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

ANNUAL REPORT 2024

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 eighth annual report of the contract covering the calendar year of 2024. The eighth year of the contract focused on maintaining the existing standing stocks and conducting research while facing a significant a drought and undergoing staff changes.

The major objectives of the USFWS Refugia Program are to 1) develop and provide 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 2024. 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 2024; all other covered species were collected in 2024.

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 2024 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). 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	0/0	0/0	0/0	26/63	0/0	0/0	0/0	0/0	0/0
FEB	31/9	0/0	5/0	0/0	0/0	5/0	0/0	2/0	0/0
MAR	6/15	0/0	25/30	166/253	11/184	3/0	0/0	15/0	10/10
APR	0/0	0/0	0/0	477/0	294/0	4/0	0/0	18/0	14/15
MAY	0/12	0/0	89/0	0/0	0/0	12/0	0/0	177/118	0/0
JUN	44/0	5/0	82/0	0/0	0/0	6/0	27/0	27/0	30/10
JUL	67/40	1/12	0/85	0/301	0/0	2/0	0/20	29/0	0/0
AUG	231/0	20/0	6/0	0/0	0/231	4/0	20/0	27/0	0/0
SEP	139/0	13/0	16/68	0/0	0/0	0/0	11/0	17/0	0/0
OCT	230/0	16/0	9/0	145/0	332/0	0/0	0/0	55/0	0/0
NOV	166/0	23/0	2/0	0/0	0/0	3/0	0/0	0/0	0/0
DEC	51/0	8/0	52/48	0/0	0/0	4/0	0/0	0/0	20/15

RESEARCH

We conducted eight research projects in 2024, several with external partners. These research projects focused on species covered by the Edwards Aquifer Habitat Conservation Plan, including three invertebrates (Comal Springs riffle beetle, Comal Springs dryopid beetle, and Peck's cave amphipod), and the San Marcos and Texas blind salamanders. Research areas included genetic assessments of wild populations, improved collections and captive propagation, 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 concluded a mark-recapture study examining the recapture rate, movement, and demographics of wild San Marcos salamanders. Tagging, using p-Chip transponder tags, and recaptures were conducted at three sites across Spring Lake and the San Marcos River. No movement between locations was observed and there was no significant difference in sex ratio among locations. There is a significant difference in snout to vent length (SVL) where the largest salamanders are located at Eastern Spillway and the smallest are at Hotel Springs. The final report for this study is included in Appendix B.

BIO-WEST led an effort to determine better methods of collecting and housing Comal Springs dryopid beetles. Experimental questions examined the housing preferences of dryopid beetles in captivity. Dryopid beetles prefer wood over other materials and did not show a preference for different species of tree leaves provided as a biofilm food source. Field collection efforts tested two wood lures and compared the performance of these wood lures to currently used cotton lures. Wood disks outperformed wood stakes and cotton lures and was a reliable method for collecting dryopid beetles. The final report for this research is in Appendix C.

A study developing tagging methodology for invertebrates was led by Dr. Shannon Brewer of the U.S. Geological Survey, Alabama Cooperative Fish and Wildlife Research Unit. 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 in 2024. 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 and reporting are planned for 2025. An interim report for this study is included in Appendix D.

USFWS staff, Dr. Kate Bell and Dr. Chris Nice (Texas State University) completed a genetic assessment of the Peck's cave amphipod (PCA) in the Comal Springs system. Amphipods were collected as bycatch during Comal Springs riffle beetle collections across Spring Runs 1-3, Spring Island and Western Shore in the Comal Springs system. Collections were carried out from 2023-2024 and genetic analysis was concluded in 2024. There is no evidence of isolation or restrictions to gene flow across sampling locations. Genetic diversity remains relatively high and PCA populations do not appear to be significantly impacted by repeated droughts and low flow conditions. The final report for this research is in Appendix E.

Ruben Tovar and Dr. David Hillis of the University of Texas Austin led a project using comparative gene expression in San Marcos and Texas blind salamanders to identify potential reproductive triggers in captivity. Salamanders were preserved in a fixative allowing for molecular work and microCT scanning to create a transcriptome and developmental time series for each species. There were significant gene expression differences between gonad tissues and other tissues. There is also significant difference in gene expression profiles between reproductively active and inactive individuals. Seven genes were identified to be highly correlated to reproductive state. These genes are not associated with other tissue types, suggesting there is no obvious correlation to sight, smell, or other sensory stimuli that may induce reproduction. The final report for this project is available in Appendix F.

A genetic assessment of the CSRB in Landa Lake concluded in 2024 in partnership with BIO-WEST. BIO-WEST set lures at 80 biomonitoring sites at four seasonal time points to gather data for an occupancy study. A portion of the adult CSRB observed on each lure was retained for genetic assessment. All larval CSRB were retained for genetic assessment. There is significant genetic structure between Spring Runs 1 and 3 and Western Shore and Spring Island, where Spring Runs 1 and 3 share genetic diversity, Western Shore and Spring Island share genetic diversity but the two groups of locations do not share genetic diversity. Additionally, Spring Runs 1 and have very low genetic diversity relative to Western Shore and Spring Island; a signature indicative of significant reductions in population size at Spring Runs 1 and 3, likely due to repeated low flow conditions and loss of habitat. The final report for this research is included in Appendix G.

A genetic assessment of San Marcos salamanders was started in 2024. Tail clips were collected from the 453 San Marcos salamanders collected for p-Chip tagging during the Mark and Recapture study. A RADSeq library was generated from the 453 tail clips and the library was sequenced to produced thousands of variable single nucleotide polymorphisms (SNPs), or genetic data points. Thus far, tail clips were collected from 29 F1 captive bred San Marcos salamanders at SMARC for additional genetic analysis. Sequencing of the captive populations (wild stock and captive breed) and data analysis of all data will occur in 2025. The interim report for this research is included in Appendix H.

A genetic assessment of Texas blind salamanders was started in 2024. The EARP has the largest captive population of Texas blind salamanders and regularly produces captive breed offspring. It is important to determine the diversity of wild caught individuals and their Fx offspring. Thus far, tail clips were taken from 68 wild stock TBS and 4 Fx captive bred TBS. To assess wild populations, tail clips from TBS encountered in traps but not retained for the refugia will also be included in the study. Collection locations include Purgatory Natural Area wells (Primer's Fissure, Johnson's Well and Rattlesnake Cave. Sequencing and Data analysis will occur in 2025. The interim report is located in Appendix I.

BUDGET

The Aquifer Refugia Program did not exceed the allocated budget defined in the 2024 Refugia Work Plan previously approved by the EAA Board of Directors. The Refugia Program spent approximately $1,323,005 in 2024. Research activities accounted for $396,994, and approximately $868,808 was spent on collections, husbandry, and propagation. Approximately $57,203 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 2021 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 Table 2 for list of the 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 with back-up populations in 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 (Table 2), 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*	Petitioned
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 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 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 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 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 apprised 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. David Britton with the assistance of the Deputy Center Director, Dr. Jennifer Howeth. Dr. Scott Walker is the Project Leader at the Uvalde National Fish Hatchery. Adam Daw, based at the UNFH, led the Refugia Husbandry and Collections team for both facilities in 2024. Dr. Katie Bockrath, the Refugia Research Lead, serves as the point of contact for the Edwards Aquifer Refugia Program, coordinates all research activities, project plans, reporting and budgets in 2024. The Edwards Aquifer Refugia Program underwent staff changes in 2024. The program welcomed four new employees, Jonathan Donahey and Heidi Meador at UNFH, along with Shawn Moore and Richelle Jackson at the SMARC. Table 3 USFWS Refugia Program Staff

San Marcos Aquatic Resources Center	
Dr. David Britton	Center Director
Dr. Jennifer Howeth	Deputy Center Director
Dr. Katie Bockrath	Refugia Research Team Lead
Desiree Moore/Vacant	Research Biologist
Braden West	Refugia Biologist
Shawn Moore	Biological Science Technician
Richelle Jackson	Biological Science Technician
Uvalde National Fish Hatchery	
Scott Walker	Uvalde National Fish Hatchery Project Leader
Adam Daw/ Kallan Padget	Refugia Husbandry and Collections Team Lead
Dominique Alvear	Refugia Biologist
Heidi Meador/ Matthew Donelon	Biological Science Technician
Jonathan Donahey/ Vacant	Biological Science Technician

Day-to-day operations were managed by two Lead Biologists providing supervision, mentorship, and training to the Fish Biologist and Biological Technicians (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 related to the contract. Leads 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 input of supporting staff, prepared the annual report, annual work plans, and monthly reports, developed research activities and reports, developed and managed the Refugia Program budget, and established and oversaw outside research agreements.

Adam Daw, Refugia Husbandry and Collections Lead, coordinated the husbandry and collections across stations. Daw, with input from supporting staff, prepared the annual report, annual work plans, and monthly reports, developed and managed the Refugia Program budget, oversaw development and implementation of husbandry standard operating procedures, designed and oversaw construction of refugia system improvements and coordinated collection activities.

Desiree Moore, Research Biologist, worked with Dr. Bockrath to design and implement research projects across stations. D. Moore contributed to the annual report and monthly reports, developed research activities and reports, contributed to annual work plans, husbandry, and collections, and coordinated with external research partners.

Dominique Alvear and Braden West, Refugia Biologists, worked with Daw 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.

Jonathan Donahey, Heidi Meador, Matthew Donelon, Shawn Moore, and Richelle Jackson, Biological Science Technicians, carried out collections and daily husbandry duties. They constructed, maintained, and monitored holding systems for refugia species. The technicians performed daily data recording duties, 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.

BUILDING CONSTRUCTION

Staff made major improvements to the EARP building at the SMARC. We completed the Refugia room automatic bypass valve in January, and the Quarantine room valve in March. Staff made slight modifications to the controller for both valves to read the same input and thus operate simultaneously. The addition of automatic bypass valves in both rooms in the EARP building severely minimized the potential for gas-saturated well water reaching either refugia or quarantine tanks. Edwards Aquifer Refugia Program staff completed the purchase for 15 additional Walchem Intuition 9 controllers in June. The controllers were distributed between the SMARC and UNFH as needed.

Staff traveled between the UNFH and the SMARC to ensure homogeneity in system design, allowing for greater parts and knowledge interchangeability between the facilities. Quarantine and hospital racks at both facilities were retrofitted with air lines, allowing for aeration, and further decreasing the need for chilled well water. The addition of aeration added an additional layer of protection for animals if the system pump failed. The second invertebrate rack at the SMARC was fitted for system parameter monitoring via controller. Standard rack systems were modified to accept controllers, allowing system parameter monitoring capabilities. CO2 injection systems were constructed at the SMARC to better control water pH in recirculating systems. UNFH staff connected the existing CO2 system to all invertebrate systems in the UNFH invertebrate room, greatly stabilizing the pH of recirculating water. Uvalde National Fish Hatchery EARP staff completed construction and plumbing design on three new recirculating rack systems in the UNFH quarantine building. Staff continued refreshing older chillers as they failed this year, replacing three broken chillers with new Raypak models. Staff at the UNFH completed four additional equipment controller boxes, installing one at the SMARC and three on completed systems in the UNFH refugia and invertebrate room.
Staff at the SMARC worked closely with USFWS Information Resources and Technology Management (IRTM) to construct a local network. The dedicated local network was constructed

to allow every controller to be hard-wired to the internet, increasing the reliability of system parameter notifications from the controllers.

New storage and work benches were added to the SMARC Refugia room to better organize system controller equipment and provide a clean, dedicated space for construction of additional controllers. Staff at the SMARC also constructed two biosecurity curtains in the EARP quarantine building dissecting the space into “Low”, “Medium”, and “High” biosecurity areas with increasing levels of biosecurity measures such as footbaths and glove stations. Newly erected physical barriers reduced the chance of disease transmission via splashing and aerosolization.

COVERED SPECIES ANALYSIS

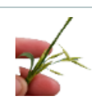
Collections of the Covered Species continued to work toward standing stock targets as outlined in the Contract and the 2024 EA Refugia Work Plan (Tables 3 and 4). For many species, the acclimation to captive systems can be achieved relatively quickly; this is particularly true for Texas wild rice, San 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 5) 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 2. Texas blind salamander

Table 3. Number of organisms incorporated in the SMARC Refugia Standing Stock in 2024, 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* 	424	288	56%
Fountain darter – Comal Springs *Etheostoma fonticola* 	494	193	30%
Comal Springs riffle beetle *Heterelmis comalensis* 	580	544	99%
Comal Springs dryopid beetle *Stygoparnus comalensis* 	50	45	90%
Peck's cave amphipod *Stygobromus pecki* 	170	110	35%
Edwards Aquifer diving beetle *Haideoporus texanus* 	0	0	-
Texas troglobitic water slater *Lirceolus smithii* 	0	0	-
Texas blind salamander *Eurycea rathbuni* 	16	101	97%
San Marcos salamander *Eurycea nana* 	159	224	70%
Comal Springs salamander *Eurycea* ***pterophila*** 	44	81	79%
Texas wild rice *Zizania texana* 	62	176	73%

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 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 4. Number of organisms incorporated in the UNFH Refugia Standing Stock in 2024, 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*		246	333	61%
Fountain darter – Comal Springs *Etheostoma fonticola*		200	439	77%
Comal Springs riffle beetle *Heterelmis comalensis*		20	36	100%
Comal Springs dryopid beetle *Stygoparnus comalensis*		23	30	97%
Peck's cave amphipod *Stygobromus pecki*		93	145	66%
Edwards Aquifer diving beetle *Haideoporus texanus*		0	0	--
Texas troglobitic water slater *Lirceolus smithii*		0	0	--
Texas blind salamander *Eurycea rathbuni*		0	58	94%
San Marcos salamander *Eurycea nana*		84	140	56%
Comal Springs salamander *Eurycea* ***pterophila***		8	73	42%
Texas wild rice *Zizania texana*		37	126	56%

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 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. Updated table showing 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 exists. 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	2	1
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

FOUNTAIN DARTER (*ETHEOSTOMA FONTICOLA*), ENDANGERED

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 were slightly below target numbers in 2024. In the summer, due to a drought, the Comal River spring flow conditions reached critically low levels. In consultation with the EAA and USFWS staff, the refugia started collecting Comal Springs fountain darters to increase refugia stocks. Numbers incorporated, end of the year census, and survival rates can be found in Table 6.

Table 6. Fountain darter refugia population figures

		Beginning of Year Census	Incorporated 2024[1]	End of Year Census	Target Goal 2024 Work Plan	Percent Survival [2]
San Marcos River	SMARC	89	424	288	500	56
	UNFH	300	246	333	500	61
Comal River	SMARC	149	494	193	500*	30
	UNFH	371	200	439	500*	77

* Prior to the Summer of 2022 collecting Comal Springs fountain darters was postponed until we have a better understanding of their mortality rates.

[1]The number of 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 2024, the collection of fountain darters was increased due to the low spring flows of both the Comal and San Marcos Rivers. Refugia staff conducted collections for San Marcos River and Comal River Fountain darters in the months of January, March, April July, August, and October. A total of 1052 San Marcos River Fountain darters and 1431 Comal River Fountain darters were collected. Of the 1052 San Marcos River Fountain darters, 1028 were retained, with 613 transferred to the SMARC and 415 to the UNFH for incorporation into refugia. Of the 1431

Comal River Fountain darters, 1423 were retained, with 807 transferred to the SMARC and 616 to the UNFH for incorporation into refugia.

Refugia staff also collaborated with BIO-WEST to collect Fountain Darters during biomonitoring efforts in April. Refugia staff received 291 San Marcos River Fountain darters and 477 Comal River Fountain darters. These collection numbers are included in the collections described above and fish were transferred to the SMARC for incorporation into the refugia.

10% of fish caught from both the Comal and San Marcos Rivers were sent to the USFWS Southwestern Fish Health Unit (SFHU) in Dexter, New Mexico in March. Subsets of Fountain darters collected by EARP staff in January, August, and October were sent directly to SFHU for parasite enumeration and viral analysis. In total, 69 Comal River Fountain darters and 72 San Marcos River Fountain darters were submitted.

QUARANTINE PROCEDURES

Fountain darters 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. A standard fountain darter intake and quarantine procedure was used at both facilities. To minimize stress, temperature acclimation progressed at a rate of one degree Celsius per hour. The fish were treated for external parasites in an aerated static bath solution of formalin at 170 ppm for 50 to 60 minutes. Darters were then transferred to clean flow-through quarantine tanks. Fish sent to the USFWS SFHU for routine parasitology and health screening were not given a formalin dip and were shipped to 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, 15 mg/L Chloramine-T, or 170 uL/L formalin 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 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 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 River fountain darters were collected again in larger numbers because of low spring flow. Survival rates of Comal River fountain darters were highly variable during their 30-day quarantine period. 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. Although survival rates of fountain darters from both rivers showed slight improvement over their 2023 levels, the 2024 survival rates for incorporated fountain darters remained relatively low. Survival percentages in the refugia at the SMARC was 56% for the San Marcos River population and 30% for the Comal River population. In previous years the San Marcos populations are relatively healthy when brought into quarantine. In 2024 necropsies reviled parasites in a majority of the mortalities. Some parasitic effects become more severe in rising water temperatures (McDonald et. al 2007). With high observed parasite load, coupled with continued exceptional drought conditions stressors, it's likely the San Marcos fountain darters arrived at the Refugia in already suboptimal condition. At the UNFH, the survival rate was 61% for the incorporated San Marcos population and 77% for the Comal River population.

MAINTENANCE OF SYSTEMS

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, 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

There were limited efforts to produce captive offspring of either San Marcos River or Comal Springs fountain darters at either facility during 2024, relying on harvesting eggs/juveniles produced in the refugia tanks. Generally, fountain darters in captivity lay eggs on the undersides of PVC and other habitat structures placed in the tanks. If offspring were not desired, staff removed the structures and disposed of the eggs. F1 generations were separated based on the river system from which their parents originated. Egg production was opportunistic and not controlled or directed by staff during periods when offspring were not needed for research or for reintroduction. A captive propagation plan is on file and available upon request for fountain darters.

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

Comal Spring riffle beetle collection by EARP staff for standing and refugia stocks occurred in February from around Spring Island. In November, BIO-WEST Inc. collected 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 7). Standing stock number will be evaluated yearly by the Comal Springs Riffle Beetle Work Group.

Table 7 Comal Springs riffle beetle refugia population figures

	Beginning of Year Census	Incorporated 2024	End of Year Census	In Quarantine End of Year	*Target Goal 2024 Work Plan	Percent Survival
SMARC	32	517	544	51	75	99
UNFH	16	20	36	-	75	100

* For 2024 the goal of 75 was not a priority due to a BIO-WEST led occupancy research project on wild population populations where Refugia collections could impact the study.

COLLECTIONS

Refugia staff collected CSRB in concert with BIO-WEST research and biomonitoring in February, March, May, June, July, September, October, November, and December. A total of 683 CSRB were retained by the EARP in 2024, of which 655 were transferred to the SMARC and 28 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. As CSRB feed predominantly on biofilm, there was 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 on a schedule 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 this 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 2024 survival rates for CSRB in refugia at the SMARC 99% and 100% at the UNFH. The extremely high survival percentages at both stations were a function of the formula used to calculate the values. Extremely high collection numbers at the end of the year coupled with extremely low stocks at the beginning of the year skewed the EARP's survival numbers very high, as most lots of CSRB were not due for their first inventory by the end of 2024. Incorporation percentages between lots of CSRB varied between 50% and 90%.

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, indicating that the recent improvements to culture systems and husbandry methods are beneficial.

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 8.

Table 8. Comal Springs dryopid beetle refugia population figures

	Beginning of Year Census	Incorporated 2024	End of Year Census	In Quarantine End of Year	Target Goal 2024 Work Plan	Percent Survival
SMARC	0	50	45	0	20	90
UNFH	8	23	30	0	20	97

COLLECTIONS

Refugia staff collected CSDB in concert with BIO-WEST research and biomonitoring in June, July, September, October, November, and December. A total of 75 CSDB were retained by the EARP in 2024, all of which were transferred to the SMARC 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 these 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.

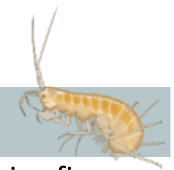
SURVIVAL RATES

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 2024 survival rates for CSDB in the refugia at the SMARC was 90% and 97% at the UNFH. Survival rates for CSDB were highly skewed due to similar reasons as CSRB. Extremely low stocks at the beginning of the year coupled with extremely high collection rates at the end of the year resulted in high survival rates by year end. Incorporation rates were consistently high across both stations, ranging between 90% and 100%.

CAPTIVE PROPAGATION

Larvae were observed in 2024 during inventories of the UNFH population.

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



Peck's cave amphipods (PCA) were collected from Comal Springs by hand during five collection events. 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 9.

Table 9 Peck's cave amphipod refugia population figures

	Beginning of Year Census	Incorporated 2024	End of Year Census	In Quarantine End of Year	Target Goal 2024 Work Plan	Percent Survival
SMARC	145	170	110	55	250	35
UNFH	203	93	145	50	250	49

COLLECTIONS

Refugia staff conducted six EARP-led collection events for PCA in 2024. These collection events took place around the Spring Island of the Comal River, New Braunfels, Texas. A total of 450 PCA were captured and transferred to the SMARC and UNFH for incorporation into the refugia. Refugia staff also collected PCA in concert with BIO-WEST research and biomonitoring activities. Refugia staff conducted these additional six collection events at Spring Runs 1, 2, and

3, the Western shore of Landa Lake, and Spring Island, of the Comal River, New Braunfels, Texas. Refugia staff transferred 50 PCA collected during these events to the SMARC and UNFH for incorporation into the refugia.

In addition to the refugia collections, during a population study in coordination with BIO-WEST, six PCA were transferred to refugia staff for incorporation into the refugia population.

QUARANTINE

Incoming PCA were quarantined in the refugia invertebrate areas in the quarantine rooms at the SMARC and UNFH. PCA 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 these 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. With completion of a dissertation at Texas State University, Dr. Parvathi Nair produced results that show PCA eat other smaller species of amphipods (Nair 2019). 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 traditional cleaning or siphoning schedule, but alternatively, were cleaned during inventory. At this 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 2024 survival rates for PCA in refugia at the SMARC was 35% and 49% at the UNFH.

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 very late-stage eggs from a gravid female and placing in a separate container to hatch. Although somewhat laborious, the eggs hatched successfully.

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

No Edwards Aquifer diving beetles were collected during 2024. 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 2024. 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 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 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 10.

Figure 3. Shawn Moore pulling up the Diversion Spring net in Spring Lake.

Table 10 Texas blind salamander refugia population figures

	Beginning of Year Census	Incorporated 2024	End of Year Census	In Quarantine End of Year	Target Goal 2024 Work Plan	Percent Survival
SMARC	88	16	101	1	250	97
UNFH	62	0	58	0	60	94

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 2024, Primer's Fissure and Johnson's Well were both sampled in February, August, and November. Only Primer's Fissure was sampled in May due to low water during the month. In total, 18 TBS were captured from Primer's Fissure, 7 new individuals and 11 recaptures, with seven (3 new individuals and 4 recaptures) retained and transferred to the SMARC for incorporation into the refugia. Nine TBS were captured from Johnson's Well, 5 new individuals and 4 recaptures, with four (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 2024, the drift net over Diversion Spring was deployed from February to November. In total, 16 TBS, all larval individuals, were captured in the net. Six individuals were retained and transferred to the SMARC for incorporation into the refugia. Of the ten animals released, seven were dead on capture. Neither Rattlesnake Cave nor Rattlesnake Well were sampled in 2024.

Figure 4. Braden West and Shawn Moore processing Texas blind salamanders caught from the trap set in Johnson's Well.

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. Salamanders were not incorporated into the refugia until the results from the Bsal/Bd test were received.

Figure 5. Braden West scanning a p-Chip after tagging a Texas blind salamander at the SMARC.

Texas blind salamanders from all collection locations were housed together; however, 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 to access sex and 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 97% at the SMARC and 94% at the UNFH in 2024. 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

Male and female salamanders were tagged so that collection information is known and were housed in group systems to encourage production of offspring for future research. Females were checked periodically for presence of visible eggs. Genetic analysis shows that collection locations are part of one panmictic population (Corbin 2020), thus these offspring could be employed should a restocking event occur.

In total, Texas blind salamanders at the SMARC produced 47 clutches of eggs and 8 clutches were produced at the UNFH in 2024. At SMARC 20 clutches of eggs were collected for propagation, 7 clutches of eggs were collected and preserved, and 10 clutches of eggs were eaten by adults in the tank before they could be collected. Clutch data are reported in Table 11.

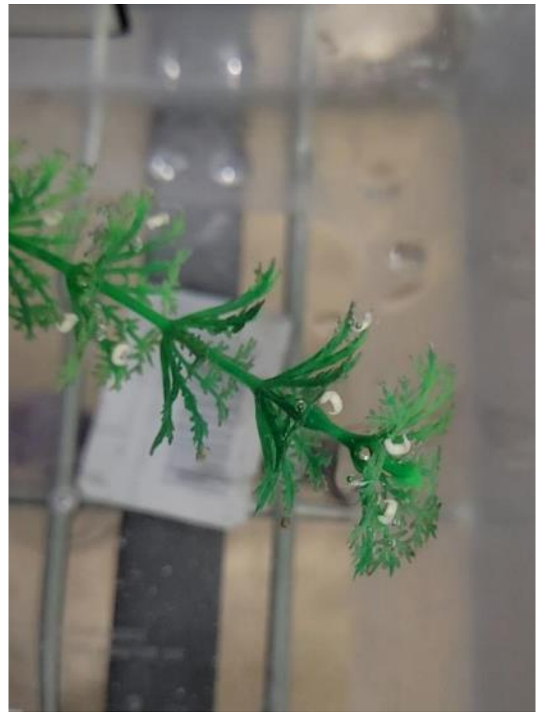


Figure 6. A clutch of partially developed Texas blind salamander eggs on an artificial plant.

Table 11. Texas blind salamander clutches produced during 2024. Percent Survival is listed as "NA" for clutches that have not fully hatched. Percent hatched is defined as the percentage of eggs that hatched into larval TBS. Percent survival is defined as the percentage of captively propagated TBS that survived until at least 12/31/2024.

Site	**Date**	***Parent Generation***	***Offspring Generation***	***# Deposited***	***# Hatched***	***(%) Hatched***	***(%) Survival***
UNFH	1/10/2024	WS	F1	39	6	4	50
UNFH	1/29/2024	WS	F1	1	0	0	0
UNFH	2/12/2024	WS	F1	1	0	0	0
UNFH	2/15/2024	WS	F1	22	2	100	100
UNFH	2/23/2024	WS	F1	12	0	0	0
UNFH	2/26/2024	WS	F1	9	8	88	50
UNFH	4/10/2024	WS	F1	34	11	2	100
SMARC	1/23/2024	WS	F1	18	3	16.7	6

SMARC	2/27/2024	WS	F1	7	3	42.9	43
SMARC	2/28/2024	WS	F1	7	3	42.9	29
SMARC	3/14/2024	WS	F1	8	8	100	88
SMARC	4/24/2024	WS	F1	17	17	100	76
SMARC	4/24/2024	F1	F2	21	18	86	34
SMARC	4/28/2024	WS	F1	6	5	83.3	50
SMARC	4/30/2024	WS	F1	11	2	18.2	9
SMARC	4/30/2024	F1	F2	18	6	33.3	22
SMARC	5/1/2024	WS	F1	10	10	100	80
SMARC	5/2/2024	WS	F1	21	19	90.5	62
SMARC	5/13/2024	WS	F1	5	5	100	80
SMARC	5/13/2024	WS	F1	15	15	100	100
SMARC	5/20/2024	WS	F1	35	18	51.4	14
SMARC	5/21/2024	WS	F1	15	6	40	7
SMARC	7/10/2024	F1	F2	16	8	50	31
SMARC	8/21/2024	F1	F2	15	6	40	27
SMARC	9/11/2024	WS	F1	28	12	42.9	32
SMARC	9/27/2024	WS	F1	6	6	100	100
SMARC	12/15/2024	WS	F1	5	4	80	80

Notes: Clutches experience some degree of loss after hatching, therefore the number that hatched does not represent the number of offspring present at the facility.

SAN MARCOS SALAMANDER (*EURYCEA NANA*), THREATENED

The Standing Stock goal for the San Marcos salamander is 500 individuals, divided between the two facilities. Typically, staff collect San Marcos salamanders twice each year in amounts sufficient to cover the expected loss given average mortality. In 2024, the number of collections for the refugia was reduced due to a mark-recapture study being conducted. 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 2024	End of Year Census	In Quarantine End of Year	Target Goal 2024 Work Plan	Percent Survival
SMARC	163	159	224	0	250	70
UNFH	164	84	140	0	250	56

COLLECTIONS

In 2024, refugia staff conducted eight collection events from Spring Lake and the Eastern spillway of the San Marcos River. The Eastern spillway was sampled twice, in May and October. A total of 86 SMS were captured, retained, and transferred to the SMARC for incorporation into the refugia. Refugia staff utilized USFWS Divers Justin Crow, Randy Gibson, Somerley Swarm, and Matthew Johnson to conduct six collection events throughout Spring Lake, San Marcos, Texas in May. A total of 163 SMS were captured. Of the 163 SMS, 121 were transferred to the SMARC and 42 to the UNFH for incorporation into the refugia. San

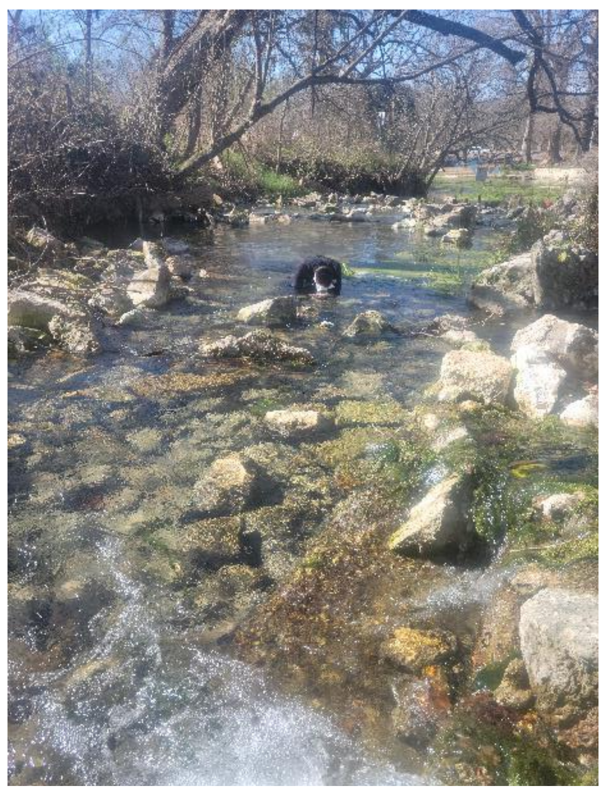


Figure 7. Shawn Moore snorkeling in the San Marcos River to collect San Marcos salamanders.

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 2024. A total of 157 SMS were captured in the drift net, 2 of which were retained and transferred to the SMARC for incorporation into the refugia.

QUARANTINE

Figure 8. 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 22 ±1 °C. 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 70% at the SMARC and 56% at the UNFH. Survival rates during their quarantine period are not included in the annual survival rates. The mortality of egg-bound females continued at both refugia facilities, albeit diminishing greatly at the 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

In 2024, wild-stock salamanders produced ten clutches at the SMARC and seven clutches at the UNFH. Clutch information is presented in Table 13.

Table 13. Clutches of San Marcos salamanders. Percent Survival is listed as "NA" for clutches that have not fully hatched. Percent hatched is defined as the number of SMS eggs that hatched into larval salamanders. Percent survival is defined as the number of SMS that survived until at least 12/31/2024.

Site	Date	Parent Generation	Offspring Generation	Eggs Deposited	# Hatched	(%) Hatched	(%) Survival
UNFH	1/18/2024	WS	F1	11	0	0	0
UNFH	3/2/2024	WS	F1	20	8	40	37.5
SMARC	1/31/2024	WS	F1	18	18	100	100
SMARC	2/7/2024	WS	F1	4	1	25	25
SMARC	2/24/2024	WS	F1	12	6	50	50
SMARC	2/28/2024	F1	F2	1	1	100	100
SMARC	3/2/2024	WS	F1	21	10	47.6	47.6
SMARC	3/10/2024	F1	F2	11	0	0	0
SMARC	3/27/2024	WS	F1	16	NA	NA	NA

SMARC	5/3/2024	F1	F2	17	3	17.6	17.6
SMARC	6/25/2024	WS	F1	9	7	77.8	77.8
SMARC	7/3/2024	WS	F1	24	22	91.7	91.7

Notes: Clutches experience some degree of loss after hatching, therefore the number that hatched does not represent the number of offspring present at the facility.

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

The Comal Springs salamander is a species covered in the Edwards Aquifer Habitat Conservation Plan (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 14.

Table 14 Comal Springs salamander refugia population figures

	Beginning of Year Census	Incorporated 2024	End of Year Census	In Quarantine End of Year	Target Goal 2024 Work Plan	Percent Survival
SMARC	50	44	81	1	150	79
UNFH	83	8	73	0	135	42

COLLECTIONS

In 2024, staff conducted sampling events for CSS in June, July, and September. Fifty-seven CSS were captured during these events, of which thirty-seven were transferred to the SMARC and twenty to the UNFH for incorporation into the refugia. Staff transferred two CSS captured during a BIO-WEST drift net biomonitoring event in May to the SMARC for incorporation into the refugia.

QUARANTINE

In 2024, 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 22℃ (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 2024 were 79% at the SMARC and 42% at the 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 2024, Comal Springs salamanders were housed in mixed-sex groups to encourage reproduction in refugia systems at both facilities. Reproduction can occur year-round as female salamanders come in and out of gravidity. Four clutches of eggs were produced at the SMARC and two clutches at the UNFH (Table 15).

Table 15. Propagation of Comal Springs salamanders.

Site	***Date***	***Parent Generation***	***Offspring Generation***	***# Deposited***	***# Hatched***	***(%) Hatched***	***(%) Survival***
UNFH	3/22/2024	WS	F1	15	5	33	80
UNFH	3/29/2024	WS	F1	40	26	65	50
SMARC	4/16/2024	WS	F1	16	9	56.3	25

Notes: Clutches experience some degree of loss after hatching, therefore the number that hatched does not represent the number of offspring present at the facility.
*Clutches have not hatched yet

TEXAS WILD RICE (*ZIZANIA TEXANA*), ENDANGERED

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 16.

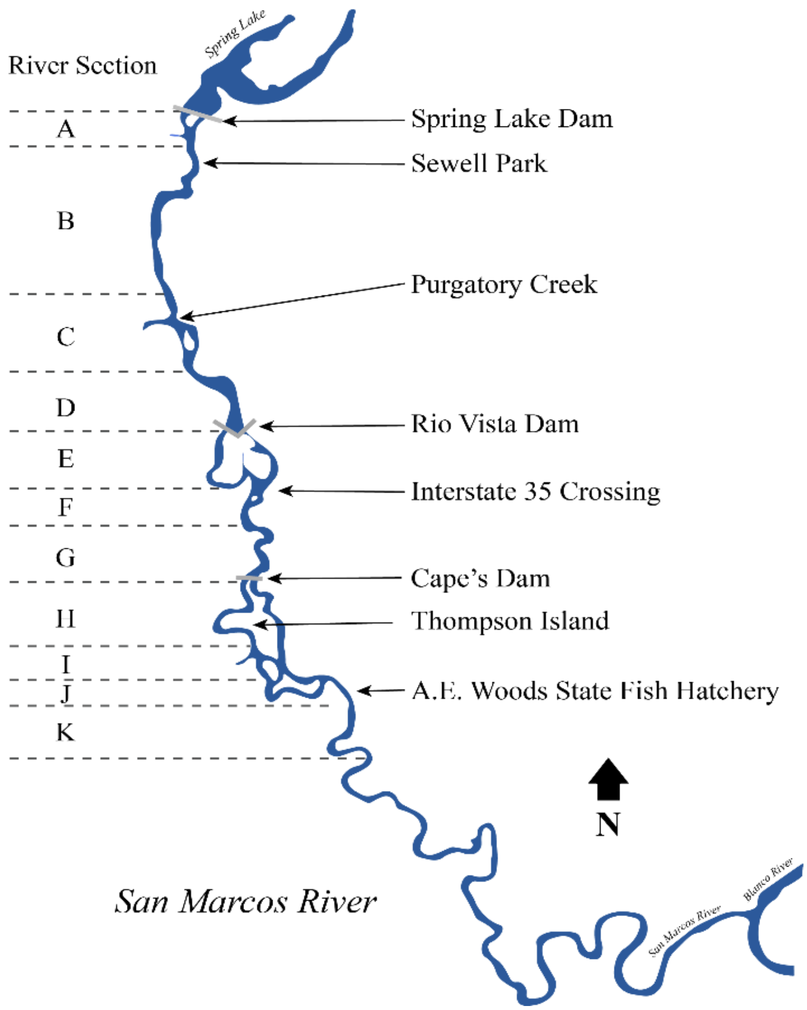


Figure 9. Lettered sections of the San Marcos River designating Texas wild rice habitat established by Texas Parks and Wildlife Department.

Table 16. Texas wild rice refugia population figures

	Beginning of Year Census	Incorporated 2024	End of Year Census	In Quarantine End of Year	Target Goal 2024 Work Plan	Percent Survival
SMARC	178	62	176	20	215	73%
UNFH	188	37	126	15	215	49%

COLLECTIONS

In 2024, refugia staff conducted four collection events for TWR in March, April, June, and December. Staff collected tillers from 121 plant stands. Of the 121 plant stands collected from, 73 were transferred to the SMARC and 48 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

Quarantine procedures differ by station. 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. Salt treatments of incoming tillers (2% salt dip) have been discontinued. Incoming quarantine plants were kept in their respective mesh bags or lightly potted in a mesh cylinder with loose gravel and placed in a quarantine tank. 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.

Figure 10. Journey Moreno (Student Conservation Association intern) and Shawn Moore repotting Texas wild rice.

HUSBANDRY

We continued to investigate different soil, potting techniques, and water flow/velocity regimes for TWR plants at the SMARC and UNFH. When plants are potted, we add a layer of lava rock at the bottom of the pot (space in the dirt we have previously not found roots to reach) to reduce anoxia forming in the soil. 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 73%. The overall survival rate of TWR plants at the UNFH was 49%. Survival numbers at the UNFH were lower than expected due to a high number of mortalities during the months of August-November. Staff rectified potential issues by re-potting and moving unhealthy TWR to a new tank.

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 1/4HP 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.

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 as needed 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 2024. 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 captive holding and propagation of Comal Spring dryopid beetle, genetic assessments of covered invertebrate species, and mark-recapture studies on invertebrates and the San Marcos Salamander. Much of this research was built on knowledge gained in previous studies. Below are summaries for each project approved within the 2024 Work Plan (Appendix A).

MARK AND RECAPTURE OF SAN MARCOS SALAMANDERS

The objective of this study is to examine the recapture rate, movement rate, and demographics of wild San Marcos salamanders tagged with 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. Recapture collections occurred 1-2 times each month at each of the sites for a year (May 2023-May 2024). The recapture rate across sites was 14%, varying 10-21%. A total of 3,469 San Marcos salamanders were collected for this study. No movement was detected across

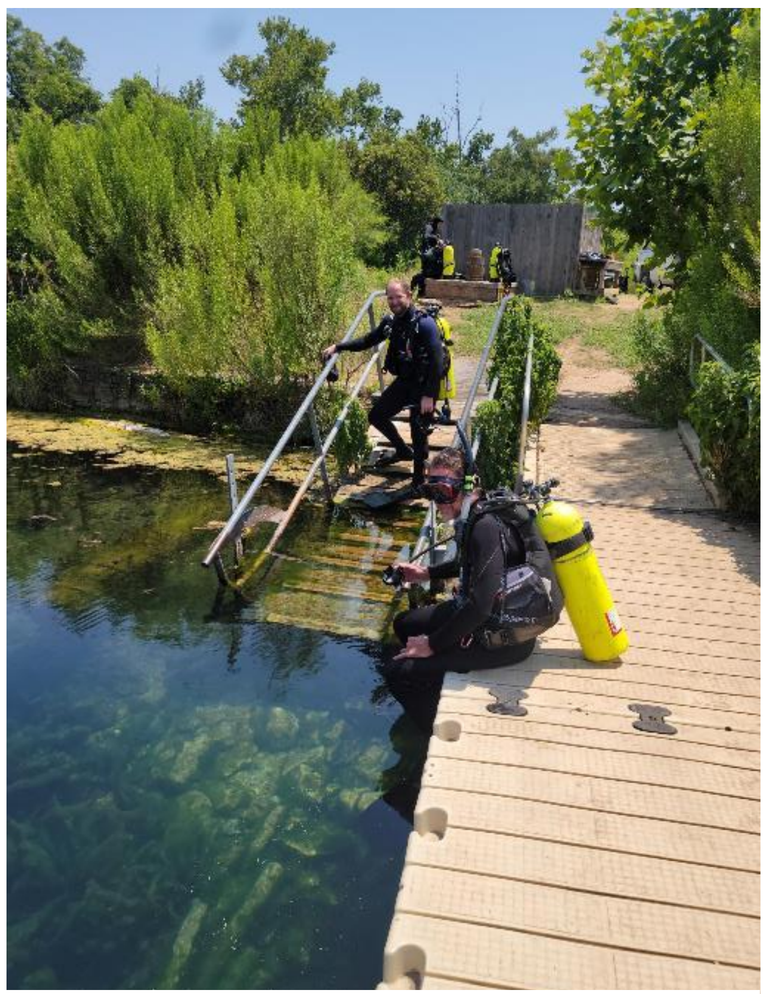


Figure 11. Justin Crow and Randy Gibson (SMARC biologists) preparing to dive to collect San Marcos salamanders in Spring Lake.

sites. On average, the salamanders collected at the San Marcos River site (Eastern Spillway) were larger (28.2 ± 4.0 mm) than the salamanders collected at the two Spring Lake sites (Hotel Springs and Diversion Spring; 26.6. ± 3.3 and 27.0 ± 3.6, respectively). There is no significant difference in sex ratio across sites (P=0.92) and neither sex was more abundant than the other (P=0.6967). The final report is in Appendix B.

CAPTIVE HUSBANDRY AND PROPAGATION OF THE COMAL SPRINGS DRYOPID BEETLE

The Edwards Aquifer Refugia Program houses Comal Springs dryopid beetles in captivity under the same conditions as the Comal Springs riffle beetle with the assumption that because they are found in the same or very similar locations, dryopid beetles utilize very similar habitat and food sources as riffle beetles. The dryopid beetle has very long egg and larval stages, which makes determining their captive needs difficult. Dryopid beetles survive captive holding in riffle beetle housing, but survival is low and larval production is rare, suggesting captive housing can be improved. This effort, led by BIO-WEST, uses challenge experiments to determine larval and adult dryopid beetle captive housing preference using riffle beetle housing as a reference and a cooccurring surrogate species as a comparison. Flow, light, habitat materials, the availability of interstitial space, and food sources have been compared. Although some habitat preferences have been determined, additional challenge experiment replicates are required because few individuals were included in the challenge experiments due to limited dryopid availability. In addition to captive holding challenges, extensive field work aimed at improving collections occurred across the Comal Springs system. Wood disks and stakes were placed in spring openings alongside cotton lures. Wood disks significantly improved dryopid beetle collections and increased the refugia stock from 8 individuals to 75; a 106% increase that surpassed work plan goals. The final report is in Appendix C.

TAGGING AQUATIC INVERTEBRATES

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. 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. Internal tagging of Peck's cave amphipod was unsuccessful thus far, but additional tagging methods were identified for testing in year 2 (e.g., external tagging). The interim report is in Appendix D.

Figure 12. A Comal Springs riffle beetle tagged with a p-Chip.

GENETIC ASSESSMENT OF PECK'S CAVE AMPHIPOD

The objective of this study is to assess the genetic diversity of the Peck's cave amphipod (PCA) in the Comal Springs System to determine the distribution of genetic diversity across sampling locations and to better inform Refugia collection efforts and captive breeding and reintroduction strategies. PCA were collected as bycatch during Comal Springs riffle beetle collection efforts in 2023 and 02, as they are often observed on the same lures. PCA were collected using dip nets in locations where less than 30 individuals were collected. All collected PCA were preserved in 95% ethanol and transferred to Dr. Chris Nice at Texas State University for genetic analysis. Preserved samples from as far back as 2008 were also included in the study to investigate potential changes in genetic diversity over multiple drought seasons and low flow conditions. There is no genetic structure for Peck's cave amphipod across the Comal Springs System when compared to other species/populations. When the data is analyzed for just the PCA group (excluding non PCA species) PCA still break out as one group for the Comal Springs system, but we see more genetic diversity represented and it is evenly represented across Spring Runs. There is no change in population or genetic structure in Peck's cave amphipod between time points, suggesting PCA populations do not seem to be significantly impacted by droughts and the collection locations (Spring Run 1-3, Spring Island, and Western Shore) are all well connected. Additional population genetic assessments (Tajima's D) show values very close to 0, suggesting PCA is not under a lot of genetic selection pressure and has not undergone a lot of population size changes. PCA are distinct from other *Stygobromus* species, which means their population is mainly in the Comal Springs system, with some other populations in nearby springs fed by the Edwards Aquifer. The final report for this study is available in Appendix E.

COMPARATIVE GENE EXPRESSION IN SAN MARCOS SALAMANDERS TO TARGET REPRODUCTIVE TRIGGERS IN CAPTIVITY

Captive propagation for the San Marcos salamander is challenging. Multiple methods have been used to induce courtship and reproduction with little success. A comparative gene expression study was deployed to guide SMARC biologists in future attempts to improve captive propagation. Led by Ruben Tovar and Dr. David Hillis of the University of Texas Austin, the objective of this study was to 1) determine which genes are important for reproductively active/gravid salamanders versus non-reproductive salamanders and 2) determine which sensory

Figure 2013. Ruben Tovar (University of Texas Austin), Nisa Sindhi (Texas State University), and Brittany Dobbins (Texas State University) processing salamanders for genetic analysis.

organs correlated to reproduction and how this may play a role in mating cues. There are significant differences in gene expression profiles between salamander species, tissue types, sex and reproductive state. Seven genes were identified that are tied to gravidity in female salamanders. These genes are expressed move in non-gravid females, thus under expressed, or down regulated, in gravid females. These genes are fairly ubiquitous in function and are associated with maintaining homeostasis, ion transport, mitochondrial function, protein synthesis, cilia formation and general regulation of other genes. There were no significant differences in gene expression profiles between reproductive state and different of sensory tissue types. This suggests there is no strong indicator that reproduction is tied to sensory tissues, thus no obvious focus for future research to induce reproduction in San Marcos salamanders. The interim report is in Appendix F.

GENETIC ASSESSMENT OF THE COMAL SPRINGS RIFFLE BEETLE IN LANDA LAKE

The objective of this study is to assess the genetic diversity of the Comal Spring riffle beetle in the Comal Springs system to determine the distribution of genetic variation, identify locations with unique genetic diversity, and determine the minimum number of individuals required in the refugia to maintain a representative captive population. Poly-cotton lures were placed in 100 spring openings across the Comal Springs system including Spring Runs 1 – 3, Spring Island, Western Shore, and Upper Spring Run 4. A subset of the adult beetles and all larvae on each lure were collected and preserved in 95% ethanol for genetic analysis. A total of 242 adult and larval Comal Springs riffle beetles were collected for this study and over 500 million sequences were analyzed. There is significant genetic isolation between sampling locations and genetic diversity is not shared across locations. Genetic structure located at Spring Island and Western Shore is distinct from Spring Run 2 and Spring Run 3. Additionally, genetic lineages represented in Spring Runs 2 and 3 are absent from Spring Island and Western Shore, and vice versa (Figure 4). The representation of a unique genetic lineage and relative uniformity in Spring Runs 2 and 3 indicates unique subpopulations relative to the main river channel and the potential of a reduction in genetic diversity due to reduction in population size (bottleneck) from a decrease in habitat availability. These results suggest that spring flows in the Spring Runs must be maintained at a sufficient minimum flow rate to prevent the

Spring Runs from drying to prevent further reductions in population size, the loss of unique genetic (and thus adaptive) diversity, and potential local species extirpation from habitat loss. The final report is located in Appendix G.

GENETIC ASSESSMENT OF SAN MARCOS SALAMANDERS

Tail clips collected from the 2023-2024 San Marcos salamander p-Chip mark-recapture study and were used to conduct this population genetic assessment of wild individuals across three regularly monitored and sampled sites. The three sites include Hotel, Diversion, and Eastern Spillway and a total of 453 salamanders were sampled. 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). Data analysis will occur in 2025 using the same methods used for the PCA and CSRB genetic assessments. The interim report for this research is in Appendix H.

GENETIC ASSESSMENT OF TEXAS BLIND SALAMANDERS

The EARP has the largest captive population of Texas blind salamanders and regularly produces captive breed offspring. It is important to determine the diversity of wild caught individuals and their Fx offspring. Thus far, tail clips were taken from 68 wild stock TBS and 4 Fx captive bred TBS. To assess wild populations, tail clips from TBS encountered in traps but not retained for the refugia will also be included in the study. Collection locations include Purgatory Natural Area wells (Primer's Fissure, Johnson's Well and Rattlesnake Cave. Sequencing and Data analysis will occur in 2025 following the same protocols used for the CSRB, PCA and SMS genetic assessments. The interim report for this research is in in Appendix I.

BUDGET

Task	U.S. Fish and Wildlife Service 2024	Budget Spent	Total Task Budget Spent
1	**Refugia Operations**		**$778,594.73**
	SMARC Refugia & Quarantine Bldg.		
	Construction	-	
	Equipment	$437.75	
	Utilities	$8,870.12	
	UNFH Renovation Refugia & Quarantine Bldg.		
	Construction	-	
	Equipment	$4,649.77	
	Utilities	$27,382.75	
	SMARC Species Husbandry and Collection	$220,475.28	
	UNFH Species Husbandry and Collection	$218,036.43	
	Water Quality Monitoring System	$20,28.40	
	SMARC Reimbursables	$86,586.17	
	UNFH Reimbursables	$52,067.06	
	Subtotal	*$639,033.73*	
	Admin Cost	*$139,561.00*	
2	**Research**		**$632,462.71**
	BIO-WEST: Dryopid 2023 Rollover	$52,800.00	
	BIO-WEST: Dryopid 2024	$62,594.49	
	Texas State: PCA Genetics 2023 Rollover	$31,074.00	
	Texas State: PCA Genetics 2024	$61,423.40	
	University of Texas: Salamander Gene Expression 2023 Rollover	$43,745.00	
	University of Texas: Salamander Gene Expression 2024	$42,226.97	
	Auburn University: Invertebrate Tagging 2023 Rollover	$37,590.00	
	Auburn University: Invertebrate Tagging 2024	$33,159.93	
	Student Conservation Association	$7,520.97	
	USFWS Salary	$144,680.13	
	Materials	$21,674.21	
	Subtotal	$538,489.10	
	Admin Cost	*$93,973.61*	
3	**Species Propagation and Husbandry**	-	-
4	**Species Reintroduction**	-	-
5	**Reporting**		**$44,110.78**
	SMARC Staff	$23,374.17	
	UNFH Staff	$14,283.45	
	Subtotal	*$37,657.62*	

	Admin Cost	*$6,453.16*	
6	**Meetings and Presentations**		**$12,189.89**
	SMARC Staff	$9,591.04	
	UNFH Staff	$1,054.68	
	Subtotal	*$10,645.72*	
	Admin Cost	*$1,544.17*	
		TOTAL	**$ 1,467,358.11**

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

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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

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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. 2024 EA Refugia Work Plan
B. Mark and recapture of San Marcos salamanders - final report
C. Comal Springs dryopid beetle (*Stygoparnus comalensis*) research 2023–2024: laboratory studies of habitat preferences and development of field methods for detection, collection, and monitoring - final report.
D. Evaluating survival and tag retention of cave amphipods and Comal Spring Riffle Beetles - interim report
E. Conservation Genetics of *Stygobromus* in Texas – final report
F. Establishing a developmental atlas and de novo transcriptome for *E. rathbuni*, *E. nana*, and *E. pterophila*
G. Genetic assessment of the Comal Springs riffle beetle in Landa Lake – final report
H. Genetic assessment of the San Marcos salamander – interim report
I. Genetic assessment of the Texas blind salamander – interim report
J. Monthly reports
K. USFWS Southwestern Native Aquatic Resources and Recovery Center Fish Health Unit reports