acrylic panels have been mended together (Figure 1) and the hole for the Standpipe/overflow drain has been cut and the hardware for the drain has been attached to the back wall (Figure 2). The removeable bulkhead wall and the gaskets used to seal the bulkhead have also been constructed and implemented, giving us a perfectly waterproof end of the trough. The modular arena inserts have also been mended together, and the bubble chimneys have been attached to the arena floor and to the air pump (circulating the water in the trough). The last thing we need to do with the arena inserts to make them organism ready is screen in all the openings.

The shroud frame has been finished (Figure 3) and we are currently working on the cover that will go over the cart and frame to be able to control the light sensitivity. The cover is made of thick canvas material and will have a double layered opening under an awning giving room for researchers to stand under. A sliding shelf/board has also been attached to the cart to house the cameras and lights, for testing and recording purposes, has also been completed (Figure 3). The back half of the camera rack are PVC sliders that allow the 2 x 4 board to extend over the trough or retract back under the top shelf when access to the inside and top of the trough is necessary. The camera rack will also have the flow in system attached to it to allow us to introduce fresh water to the testing arena, but these plans are still under development.

Figure 1. Trough panels being supported by clamps and wooden struts while the mending process was occurring.

Figure 2. The stainless-steel fitting used as the overflow drain fixed in the hole with JB-weld.

Figure 3. Modular arena inserts held together with H connectors that span the whole length of the arena. PVC frame held to the cart with pipe clamps

San Marcos Salamander Photo Mark and Recapture

Marina Dreager (Student Conservation Association) continued to crop and standardized the orientation of photos of San Marcos salamander taken during the 2023-2024 mark and recapture effort. Photos from collection days with and without recaptured salamanders have been processed and are ready to be run through wild.ID to continue verifying this method and to begin assessing recapture rates, movement, and population estimates.

Peck's Cave Amphipod Offspring Exclusion

Marisol Farias (Student Conservation Association) has inventoried PCA boxes every 10 days to isolate gravid females into passive exclusion chambers and individual boxes for manual neonate separation removal once neonates have developed. Farias has 10 passive exclusion tubes set up on a flowthrough rack (Figure 4). Farias checked the tubes every two weeks and monitored the

state of neonate development and if the gravid females had dropped their neonates. Neonates develop and release in 1-2 months. This passive exclusion trial just hit its one-month mark. Neonates are still developing and should begin releasing soon. Farias successfully manually isolated one neonate from a gravid female. That neonate was placed in its own box (black and yellow box, Figure 4) and is being monitored.

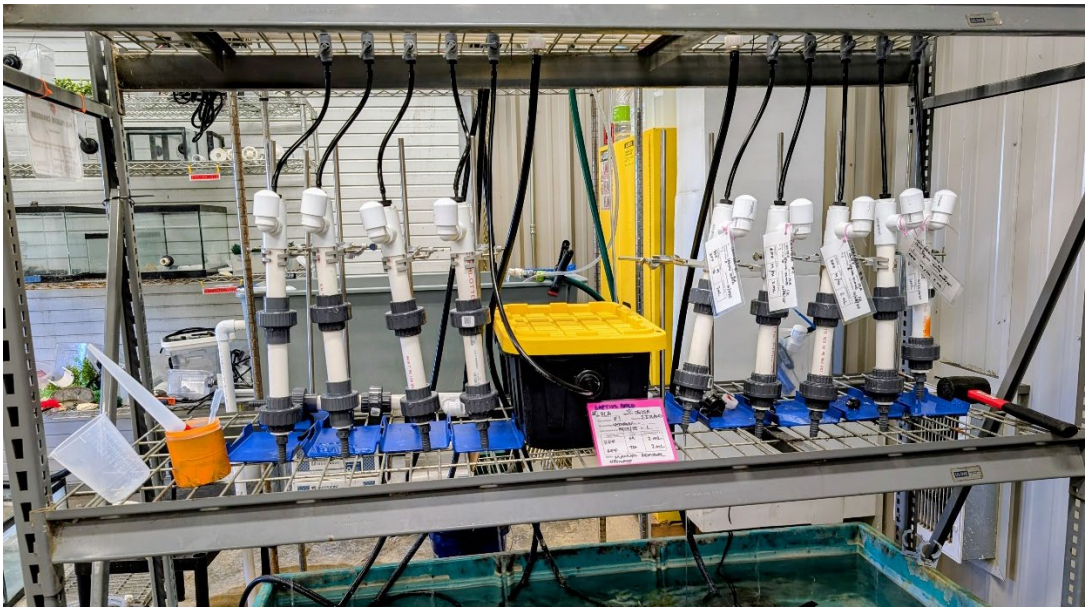

Figure 4. Rack holding 10 passive exclusion set ups (two different trials) and one box containing a First Generation (F1) captive bred PCA that was manually removed from a gravid female.

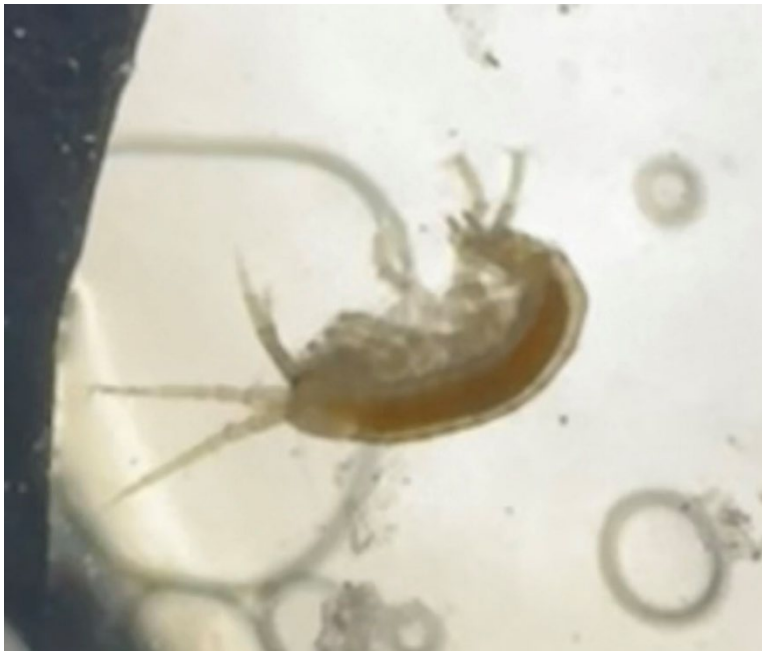

Figure 5. F1 PCA neonate that was manually removed from a gravid female.

Tagging Aquatic Invertebrates
No updates to report.

Genetic Assessment of San Marcos Salamanders
This project is currently on hold as we attempt to negotiate with Texas State University on the overhead rate.

Task 4 Species Reintroduction
No work has been completed for this task area.

Task 5 Reporting
EARP Staff contributed to the monthly report.

Task 6 Meetings and Presentations
EARP staff met weekly to discuss Husbandry needs, Collection plans, and Research updates.

November 2025 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear, Kallan Padget and Dr. Katie Bockrath

With contributions from

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Task 1 Refugia Operations

Species Collection

Collections last month were focused on gathering San Marcos fountain darters and Texas wild-rice for both refuge facilities. EARP staff successfully gathered quality rice plants at Eastern Spillway, Ramon Lucio Park and Pyramid Park. In total, 140 plants were collected and will be incorporated into the refuge throughout the month of December. Towards the end of last month staff switched to collecting fountain darters. Collection numbers were low at Eastern Spillway and Spring Lake. At Eastern Spillway, 76 fountain darters were collected in October and 8 collected in November. At Spring Lake, 76 fountain darters were collected in November. Collection numbers are higher downstream the dam (225 collected in October and 124 collected in November). In total 455 darters were collected between October and November. All darters are now being held at SMARC temporarily while they adjust to their new captive environment before transferring to UNFH. Holding fish at SMARC before transferring to UNFH has minimized post collection mortality significantly in comparison to past collection years.

Husbandry

SMARC

For a majority of November, SMARC has held all 593 collected San Marcos fountain darters in quarantine. West focuses a large chunk of his time on husbandry for the 1230 fountain darters that are now at San Marcos. West and Farias worked on incorporating 128 PCA into the refuge population. SMARC's total number of PCA is now 243, bringing the program up to the Refugia stock goal of 500 PCA between both refuges. West also incorporated 9 San Marcos Salamanders that were collected from the Diversion net. These numbers help to keep the refuge population at SMARC within its target number. West has stabilized Comal fountain darter numbers through use of salt treatments and improved husbandry. More thorough scrubbing and water exchanges are now being performed on all fish tanks and aquaria. This has resulted in a decline in mortality over the last 3 months.

UNFH

Uvalde incorporations were plentiful this month. Staff incorporated 498 Comal fountain darters. These fish finished their 60-day quarantine period that started in September. This incorporation puts the total number of darters between both refuge facilities at 1,136. It is a major recovery of the refuge population considering the loss of darters in May due to the gasification event and was possible due to the late spring salvage response by EARP staff. Alvear also incorporated 72 beetles this month which helps achieve the programs target numbers for the end of the year for both Comal Springs dryopid and Comal Springs riffle beetles. Padget and Alvear incorporated 166 Pecks cave amphipods this month. Survival was 100 percent, and no amphipods were lost during this quarantine period. Refuge numbers for amphipods are now within the programs target number for the end of the year. Valenzuela-Charro and Padget also incorporated 60 San Marcos

Salamanders into the refuge from October collections. This was the end of an effort that spanned the months of September and October to collect 150 San Marcos salamanders. Staff finished by collecting 158 and have successfully met the programs target number for the end of the year.

Donelon, Padget and Valenzuela-Charro continued building systems this month. Work was completed on a new aquarium rack system that is now accommodating 215 San Marcos fountain darters while they pass their 60-day quarantine period before refuge incorporation. Donelon, Alvear and Valenzuela-Charro are also working to finish new filters for refuge invertebrate boxes that will improve water exchange and reduce waste buildup. Padget finished restructuring and plumbing on three new refuge systems. Tanks 3,4,5 are now done and ready for salamander transfers this winter. As staff have built more of these systems there has been a steady decline in mortality. Use of biofiltration and UV treatment has improved water quality, and the systems new water exchange system keeps recirculating water fresher and cleaner than it was before.

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and for November 2025. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month. "WS" indicates Wild Stock or non-captive bred.

Species	Collections	Incorporated	Reintroductions	SMARC Transfer to UNFH	Mortalities	SMARC WS Census	UNFH WS Census
Fountain darter: San Marcos	593	0	-	215	5	226	110
Fountain darter: Comal	0	498	500	0	15	638	498
Comal Springs riffle beetle	68	71	-	0	43	98	92
Comal Springs dryopid beetle	0	1	-	0	64	4	25
Peck's cave amphipod	0	294	-	0	0	243	353
Edwards Aquifer diving beetle	-	-	-	0	-	-	-
Texas troglobitic water slater	-	-	-	0	-	-	-
Texas blind salamander	3	0	-	0	0	94	55
San Marcos salamander	0	69			9	259	251
Comal Springs salamander	0	0			1	74	1
Texas wild-rice	139	0			15	160	131

Task 2 Research

Developing tools to determine thermal tolerances and preferences of current and future covered HCP species

Major progress has been made in the development of the TBAD. The troughs acrylic panels have been mended together (*Figure 1*) and the hole for the Standpipe/overflow drain has been cut and the hardware for the drain has been attached to the back wall (*Figure 2*). The removeable bulkhead wall and the gaskets used to seal the bulkhead have also been constructed and tested, resulting in a perfectly waterproof end of the trough (*Figure 3*). The modular arena inserts have also been mended together (*Figure 4*), and the bubble chimneys have been attached to the arena floor and to the air pump (circulating the water in the trough). The last thing need to complete the arena inserts to make them organism ready is to screen in all the openings.

The shroud frame has been finished (*Figure 5*) and the light blocking cover is being constructed. The cover is made of thick canvas material and will have a double layered opening under an awning giving room for researchers to stand under. A sliding shelf/board has also been attached to the cart to house the cameras and lights, for testing and recording purposes, has also been completed (*Figure 6*). The back half of the camera rack are PVC sliders that allow the 2 x 4 board to extend over the trough or retract back under the top shelf when access to the inside and top of the trough is necessary. The camera rack will also have the flow in system attached to it to allow us to introduce fresh water to the testing arena, but these plans are still under development.

The preliminary wet testing with the conduits in water will begin soon. The conduits will be run under the same conditions as the dry tests to determine how the device functions when it is submerged in water.

Dr. Huffman and Enzo Silvagni worked on writing the draft final report.

Figure 1. Trough panels being supported by clamps and wooden struts while the mending process was occurring.

Figure 2. Figure 2: Left photo: The hole we drilled in an 8.75-foot-long sheet of acrylic that the overflow drain hardware will go through. Right photo: The stainless-steel fitting used as the overflow drain fixed in the hole with JB-weld.

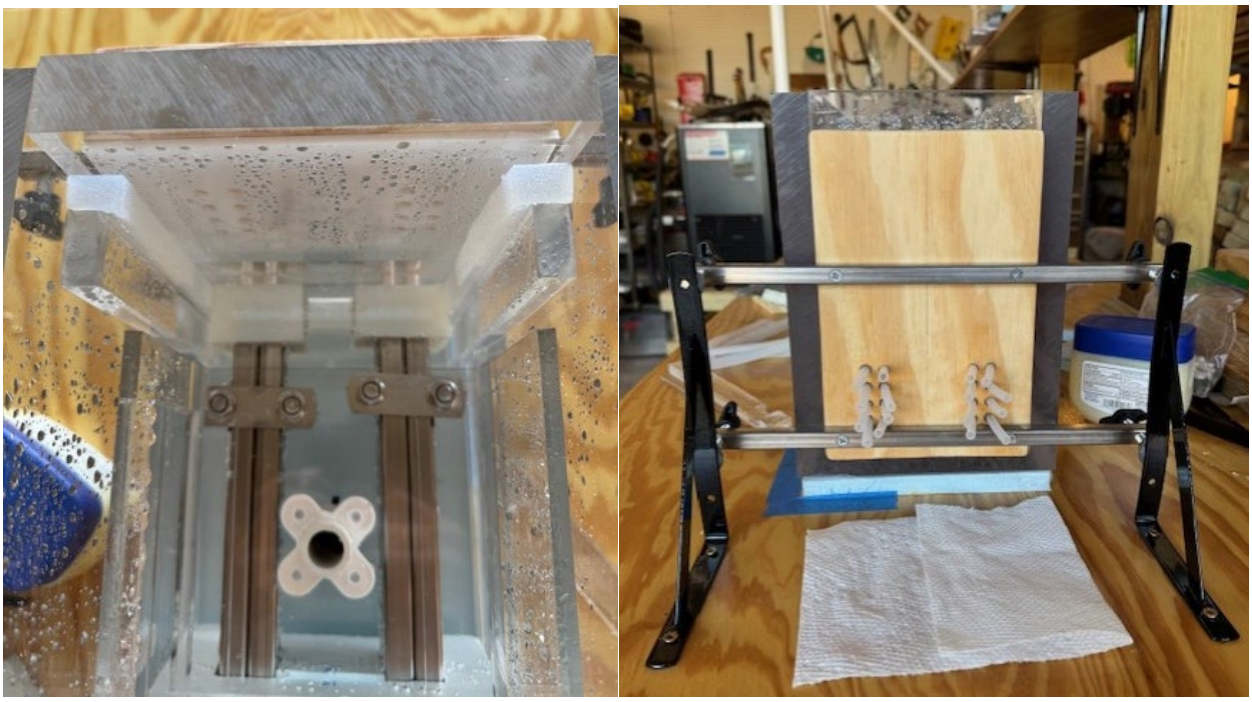

Figure 3. Left photo: The inside of the removable bulkhead wall with the gaskets squished against the acrylic strips mended to the outer walls of the trough. Right photo: The outside of the removable bulkhead with the compression hardware exposed.

Figure 4. Modular arena inserts held together with H connectors that span the whole length of the arena.

Figure 5. PVC frame held to the cart with pipe clamps.

Figure 6. Side view of the front of the camera rack over the trough.

San Marcos Salamander Photo Mark and Recapture

Dr. Bockrath ran a subset of the salamander photos through Wild.ID. 879 photos across three sampling locations (Hotel, Diversion and Eastern Spillway) from May 9 – June 14, 2023 and March 13, 2024 were analyzed. During these months there were 21 recaptures of p-chip tagged salamanders. Wild.ID returned 27 matched photos, showing that more recaptures are recorded using photos. The recapture rate and salamander movement between sites are being assessed.

Peck's Cave Amphipod Offspring Exclusion

Marisol Farias (Student Conservation Association, SCA) conducted regular inventories of PCA boxes and moved gravid females to passive exclusion housings. While there were many gravid females in September and October, the number of gravid females has declined in November, suggesting reproduction may slow down over the winter months. Farias also checked the passive exclusion housings for neonates and mortalities. There was only a single mortality over the two trials. No neonates have been released yet, but egg development has been observed. Gravid females are carrying between 5-10 eggs each. Farias monitored the single neonate that was manually removed from a gravid female. The neonate is growing and doing well post removal. The female unfortunately died two weeks post removal. Farias wrote the protocol for manual removal of neonates from gravid females and post removal husbandry.

Tagging Aquatic Invertebrates

Dr. Shannon Brewer and Brian De La Torre worked on writing the final report.

Genetic Assessment of San Marcos Salamanders

This project is currently on hold as we attempt to negotiate with Texas State University on the overhead rate. A meeting to discuss the overhead rate and the CESU agreement was set for December 9. This project was moved to the 2026 work plan.

Task 4 Species Reintroduction

Dr. Bockrath, West and Farias re-introduced 500 Comal fountain darters back into the Comal River on November 11. This is a successful conclusion to a late spring salvage event that was triggered by dramatic reductions in flow within the Comal River. Fountain darters were transported in bags with aquifer water and oxygen. The bags were placed in coolers to maintain water temperatures. The fountain darters were acclimated to the Comal River by adding river water to the coolers to allow the water temperatures in the bag to equalize with the water temperatures of the river. Comal River water was then added to each bag to equalize the water chemistry prior to releasing the fountain darters to the Comal River. Kristy Smith (EAA) and Megan Bean (USFWS) assisted with the reintroduction. This salvage has left the program with its target goal of 1000 Comal fountain darters and the ability to release 500 more back into the Comal River.

Figure 7. EAA and USFWS staff reintroducing 500 fountain darters to the Comal River. Shown from right to left: Kristy Smith (EAA), Dr. Katie Bockrath (USFWS), Braden West (USFWS), Marisol Farias (SCA) and Megan Bean (USFWS).

Figure 8. Dr. Katie Bockrath and Braden West acclimating fountain darters before releasing them to the Comal River.

Figure 9. Braden West gently releasing fountain darters to the Comal River.

Task 5 Reporting

EARP Staff contributed to the monthly report.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss Husbandry needs, Collection plans, and Research updates.

December 2025 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Braden West, Dominique Alvear, Kallan Padget and Dr. Katie Bockrath

With contributions from

Matthew (Tanner) Donelon

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

Uvalde National Fish Hatchery
754 County Rd 203
Uvalde, Texas 78001
Phone: 830-278-241

Task 1 Refugia Operations

Species Collection

Refugia Staff focused on collecting San Marcos fountain darters and Texas wild-rice this month. Noel Valenzuela-Charro, Braden West and Kallan Padget collected 130 darters from the Upper and Middle San Marcos River this month. The fish were concentrated in localized pockets near the banks in thick vegetation and leaf litter. Compared to years past, staff were able to collect less darters this year. Collection efforts were high, but fountain darter numbers were consistently low. Valenzuela-Charro and Padget also collected 7-Texas wild-rice plants last month. These plants bring UNFH very close to reaching their 215-plant goal.

Husbandry

SMARC

Work at SMARC this month focused on cleaning and maintaining aquarium habitat. Padget and West observed an increase in mortality with San Marcos Salamanders towards the middle of the month and responded by deep cleaning tanks regularly (Figure 1). West is also performing water exchanges on tanks to ensure fresh water is provided to the salamanders each day (Figure 2). As a precautionary action, this practice has been extended to include all fountain darter and Texas blind salamander tanks. West began assembling new systems in the refugia. The purpose of these new systems is to utilize partial recirculation to decrease the risk of gasification events, reduce water discharge from the refugia and give staff the ability to better control other water quality parameters such as utilizing CO2 to counter water hardness.

UNFH

Valenzuela-Charro's last day of work was on December 19th. He was a very important team member to the refuge this year and we thank him for his time. Padget, Alvear, Donelon and Valenzuela-Charro incorporated 74-plants into the Refugia, bringing the total number of plants in the Refugia to 205. Alvear has been working to disinfect and sterilize the invertebrate rack systems to accommodate the increase in invertebrate boxes that were added in the second half of the year. Rack 2 was restored to working capacity and will now accommodate Comal Springs riffle beetle and Dryopid beetle refugia populations. Donelon and Padget continue to deep clean large tanks in both the refugia and quarantine buildings. UNFH continue to incorporate improved husbandry techniques resulting in overall improvement in survival of all species (Figure 3).

Figure 1. Dirty tank vs clean tank. Right shows calcium build up prior to cleaning and a water change. Left shows the tank post cleaning.

Figure 2. Braden West cleans a fountain darter Tank.

Figure 3. Multiple fountain darters enjoying custom aquarium habitat.

Table 1. New collections and total wild stock census of Edwards Aquifer organisms taken to facilities for refugia by species and for December 2025. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month. "WS" indicates Wild Stock or non-captive bred.

Species	Collections	Incorporated	SMARC Transfer to UNFH	Mortalities	SMARC WS Census	UNFH WS Census
Fountain darter: San Marcos	130	0	-	4	224	108
Fountain darter: Comal	0	0	-	13	634	489
Comal Springs riffle beetle	68	0	-	0	98	92
Comal Springs dryopid beetle	0	0	-	0	4	25
Peck's cave amphipod	0	0	-	0	243	353
Edwards Aquifer diving beetle	-	-	-	-	-	-
Texas troglobitic water slater	-	-	-	-	-	-
Texas blind salamander	0	0	-	0	97	55
San Marcos salamander	0	0	-	19	242	249
Comal Springs salamander	0	0	-	0	74	1
Texas wild-rice	7	74	-	0	160	205

Task 2 Research

Developing tools to determine thermal tolerances and preferences of current and future covered HCP species

Dr. Huffman and Enzo Silvagni submitted the draft final report to Dr. Bockrath. Dr. Bockrath has reviewed the report and submitted to the EAA for review.

San Marcos Salamander Photo Mark and Recapture

Dr. Bockrath drafted the report and submitted the report to the EAA for review.

Peck's Cave Amphipod Offspring Exclusion

Dr. Bockrath drafted the report and submitted the report to the EAA for review.

Tagging Aquatic Invertebrates

Dr. Shannon Brewer and Brian De La Torre submitted the final report to Dr. Bockrath. Dr. Bockrath has reviewed the report and submitted to the EAA for review.

Genetic Assessment of San Marcos Salamanders

This project was moved to the 2026 work plan.

Task 4 Species Reintroduction

No reintroduction activities occurred this month.

Task 5 Reporting

EARP Staff contributed to the monthly report. Dr. Bockrath drafted research reported and submitted them to the EAA for review. Dr. Bockrath reviewed partnered research reports and submitted them to the EAA for review.

Task 6 Meetings and Presentations

EARP staff met weekly to discuss Husbandry needs, Collection plans, and Research updates.

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/1093 June 12, 2025

Memorandum

To: Kathie Bockrath, PhD, San Marcos Aquatic Resource Center

From: Sara Hamilton, Southwestern Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the Comal River, Texas (Case Number 25-29).

On March 7th, 2025, Southwestern Fish Health Unit (SFHU) staff received 62 fountain darters (*Etheostoma fonticola*) collected from the Comal River (GNIS ID: 1372140). These fish were collected using a purse seine by staff from the San Marcos ARC and shipped live to the SFHU laboratory. The location was recorded at latitude 29.7175° and longitude -98.1318° Comal County, Texas, and river water temperature at the time of collection was 22°C

Assays and examinations for the sampled fish included virology and parasitology. Viral screening included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected in the standard cell lines. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC for the Edwards Aquifer Refugia Program. Screening for *Centrocestus formosanus* was conducted by examination of all left side gills from 10 fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2020 edition) and standard SFHU protocols.

Results: *Centrocestus formosanus* was found in all 10 fish examined, while monogenean parasites were detected in 4 out of 10 fish. A detailed parasite data sheet, listing the exact number and type of parasites identified in each fish, is included at the end of this memo. No viruses were detected through cell culture testing.

If you have any questions about test methodology and results, or if the SFHU can be of additional assistance, feel free to reach out to our staff. Please refer to case history number 25-29 for all follow up correspondence.

cc: Huseyin Kucuktas, PhD, Southwestern Fish Health Unit
Scott Walker, PhD, Uvalde National Fish Hatchery

FOD Parasite Data Sheet - Form P-03

Case History No. 25-29

Date examined: 5/7/2025 Date Collected: 4/29/2025

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)	249	269	267	198	226	194	204	230	219	243
Total Length (mm)	32	29	29	28	31	29	29	31	31	31

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

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

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

Examiner signature MB, IJK 5/7/2025

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/1094 June 12th, 2025

Memorandum

To: Kathie Bockrath, PhD, San Marcos Aquatic Resource Center

From: Sara Hamilton, 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 25-30).

On May 7th, 2025, Southwestern Fish Health Unit (SFHU) staff received 56 fountain darters (*Etheostoma fonticola*) collected from the San Marcos River (GNIS ID: 1375972), Texas. These fish were collected by purse seine by staff at the San Marcos ARC and shipped live to the SFHU laboratory for fish health testing. San Marcos ARC staff recorded collection of fountain darters at latitude 29.8766° and longitude -97.9321° in Hayes County, Texas. The river water temperature at the time of collection was 22°C.
Assays and examinations for the sampled fish included virology and parasitology. Viral screening included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected in the standard cell lines. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC for the Edwards Aquifer Refugia Program. Screening for *Centrocestus formosanus* was conducted by examination of all left side gills from 10 fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2020 edition) and standard SFHU protocols.

Results: *Centrocestus formosanus* was detected in 6 out of 10 fish examined, while monogenean parasites were found in 8 of the 10 fish. A detailed parasite data sheet, outlining the number and types of parasites identified in each fish, is included at the end of this memo. No viruses were detected through cell culture testing.

If you have any questions about test methodology and results, or if the SFHU can be of additional assistance, feel free to reach out to our staff. Please refer to case history number 25-30 for all follow up correspondence.

cc: Huseyin Kucuktas, PhD, Southwestern Fish Health Unit
Scott Walker, PhD, Uvalde National Fish Hatchery

FOD Parasite Data Sheet - Form P-03

Case History No. 25-30

Date examined: 5/7/2025 Date Collected: 4/22/2025

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)	100	130	198	288	180	260	250	184	265	218
Total Length (mm)	15	25	30	33	29	34	37	30	32	30

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

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

Examiner signature HK, MB 5/7/2025

Revised on 9/20/2017