Appendix F | **2023 Edwards Aquifer Refugia Program Annual Report**

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

ANNUAL REPORT 2023

CONTRACT NO. 16-822-HCP

Katie Bockrath, Adam Daw, Desiree Moore, Dominique Alvear, and Braden West

U.S. Fish and Wildlife Service

San Marcos Aquatic Resources Center
500 E. McCarty Ln, San Marcos, TX 78666

Uvalde National Fish Hatchery
754 County Road 203, Uvalde, TX 78801

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.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

We would like to thank The Edwards Aquifer Authority who provided financial assistance for this program and R. Ruiz, S. Storment, C. Furl, J. Childers, D. Childs, K. Smith, K. Tolman, and O. Ybarra Lopez of the Edwards Aquifer Authority for their support, coordination, and direction. The Austin Ecological Field Office staff and the Texas Parks and Wildlife Department provided assistance, coordination, and support. We thank the Cities of San Marcos and New Braunfels, the Edwards Aquifer Research & Data Center and the Meadows Center for Water and the Environment. BIO-WEST, Incorporated, Dr. Chris Nice of Texas State University, Ruben Tovar and David Hillis of University of Texas Austin, and Dr. Shannon Brewer of U.S. Geological Survey, Alabama Cooperative Fish and Wildlife Research Unit contributed to refugia program research. We thank all U.S. Fish and Wildlife staff at the Southwest Regional Office, San Marcos Aquatic Resources Center, and Uvalde National Fish Hatchery for their significant contributions and expansive knowledge.

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 seventh annual report of the contract covering the calendar year of 2023. The seventh 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 2023. 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 2023; all other covered species were collected in 2023.

Figure 1. Dominique Alvear and Jonathan Donahey collecting San Marcos fountain darters at Eastern Spillway, San Marcos, Texas.

Table 1. Counts of individuals captured in 2023 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				0/36					
FEB	0/32	0/9						30/0	
MAR			0/138	0/10	65/10			75/0	
APR					182/0			53/0	
MAY				501/0	0/88	15/0			12/0
JUN			76/0	0/160					
JUL				466/0	177/28			8/0	
AUG			0/108	0/118	0/73	4/0	18/0	7/0	0/10
SEP			105/0		206/105			4/0	
OCT					133/0				0/3
NOV	36/0		6/0	159/0		3/0		2/0	
DEC			50/49						10/10

RESEARCH

We conducted six research projects in 2023, 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 salamander. 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 began 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. Tagging was completed and recaptures began in 2023. Recaptures are planned to continue into 2024. An interim 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 for captive assurance. Collections and challenge experiments for larvae and adults were conducted in 2023. Experimental questions examined the housing preferences of dryopid beetles in captivity. Collections and experiments are planned to continue in 2024. An interim 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 tagging protocol was developed for Comal Springs riffle beetle using superglue to affix a p-Chip tag to the elytra. Initial internal tagging of Peck's cave amphipod was unsuccessful thus far, but additional tagging methods were identified for testing in 2024. Survival and retention of tagged beetles is planned to be investigated in 2024. An interim report for this study is included in Appendix D.

USFWS staff and Dr. Chris Nice (Texas State University) began a genetic assessment of the Peck's cave amphipod in the Comal Springs system. Amphipods were collected as bycatch during Comal Springs riffle beetle collections and by dip nets in areas where more were needed. Collections concluded in 2023 and genetic analysis is planned to be conducted in 2024. An interim 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 salamanders to target reproductive triggers in captivity. Salamanders were preserved in a fixative allowing for molecular work microCT scanning to create a transcriptome and developmental time series. Tissue fixing and transcriptomics are planned to continue in 2024. An interim report for this project is available in Appendix F.

A genetic assessment of the CSRB in Landa Lake continued through 2023 in partnership with BIO-WEST. Lure deployment was delayed until 2023 due to drought conditions. BIO-WEST set lures at 80 biomonitoring sites at three time points to gather data for an occupancy study. A portion of the CSRB observed on each lure was retained for genetic assessment. Collections concluded in 2023 and genetic analysis will be carried out in 2024. An interim report for this research is included in Appendix G.

BUDGET

The Aquifer Refugia Program did not exceed the allocated budget defined in the 2023 Refugia Work Plan previously approved by the EAA Board of Directors. The Refugia Program spent approximately $1,323,005 in 2023. 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*	None[†]
Texas troglobitic water slater	*Lirceolus smithii*	None[‡]

* 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 2023. 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 2023. The Edwards Aquifer Refugia Program underwent staff changes in 2023.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	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	Refugia Husbandry and Collections Team Lead
Dominique Alvear	Refugia Biologist
Heidi Meador	Biological Science Technician
Jonathan Donahey	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,

Figure 2. Adam Daw, Heidi Meador, Jonathan Donahey, and Dominique Alvear in a work vehicle.

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.

Figure 3. Edwards Aquifer Refugia Program staff at the Edwards Aquifer Authority Education Outreach Center. From left to right, Braden West, Adam Daw, Dominique Alvear, Shawn Moore, Desiree Moore, and Dr. Katie Bockrath.

Jonathan Donahey, Heidi Meador, 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

Significant improvements to the EARP building occurred in 2023. We started the transition of water quality monitoring systems from Hydrolab sondes to Walchem controllers/monitors at both the UNFH and SMARC. With the use of the controllers, water quality probes (total gas pressure, water temperature, water pressure), and an automated bypass valve, the main well water supply line was redesigned for both refugia locations to minimize the potential for well water supersaturated with gas reaching refugia tanks. The well water line modification for the UNFH refugia room was completed and the one for the SMARC refugia room was under construction at the end of the year. With the use of the controllers, CO_2 injection systems were added to more tanks at the UNFH to better control water pH in the systems.

Figure 4. EARP staff in the SMARC refugia room learning about the controller units from Adam Daw.

Refugia room hospital tank racks at both facilities were modified to improve function and to standardize the design with the quarantine tank racks. The last three hospital racks in the SMARC quarantine room were constructed. The second invertebrate rack in the refugia at the SMARC was constructed and two invertebrate racks at the UNFH were modified to improve the design and allow for monitoring of systems parameters via the new controllers. A filter system was added to a Texas wild rice tank at the SMARC to evaluate if it would improve the health of the system. Multiple refugia tanks in the SMARC and UNFH refugia rooms were redesigned from flow through to partially recirculating systems. The new system design allows for the recirculation of the system water and requires up to 50% less chilled well water than previous flow-through tank designs used at the SMARC. The redesign also allowed for the addition of the new system controllers/monitors.

Four tanks were added to both facilities to culture *Daphnia magna,* which have shown to be an easily cultured live food for the Fountain darters and salamanders. The Peck's cave amphipods have also been observed eating them.

New storage and work benches were added to the refugia rooms to better organize equipment and provided dedicated space for various tasks.

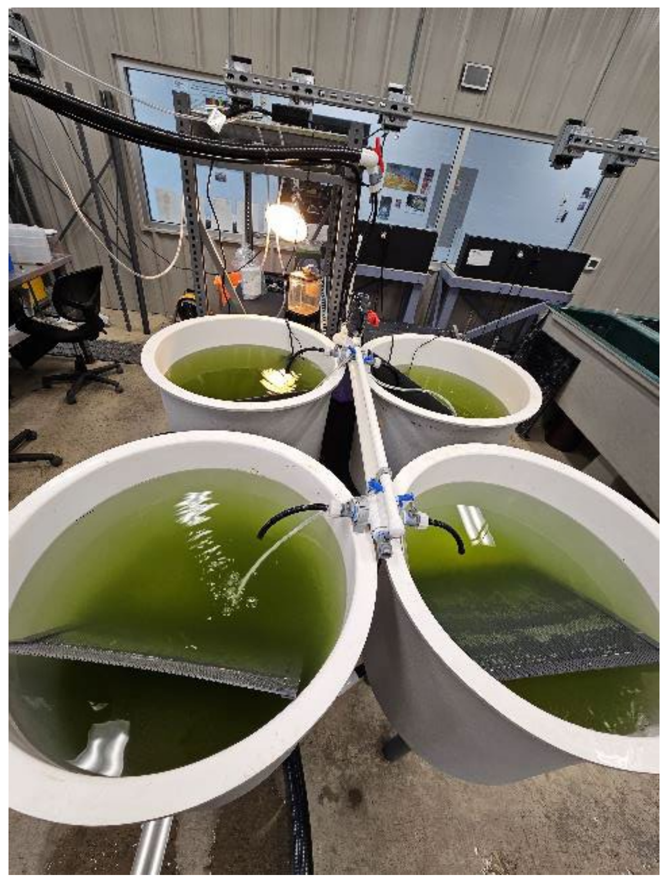


Figure 5. One of the four-tank *Daphnia magna* culture systems.

COVERED SPECIES ANALYSIS

Collections of the Covered Species continued to work toward standing stock targets as outlined in the Contract and the 2023 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 6. Texas blind salamander

Table 3. Number of organisms incorporated in the SMARC Refugia Standing Stock in 2023, 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*		466	89	19%
Fountain darter – Comal Springs *Etheostoma fonticola*		314	149	24%
Comal Springs riffle beetle *Heterelmis comalensis*		32	32	47%
Comal Springs dryopid beetle *Stygoparnus comalensis*		0	0	0%
Peck's cave amphipod *Stygobromus pecki*		73	145	68%
Edwards Aquifer diving beetle *Haideoporus texanus*		0	0	--
Texas troglobitic water slater *Lirceolus smithii*		0	0	--
Texas blind salamander *Eurycea rathbuni*		9	88	49%
San Marcos salamander *Eurycea nana*		129	163	72%
Comal Springs salamander *Eurycea* ***pterophila***		16	58	45%
Texas wild rice *Zizania texana*		12	178	82%

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.

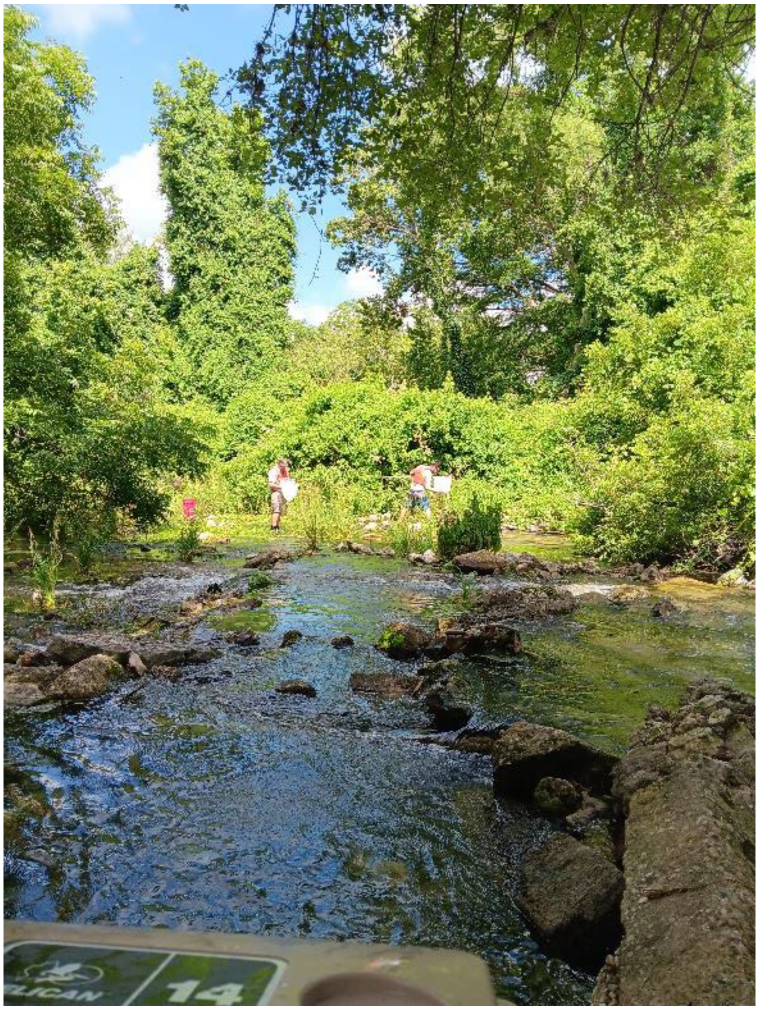
Table 4. Number of organisms incorporated in the UNFH Refugia Standing Stock in 2023, the end of year census, and overall survival rate.

Species		UNFH Incorporated into Refugia	UNFH End of Year Census	UNFH Survival Rate
Fountain darter - San Marcos *Etheostoma fonticola*		178	300	52%
Fountain darter – Comal Springs *Etheostoma fonticola*		417	371	63%
Comal Springs riffle beetle *Heterelmis comalensis*		17	16	25%
Comal Springs dryopid beetle *Stygoparnus comalensis*		6	8	50%
Peck's cave amphipod *Stygobromus pecki*		115	202	58%
Edwards Aquifer diving beetle *Haideoporus texanus*		0	0	
Texas troglobitic water slater *Lirceolus smithii*		0	0	
Texas blind salamander *Eurycea rathbuni*		0	62	94%
San Marcos salamander *Eurycea nana*		48	164	76%
Comal Springs salamander *Eurycea* ***pterophila***		0	83	89%
Texas wild rice *Zizania texana*		13	188	85%

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	5	5	3	3
Texas wild rice	5	5	5	5	5
Texas blind salamander	4	5	4	3	1
San Marcos salamander	5	5	4	3	1
Comal Springs salamander	5	4	3	3	1
Comal Springs riffle beetle	5	4	4	3	1
Comal Springs dryopid beetle	3	2	1	0	1
Texas troglobitic water slater	1	0	0	1	1
Peck's cave amphipod	4	4	4	3	2
Edwards Aquifer diving beetle	1	0	0	0	1



Our Standing Stock goal for fountain darters is 1,000 fish per river (San Marcos and Comal) divided between the two facilities. Standing stock goals for San Marcos fountain darters were slightly below target numbers in 2023. 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.

Figure 7. UNFH staff collecting fountain darters.

Table 6. Fountain darter refugia population figures

		Beginning of Year Census	Incorporated 2023[1]	End of Year Census	Target Goal 2023 Work Plan	Percent Survival [2]
San Marcos River	SMARC	309	466	89	500	19%
	UNFH	457	178	300	500	52%
Comal River	SMARC	313	314	149	500*	24%
	UNFH	181	417	371	500	63%

* 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 2023, the collection of fountain darters was increased due to the low spring flows of both the Comal and San Marcos Rivers. Refugia staff collected San Marcos fountain darters in March, May, July, August, and September and Comal fountain darters in January, March, and August. BIO-WEST Inc. transferred fish to refugia staff during their bi-annual surveys of the Comal and San Marcos Rivers in April/May and October/November, and a low-flow survey of the Comal River in July.

Figure 8. SMARC staff checking their nets for fountain darters during a collection.

Bi-annual testing for *Centrocestus* sp., a trematode parasite, in wild fountain darters, was conducted by the USFWS Southwestern Fish Health Unit (SFHU) in Dexter, New Mexico. Fish sent for testing were caught from both the Comal and San Marcos Rivers in March and August. In May and November, subsets of fountain darters from the BIO-WEST Inc. bi-annual surveys of the Comal and San Marcos Rivers were sent directly to the USFWS Southwestern Fish Health Unit (SFHU) in Dexter, New Mexico for parasite enumeration and viral analysis.

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. Feeding occurred 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. The 2023 survival rates for incorporated fountain darters in refugia at the SMARC was 19% for the San Marcos River population and 24% for the Comal River population. In previous years the San Marcos populations are relatively healthy when brought into quarantine. In 2023 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 drought stressors, it's likely the San Marcos fountain darters arrived to the Refugia in already suboptimal condition A well water gas supersaturation event occurred, due to a power outage, at the SMARC which resulted in a mortality event and the low overall survival for the year (Appendix J). Although we cannot fully predict the overall survival of Comal Springs fountain darters at SMARC, by removing the Comal Springs fountain darter that died as a result of the gasification event (N=180), survival at the SMARC could have been as high as 52%. At the UNFH, the survival rate was 52% for the incorporated San Marcos population and 63% for the Comal River population.

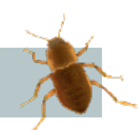
MAINTENANCE OF SYSTEMS

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 2023, 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 2023	End of Year Census	*Target Goal 2023 Work Plan	Percent Survival
SMARC	36	32	32	75	47%
UNFH	48	16	17	75	25%

* For 2023 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

On February 6, refugia staff collected 32 riffle beetles from checking in-situ submerged wood around the Northern shore of the Comal River near Spring Island, all of these were transferred to the UNFH. On November 20 and 21, riffle beetles were collected from cotton lures placed in Spring Run 3 and along the western shore of the Comal River in coordination with BIO-WEST Incorporated. In total, 36 adult riffle beetles were transferred to refugia staff and taken to the SMARC for the refugia population.

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 2023 survival rates for CSRB in refugia at the SMARC was 47% and 25% at the UNFH. The percent survival for the UNFH was lower due to a box that had F1 individuals pupate with the adult wild stock still in the box. Due to the inability to distinguish wild and F1 adults, wild individuals were counted as mortalities and all living beetles were considered as F1.

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 2023	End of Year Census	In Quarantine End of Year	Target Goal 2023 Work Plan	Percent Survival
SMARC	2	0	0	0	20	0%
UNFH	10	6	8	0	20	50%

COLLECTIONS

In 2023, sampling events occurred for CSDB at Spring Island, Comal River by checking in-situ submerged wood. Nine individuals were captured in February, with eight adults retained for the UNFH and 1 juvenile released. A collection event was conducted in March near Spring Island, but no individuals were found.

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 2023 survival rates for CSDB in refugia at the SMARC was 0% and 50% at the UNFH.

CAPTIVE PROPAGATION

Larvae were observed in 2023 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 2023	End of Year Census	Target Goal 2023 Work Plan	Percent Survival
SMARC	139	73	145	250	68%
UNFH	232	115	202	250	58%

COLLECTIONS

There were five collection events conducted in 2023 for Peck's cave amphipods (PCA) by refugia staff. These took place around Spring Island of the Comal River, New Braunfels, Texas. A total of 536 PCA were captured, with 509 of those transferred to the SMARC and the UNFH for 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 2023 survival rates for PCA in refugia at the SMARC was 68% and 58% 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 2023. 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*), 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 2023. 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 9. Shawn Moore pulling up the Diversion Spring net in Spring Lake.

Table 10 Texas blind salamander refugia population figures

	Beginning of Year Census	Incorporated 2023	End of Year Census	In Quarantine End of Year	Target Goal 2023 Work Plan	Percent Survival
SMARC	172	9	88	3	250	48%
UNFH	66	0	62	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 2023, Primer's Fissure and Johnson's Well were both sampled in May, but only Johnson's Well was sampled in August and November due to low water in Primer's Fissure in those months. In total, 20 TBS were captured from Johnson's Well, of which five were transferred to the SMARC. Eight TBS were captured from Primer's Fissure with two transferred to the SMARC. Neither Rattlesnake Cave nor Rattlesnake Well were sampled in 2023. All sites were trapped for two weeks during each collection event and biologists tagged Texas blind salamanders with a p-Chip transponder tag, scanned all collected salamanders for a p-Chip, and collected tail clips of all released salamanders for future genetic analysis. A total of 15 recaptures were observed throughout the year, where 11 occurrences were at Johnson's Well and 4 were at Primer's Fissure. The Diversion Springs driftnet was installed in July and checked two to three times a week for the rest of the year. One TBS was captured in the driftnet in November soon after a hard rain event in the area. This animal was retained for refugia at the SMARC.

Figure 10. Braden West and Shawn Moore processing Texas blind salamanders caught from the trap set in Johnson's Well.

Figure 11. Braden West scanning a p-Chip after tagging a Texas blind salamander at the SMARC.

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.

HUSBANDRY

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.

Figure 12. Dominique Alvear practicing tagging salamanders at the SMARC.

Adult salamanders were fed twice weekly and received either live amphipods, live blackworms, live red composting worms, live *Daphnia*, or frozen mysid 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 48% at the SMARC and 94% at the UNFH in 2023. Survival rates during quarantine period are not included in annual survival rates. The low survival of the SMARC TBS was a result of a well power outage causing a severe well water gas supersaturation event (Appendix J). Eighty-two wild caught Texas blind salamanders died as a result of the gasification event. We cannot fully predict what the survival rate of Texas blind salamanders at SMARC would have been without the event, but by removing the 82 that perished, the overall survival of Texas blind salamanders at the SMARC could have been as high as 94%.

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 2023. Clutch data are reported in Table 11.

Figure 13. A clutch of partially developed Texas blind salamander eggs on an artificial plant.

Table 11. Texas blind salamander clutches produced during 2023. Percent Survival is listed as “NA” for clutches that have not fully hatched.

Site	*Date*	*Parent Generation*	*Offspring Generation*	*# Deposited*	*# Hatched*	*(%) Survival*
UNFH	5/3/2023	WS	F1	38	6	NA
UNFH	7/5/2023	WS	F1	26	0	0
UNFH	7/31/2023	WS	F1	37	0	0
UNFH	7/31/2023	WS	F1	26	0	0
UNFH	9/27/2023	WS	F1	33	0	0

UNFH	11/24/2023	WS	F1	35	0	0
UNFH	12/18/2023	WS	F1	39	NA	NA
UNFH	12/18/2023	WS	F1	33	*	*
SMARC	1/31/2023	WS	F1	5	4	80
SMARC	2/6/2023	WS	F1	1	1	100
SMARC	2/8/2023	WS	F1	27	19	70.4
SMARC	2/11/2023	WS	F1	5	5	100
SMARC	2/21/2023	WS	F1	21	11	52.4
SMARC	2/28/2023	WS	F1	21	0	0
SMARC	3/3/2023	WS	F1	8	6	75
SMARC	3/10/2023	WS	F1	22	0	0
SMARC	3/24/2023	WS	F1	4	3	75
SMARC	4/7/2023	WS	F1	9	9	100
SMARC	4/17/2023	WS	F1	12	1	8.3
SMARC	4/25/2023	WS	F1	18	0	0
SMARC	4/26/2023	WS	F1	19	6	31.6
SMARC	5/1/2023	WS	F1	12	9	75
SMARC	5/1/2023	WS	F1	26	4	15.4
SMARC	5/8/2023	WS	F1	27	4	14.8
SMARC	5/10/2023	WS	F1	20	11	55
SMARC	5/15/2023	WS	F1	23	13	56.5
SMARC	6/20/2023	WS	F1	18	17	94.4
SMARC	6/20/2023	WS	F1	16	12	75
SMARC	6/20/2023	WS	F1	4	4	100
SMARC	7/4/2023	WS	F1	3	0	0
SMARC	7/5/2023	WS	F1	7	5	71.4
SMARC	7/7/2023	WS	F1	28	4	14.3

SMARC	7/10/2023	WS	F1	25	19	76
SMARC	7/14/2023	WS	F1	33	13	39.4
SMARC	7/28/2023	WS	F1	4	4	100
SMARC	7/31/2023	WS	F1	27	3	11.1
SMARC	8/21/2023	F1	F2	15	13	86.7
SMARC	10/10/2023	WS	F1	2	2	100
SMARC	10/10/2023	WS	F1	1	1	100
SMARC	10/17/2023	WS	F1	13	5	38.5
SMARC	10/23/2023	WS	F1	3	3	100
SMARC	10/30/2023	WS	F1	23	21	91.3
SMARC	11/7/2023	WS	F1	14	8	57.1
SMARC	11/14/2023	WS	F1	30	2	6.7
SMARC	11/15/2023	WS	F1	19	10	52.6
SMARC	11/21/2023	WS	F1	22	21	95.5
SMARC	11/21/2023	WS	F1	21	21	100
SMARC	11/21/2023	WS	F1	5	1	20
SMARC	11/27/2023	WS	F1	23	*	*
SMARC	11/30/2023	WS	F1	7	*	*
SMARC	11/30/2023	WS	F1	21	*	*
SMARC	12/5/2023	WS	F1	19	*	*
SMARC	12/6/2023	F1	F2	26	*	*
SMARC	12/6/2023	WS	F1	10	*	*
SMARC	12/19/2023	WS	F1	15	*	*

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

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 2023, 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 2023	End of Year Census	In Quarantine End of Year	Target Goal 2023 Work Plan	Percent Survival
SMARC	96	129	163	0	250	72%
UNFH	168	48	164	0	250	76%

COLLECTIONS

In 2023, there were San Marcos salamander collections for the refugia population in February (30 caught, 27 retained) and April (53 caught, 39 retained) in the San Marcos River at the Eastern Spillway below Spring Lake Dam. In March, there was also a collection at the Hotel Springs area in Spring Lake (75 caught, 54 retained). Thirty-three San Marcos salamanders were caught as by-catch from the Diversion Springs drift net, all of which were released.

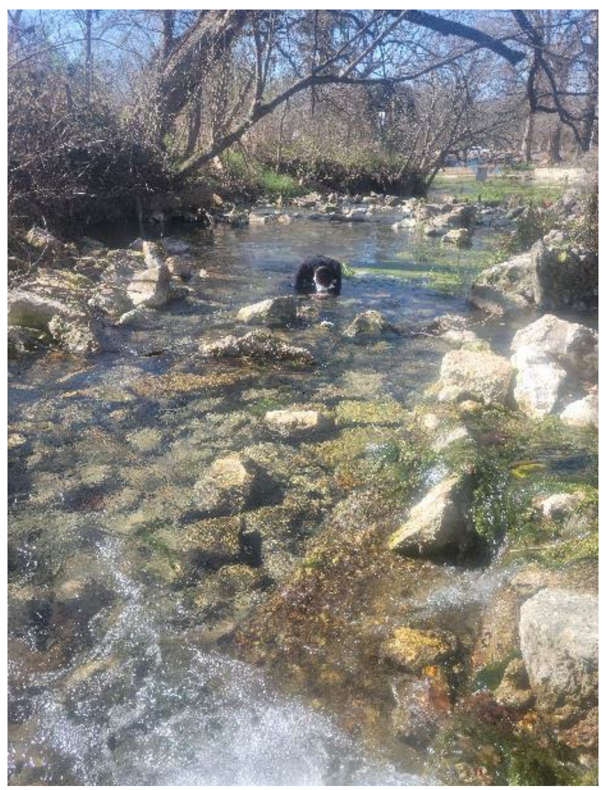


Figure 14. Shawn Moore snorkeling in the San Marcos River to collect San Marcos salamanders.

QUARANTINE

Figure 15. 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 ℃. 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 72% at the SMARC and 76% 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. A super gas saturation event occurred at the SMARC due to a power failure (Appendix J). Fifty-six San Marcos salamanders died as a result of the event. Although we are unable to verify what the overall survival of San Marcos salamanders would have been without the event, by removing the 56 salamanders that perished, survival rates may have been as high as 97%.

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 2023, 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.

Site	Date	Parent Generation	Offspring Generation	Eggs Deposited	# Hatched	(%) Survival
UNFH	1/11/2023	WS	F1	22	17	77
UNFH	3/20/2023	WS	F1	17	0	0
UNFH	4/4/2023	WS	F1	26	0	0
UNFH	4/20/2023	WS	F1	22	0	0
UNFH	7/31/2023	WS	F1	226	0	0
UNFH	8/8/2023	WS	F1	8	2	025
UNFH	9/1/2023	WS	F1	27	2	7
SMARC	1/31/2023	WS	F1	18	18	100
SMARC	2/7/2023	WS	F1	4	1	25
SMARC	2/24/2023	WS	F1	12	6	50
SMARC	2/28/2023	F1	F2	1	1	100
SMARC	3/2/2023	WS	F1	21	10	47.6

SMARC	3/10/2023	F1	F2	11	0	0
SMARC	3/27/2023	WS	F1	16	NA	NA
SMARC	5/3/2023	F1	F2	17	3	17.6
SMARC	6/25/2023	WS	F1	9	7	77.8
SMARC	7/3/2023	WS	F1	24	22	91.7
UNFH	8/01/2023	WS	F1	34	20	59%
UNFH	12/13/2023	WS	F1	15	*	NA

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

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 2023	End of Year Census	In Quarantine End of Year	Target Goal 2023 Work Plan	Percent Survival
SMARC	110	2	50	6	150	45%
UNFH	93	0	83	0	135	89%

COLLECTIONS

In August 2023, staff collected 18 individuals, 12 of which were taken to the SMARC refugia.

QUARANTINE

In 2023, 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 were high in 2023, with 45% at the SMARC and 89% at the UNFH. The low survival of the SMARC Comal salamanders was a result of a well power outage causing a severe well water gas supersaturation event (Appendix J). Fifty-two Comal Springs salamanders perished as a result of the gas supersaturation event. Although we cannot fully predict what the survival rate at the SMARC would have been without the event, by removing the 52 Comal Springs salamanders that died, survival may have been as high as 91%.

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 2023, 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*	*(%) Survival*
UNFH	8/1/2023	WS	F1	31	11	35
UNFH	12/13/2023	WS	F1	32	*	*
SMARC	2/13/2023	WS	F1	7	7	100
SMARC	2/15/2023	WS	F1	5	4	80
SMARC	2/21/2023	WS	F1	15	0	0
SMARC	2/27/2023	WS	F1	12	10	83.3

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.

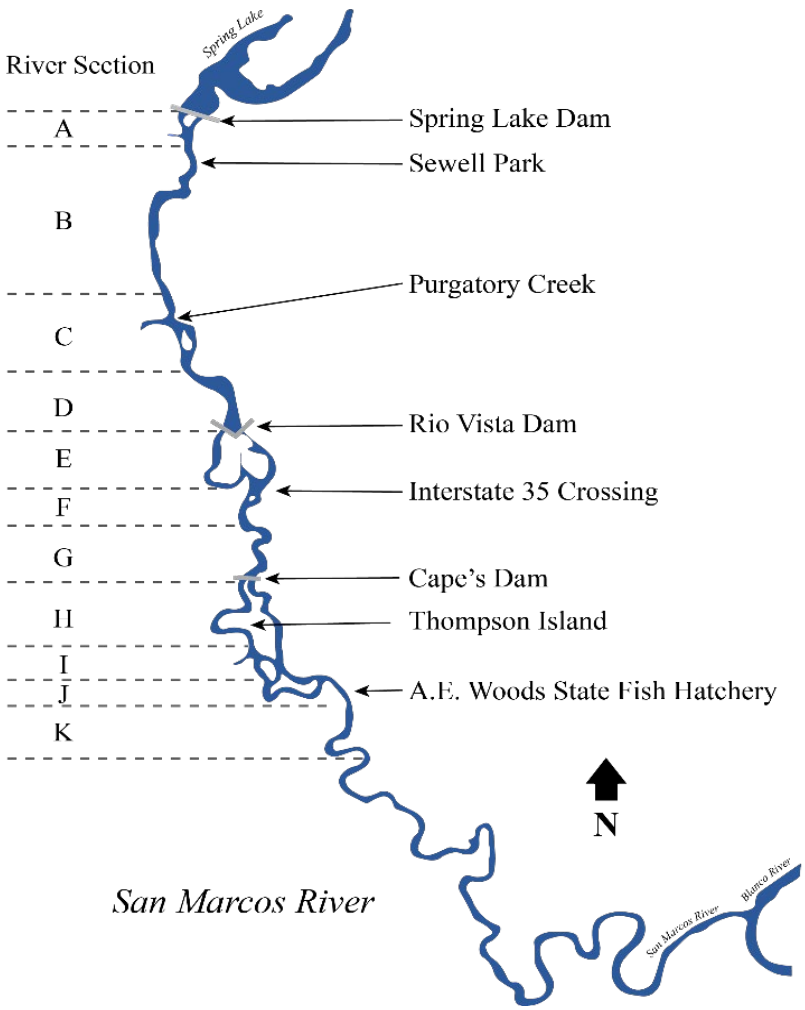


Figure 16. Lettered sections of the San Marcos River designating Texas wild rice habitat established by Texas Parks and Wildlife Department.

Table 16. Texas wild rice refugia population figures

	Beginning of Year Census	Incorporated 2023	End of Year Census	In Quarantine End of Year	Target Goal 2023 Work Plan	Percent Survival
SMARC	205	12	178	10	215	82%
UNFH	207	13	188	10	215	85%

COLLECTIONS

Tiller collections in the San Marcos River occurred in May, August, October, and December of 2023. 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 17. 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 82%, with older plants more likely to succumb to mortality. The overall survival rate of TWR plants at the UNFH was 85%. The average lifespan in captivity, based on records of the 74 plants (with known collection location by GPS) that have died since 2016 is 1.7 years.

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 2023. 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 2023 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. Thus far, the recapture rate across sites was 13%, varying 10-17%. A total of 2,013 San Marcos salamanders were collected for this study in 2023. No movement was detected yet. On average, the salamanders

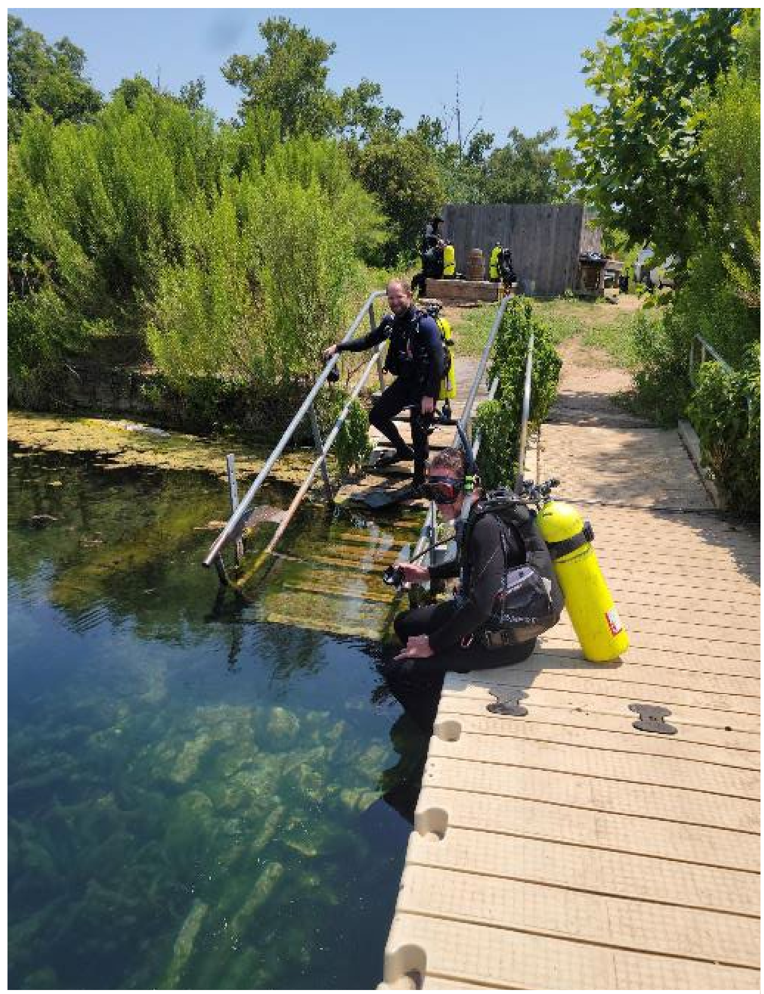


Figure 18. Justin Crow and Randy Gibson (SMARC biologists) preparing to dive to collect San Marcos salamanders in Spring Lake.

collected at the San Marcos River site were larger than the salamanders collected at the two Spring Lake sites. This study is in progress and collections are planned to continue through the end of May 2024. The interim 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. The interim report is in Appendix C.

Figure 19. A Comal Springs riffle beetle tagged with a p-Chip.

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.

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 their range. The information gathered from this study will identify locations with unique genetic diversity, inform collection and reintroduction strategies, and determine the minimum number of individuals required in the refugia to have a representative captive population. Peck's cave amphipods were collected as bycatch during Comal Springs riffle beetle collection efforts, as they are often observed on the same lures. PCA were collected using dip nets in locations where an insufficient number of individuals were collected. All collected PCA were preserved in 95% ethanol and transferred to Dr. Chris Nice at Texas State University for genetic analysis. A total of 119 PCA were collected for this study across six sampling locations. An interim 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 organs correlated to reproduction and how this may play a role in mating cues. As oviposition occurred in the captive-assurance population at the SMARC, San Marcos and Texas blind salamanders were fixed in a proprietary fixative that allows for downstream molecular work to generate a comprehensive transcriptome. RNA quality and Quantity were sufficient for RNA sequencing. The interim report is in Appendix F.

Figure 2020. Ruben Tovar (University of Texas Austin), Nisa Sindhi (Texas State University), and Brittany Dobbins (Texas State University) processing salamanders for genetic analysis.

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. DNA was extracted from the beetles using a Qiagen DNEasy Blood and Tissue DNA extraction kit. A total of 168 adult and larval Comal Springs riffle beetles were collected for this study. The interim report is located in Appendix G.

BUDGET

Task	U.S. Fish and Wildlife Service 2023		Budget Spent	Total Task Budget Spent
1	**Refugia Operations**			**$868,808.36**
	SMARC Refugia & Quarantine Bldg.			
	Construction		-	
	Equipment		$3,319.46	
	Utilities		$7,212.74	
	UNFH Renovation Refugia & Quarantine Bldg.			
	Construction		-	
	Equipment		$9,818.74	
	Utilities		$22,588.87	
	SMARC Species Husbandry and Collection		$155,785.17	
	UNFH Species Husbandry and Collection		$261,401.05	
	Diver Salaries		$0	
	Water Quality Monitoring System		$5,655.55	
	Fish Health Unit		$7950.65	
	SMARC Reimbursables		$78,484.38	
	UNFH Reimbursables		$159,921.39	
		Subtotal	*$712,138.00*	
		Admin Cost	*$156,670.36*	
2	**Research**			**$396,994.15**
	BIO-WEST: CSRB pupation (2021 Rollover)		$1,587.36	
	BIO-WEST: Dryopid Captive Holding		$72,200.46	
	Texas State: PCA Genetics		$1,826.17	
	University of Texas: Salamander Gene Expression		$41,014.19	
	Auburn University: Invertebrate Tagging		$26,650.57	
	USFWS Research Projects		$139,653.24	
		Subtotal	$325,405.04	
		Admin Cost	*$71,589.11*	
3	**Species Propagation and Husbandry**		-	-
4	**Species Reintroduction**		-	-
5	**Reporting**			**$40,019.30**
	SMARC Staff		$24,527.99	
	UNFH Staff		$8,274.71	
		Subtotal	*$32,802.70*	
		Admin Cost	*$7,216.60*	
6	**Meetings and Presentations**			**$17,183.34**
	SMARC Staff		$10,503.19	
	UNFH Staff		$3,581.51	
		Subtotal	*$14,084.70*	
		Admin Cost	*$3,098.64*	
			TOTAL	**$ 1,323,005.15**

ACRONYMS AND ABBREVIATIONS

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

WORKS CITED

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

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

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

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

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

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

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

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

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

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

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

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

PUBLISHED MANUSCRIPTS

Dobbins, B. A., **D. M. Moore**, R. U. Tovar, and D. M. Garcia. 2023. Cannibalism. Herpetological Review 54(2):237-238.

Moore, D. M., M. S. Gillis, and T. S. Funk. In Review. Evaluation of p-Chip microtransponder tags on small-bodied salamanders (*Eurycea* spp.). Amphibian and Reptile Conservation.

PROFESSIONAL PRESENTATIONS FROM STAFF AND COLLABORATORS

Daw, A. 2023. Improvements and modifications to the Edwards Aquifer Refugia Program Husbandry and Collections. Edwards Aquifer Habitat Conservation Plan Stakeholder Committee Meeting, San Antonio, Texas.

Moore, D.M. 2023. Evaluation of p-Chip tags on small-bodied aquatic salamander species. Texas Conservation Symposium, Georgetown, Texas.

Moore, D.M. 2023. Evaluation of p-Chip tags on small-bodied aquatic salamander species. R2 Fish and Aquatic Conservation Science Symposium, Region 2, USFWS.

Moore, D.M. 2023. P-Chip tagging in small-bodied aquatic organisms. Workshop taught at the San Marcos Aquatic Resources Center, San Marcos, Texas. (March 21, 2023)

Moore, D.M. 2023. The application of p-Chip tags on small-bodied aquatic organisms. The 70th Annual Meeting of The Southwestern Association of Naturalists, San Antonio, Texas.

Moore, D.M. 2023. Mark and recapture of wild San Marcos salamanders. Edwards Aquifer Habitat Conservation Plan Stakeholder Committee Meeting, San Antonio, Texas.

Moore, S.E. 2023. The historical fountain darter tissue archive. The 70th Annual Meeting of The Southwestern Association of Naturalists, San Antonio, Texas.

Moore, S.E. 2023. Archiving preserved biological samples and DNA viability testing. Second Annual Fish and Aquatic Conservation Science Symposium, Region 2, USFWS.

West, B. 2023. Improvements to culture techniques for Peck's cave amphipod. Second Annual Fish and Aquatic Conservation Science Symposium, Region 2, USFWS.

APPENDICES

A. 2023 EA Refugia Work Plan
B. Mark and recapture of San Marcos salamanders
C. Captive husbandry and propagation of the Comal Springs dryopid beetle
D. Tagging aquatic invertebrates
E. Genetic assessment of Peck's cave amphipod
F. Comparative gene expression in San Marcos salamanders to target reproductive triggers in captivity
G. Genetic assessment of the Comal Springs riffle beetle in Landa Lake
H. Monthly reports
I. Fish health unit reports
J. Supersaturation Event

Edwards Aquifer Authority
2023 Work Plan

2023 Edwards Aquifer Authority Work Plan Budget

EAHCP Section	Conservation Measure	Table 7.1	Estimated 2023 Budget[a]
5.1.1	Refugia	$1,678,597	$1,750,760[E]
5.1.2	VISPO	$4,172,000[b]	$9,987,551[c]
5.1.3	RWCP	$1,973,000	$0
5.1.4	Stage V	NA	NA
5.5.1	ASR Leasing & Forbearance	$4,759,000	$5,765,325
	ASR O&M	$2,194,000	$0
5.7.2	Water Quality Monitoring	$200,000	$65,000
6.3.1	Biological Monitoring	$400,000	$800,702[d]
6.3.3	Ecological Model	$25,000	$0
6.3.4	Applied Research	$0	$250,000
FMA §2.2	Program Management	$750,000	$1,742,628
Total		$16,151,597	$20,296,966

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

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

c. On October 1, 2022, the VISPO program was triggered, resulting in suspension payments totaling $9,987,551.

d. Includes Critical Period Monitoring if required.

e. Includes $517,282.41 of unspent funds to be used towards operational and research effort costs.

2023 Edwards Aquifer Authority (EAA) Work Plan and Funding Application Amendments

Amendment #	Date EAHCP Committee Approved	Conservation Measure Amended	Y/N Funding Application Change	Funding Application Change ($)	Date EAA Board Approved	Comments
0	5/19/2022	Original Work Plan	NA	NA	NA	Original Work Plan
1	10/13/2022	VISPO, Water Quality Monitoring, and Program Management	N	N	11/8/2022	Updated amount for VISPO suspension payments as well as updated Water Quality Monitoring and Program Management with known activities and 2023 costs
0	10/13/2022	Original Funding Application	NA	NA	11/8/2022	Original Funding Application
2	12/15/2022	Refugia	Y	$517,282.41	NA	Updated Refugia with known activities and revised 2023 costs

5.1.1 Refugia Program

Introduction

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

This annual work plan and associated cost estimate have been developed per the requirements of contract number 16-822-HCP for the Implementation of the Refugia Program under the Edwards Aquifer Habitat Conservation Plan (EAHCP). The tasks and subtasks that follow provide the details for the services to be performed in 2023, which provide for the maintenance of a refugia population of the Covered Species (Table 1), including salvage, propagation, and restocking of the species (if species-specific habitat triggers occur and species are extirpated), plus research conducted on the Covered Species.

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

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

Long-term Objective

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

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

2023 Assumptions
As work plans are developed almost a year prior to implementation, it is possible that methods described herein will be contingent on the status of the current year's activities or authorization from the HCP process. If conditions change, this work plan may need to be amended to accommodate realized outcomes.

The following potential situations could necessitate methodology adjustments.

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

Target for 2023 (Deliverables and Methods by Task):

Task 1. Refugia Operations

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

Table 2. Target refugia numbers and census by species.

Species	Standing Stock	Refugia Stock	Salvage Stock	Anticipated SMARC census (Jan 2023)	Anticipated SMARC census (Dec 2023)	Anticipated UNFH census (Jan 2023)	Anticipated UNFH census (Dec 2023
Fountain darter (Comal)	1000	1000†	2000	250	500	250	500
Fountain darter (San Marcos)	1000	1000†	2500	500	500	500	500
Texas wild-rice	430	430†	1500	215	215	215	215
Texas Blind Salamander	500	500†	500	250	250	60	80
San Marcos salamander	500	500†	500	250	250	250	250
Comal Springs salamander	500	500†	500	150	150	135	135
Peck's cave amphipod	500	500†	500	250	250	250	250
Comal Springs riffle beetle	500	500†	500	75	75	75	75
Comal Springs dryopid beetle	500	500†	500	*	20	*	20
Edwards Aquifer diving beetle	500	500†	500	*	*	*	*
Texas troglobitic water slater	500	500†	500	*	*	*	*

† Includes specimens within standing stock
# We will not collect Comal fountain darters until we have a better understanding of their mortality rates.
*Catch rates and hatchery survival are uncertain given the rarity of the species.

Collection: In 2023, the USFWS will collect Covered Species as required to reach and maintain target standing and refugia stock numbers as shown in Table 2. The USFWS will coordinate species collections with other ongoing HCP activities (e.g., Biological Monitoring Program) so that collections for refugia do not adversely impact other efforts. The USFWS will carry out species collections through a variety of passive and active collection methods and will minimize aquatic invasive species transfer by conducting collections in accordance with a Hazard Analysis Critical-Control Point Plan. The USFWS will document and report collection efforts to the EAA. The USFWS will distribute captured organisms between the SMARC and UNFH facilities to ensure redundancy and to expedite the obligation to establish and maintain two refugia populations at separate locations. The USFWS will hold all species in respective quarantine areas until their health has been assessed. Staff will incorporate quarantined organisms into the general refugia population once they have determined that such specimens are healthy and free from invasive species. The USFWS will share reports, including test results, produced as part of the quarantine process.

The following sections briefly describe planned 2023 collection, maintenance, and propagation efforts for each species.

Fountain Darters:

Collection: In 2023, the USFWS will collect fountain darters from the San Marcos River in coordination with the Spring and Fall Biomonitoring events. This will be more efficient than separate collection events and will reduce habitat disturbance. For refugia purposes, USFWS staff will retain fountain darters collected by biomonitoring staff via drop nets. Staff will collect fish proportionally from the three sections of the San Marcos River: 1) Upper = Spring Lake, 2) Middle = Spring Lake dam to Rio Vista dam, and 3) Lower = below Rio Vista dam to Cape's Dam. The USFWS will thoroughly investigate unusual mortality events. The USFWS will include summary reports to the EAA as part of the monthly reports. Collections will target sufficient fish so to account for regular, expected mortality, such that the captive population should remain at or above the target.

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

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

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

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

Texas wild-rice:

Collection: USFWS staff will collect Texas wild-rice tillers from San Marcos River segments (Figure 1), with a break during summer months when collected wild rice does not fare well due to heat stress. In 2023, staff will target stands and genetic variants that are not already part of the refugia population or require supplementation in collections for SMARC and UNFH. The refugia populations will reflect the wild populations in both their respective proportion, based on the most recent Texas wild-rice survey data, and historical genetic diversity (2021 genetic assessment and Wilson et al. 2016). During tiller collection, the USFWS will record the geographic coordinates, area coverage, and depth of the stand or individual plant. USFWS staff will collect tillers by wading and SCUBA diving. The USFWS will consider georeferenced aerial imagery to help identify distinct TWR stands used for tiller collection.

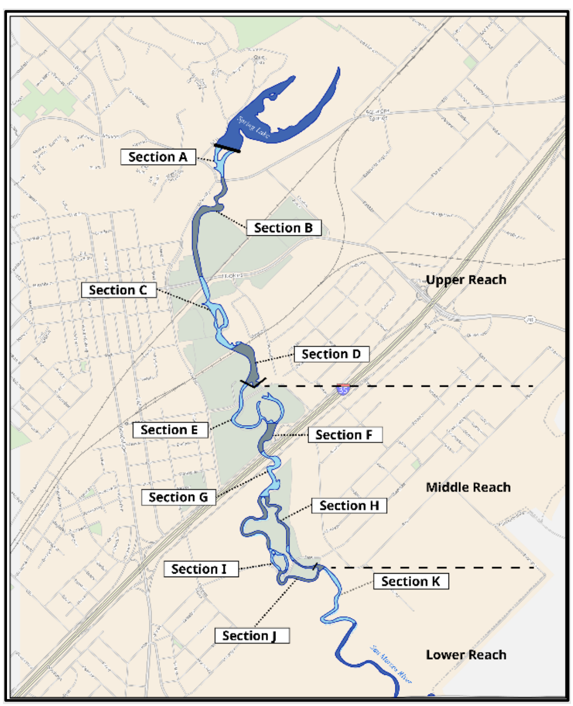


Figure 1. Letters define designated San Marcos River reaches where Texas wild rice is collected for refugia populations.

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

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

Texas blind salamanders:

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

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

As part of quarantine procedures, USFWS staff will swab all large Texas blind salamanders. If they are too small to be swabbed, then we will do a representative batch swab of group-housed salamanders once they are large enough to be safely swabbed. USFWS staff will process these samples at SMARC or other facility to screen for *Batrachochytrium dendrobatidis* (Bd, commonly referred to as chytrid fungus) and *Batrachochytrium salamandrivorans* (Bsal) prior to specimen incorporation into the general refugia population. Staff will retain duplicate swabs in case further testing is warranted. Staff will hold all salamanders in quarantine for at least 30 days and until test results have returned. Previous tests of wild caught salamanders at SMARC (both Texas Blind and San Marcos salamanders) have regularly tested positive for Bd. Positive testing for Bsal will be treated more cautiously as it has not yet been documented in North America. Staff would retain such salamanders in quarantine until further study and recommendations from FWS Fish Health.

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

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

San Marcos salamanders:

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

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

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

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

Comal Springs salamanders:

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

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

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

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

Comal Springs riffle beetle:

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

1. Staff will not sample the same spring orifice two times in a row.
2. Staff will collect all riffle beetle adults and larvae from lures.
3. Standing stock numbers will be reduced to 75 per station until USFWS has established sufficient propagation methods, and we have better understanding of population numbers to derive meaningful standing stock targets.

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

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

Propagation: Propagation methods for this species are being developed.

Peck's cave amphipod:

Collection: USFWS will conduct Peck's cave amphipod collection for standing stock four times annually (Table 5). Staff will collect adult Peck's cave amphipods with drift nets and by hand at a variety of locations (drift nets: Spring Run 3, N = 2; Spring Island and associated Spring Island habitats: hand collection).

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

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

Comal Springs dryopid beetle:

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

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

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

Edwards Aquifer diving beetle:

Collection: Staff will collect Edwards Aquifer diving beetles with drift nets (Table 5). Staff will set drift nets at a variety of locations where the species has been collected in the past (Texas State University Artesian Well N = 1; and Diversion Springs N = 1). USFWS staff will deploy and check drift nets at the Artesian Well when as Texas State University allows.

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

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

Texas troglobitic water slater:

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

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

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

Table 5. A tentative schedule for all species sampling during 2023. Collections listed here are subject to change with extenuating circumstances such as weather and coordination with external partners. USFWS will notify EAA and partners of sampling dates as they become known or changed.

Edward's Aquifer Species Collection Plan 2023			
Date (month)	**Interval**	**Location**	**Target Species**
January	14 Consecutive days with traps checked 2-3 times a week	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
January	1 day sampling event, hand pick from downed wood	Landa Lake	Comal Springs dryopid beetle
February	14 Consecutive days with traps checked 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
February	Set lures	Spring Run, Landa Lake	Comal Springs dryopid beetle, Comal Springs riffle beetle, Peck's cave amphipod
February	1 day sampling event	San Marcos River	Texas wild rice
March	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
March	Collect Lures	Spring Run, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod
March	1 day sampling event, hand pick	Landa Lake	Peck's Cave amphipod
March	1 day sampling event	Comal Springs	Comal Springs salamander
March	1 day sampling event, hand pick from downed wood	Landa Lake	Comal Springs dryopid beetle
April	Check 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
April	1-2 day sampling event	Spring Lake and below dam	San Marcos Salamander

Edward's Aquifer Species Collection Plan 2023			
Date (month)	**Interval**	**Location**	**Target Species**
April	1 day sampling event	San Marcos River	Texas wild rice
April	Throughout, coincide with bio-monitoring	San Marcos River	Fountain darters
April	Drift net, donated from bio-monitoring	Comal Springs	Peck’s cave amphipod
May	Set lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck’s cave amphipod
May	14 Consecutive days with traps check 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
May	1-day sampling event	San Marcos River	Texas wild-rice
June	Collect lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck’s cave amphipod
June	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
June	1 day sampling event, hand pick	Landa Lake	Peck’s Cave amphipod
June	1 day sampling event	Comal Springs	Comal Springs salamander
June	Set lures	Western Shore	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck’s cave amphipod
July	14 Consecutive days with traps check 2-3 times a week	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander

Edward's Aquifer Species Collection Plan 2023			
Date (month)	**Interval**	**Location**	**Target Species**
July	Collect lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod
August	Set lures	Western Shore	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod, Texas troglobitic water slater
August	14 Consecutive days with traps check 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
August	1-2 day sampling event	Spring Lake and below dam	San Marcos salamander
September	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
September	1 day sampling event, hand pick	Landa Lake	Peck's Cave amphipod
September	1 day sampling event	Comal Springs	Comal Springs salamander
September	Collect lures	Western Shore	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod
October	14 Consecutive days with traps checked 2-3 times a week	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
October	Throughout, coincide with bio-monitoring	San Marcos River	Fountain darters
October	Drift net, donated from bio-monitoring	Comal Springs	Peck's cave amphipod
October	1 day sampling event	San Marcos River	Texas wild rice

Edward's Aquifer Species Collection Plan 2023			
Date (month)	**Interval**	**Location**	**Target Species**
October	1 day sampling event, hand pick from downed wood	Spring Runs, Landa Lake	Comal Springs dryopid beetle
November	14 Consecutive days with traps checked 2-3 times a week	Primer's Fissure & Johnson's Well	Texas blind salamander
November	1 day sampling event, hand pick	Landa Lake	Peck's cave amphipod
November	1 day sampling event	Comal Springs	Comal Springs salamander
November	Set lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod
December	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander
December	1 day sampling event	San Marcos River	Texas wild rice
December	Collect lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle, Peck's cave amphipod

Refugia Stocks:

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

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

Propagation: Propagation for stocking is not anticipated during 2023.

Salvage Stocks:

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

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

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

Construction/Renovation/Infrastructure/Facility:

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

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

Staffing/Labor/Personnel:

The two Program Leads (Research and Husbandry/Collections) will mentor and train lower-graded employees, oversee facility maintenance and repair, develop, and implement budgets, and organize activities that relate to all contract activities. The program leads will manage and coordinate research, propagation, culture, and field activities related to the refugia. The leads are expected to provide proper and efficient use of facilities and staff resources. These leads will work with the Center Director and the Deputy Director to ensure that contractual obligations are met in a timely manner. In coordination with the Deputy Center Director, they will prepare all the written materials required for the reimbursable agreement reporting. Likewise, the leads will also prepare oral presentations to be used as briefing statements, outreach presentations, internal reports, work summaries, and technical presentations at professional meetings. The two leads will continue to work and communicate regularly with partners, USFWS personnel and other researchers to meet USFWS and contract goals.

Under the direction of the Program Leads, biologists and biological science technicians, split between SMARC and UNFH, will assist with the collection, daily upkeep, maintenance, propagation, and research efforts for the ten species at the SMARC and UNFH. This includes maintaining culture and experimental production systems, keeping records along with entering, filing, and collating data. The biologists and technicians will also generate basic summary statistics and graphic analyses of data and document program accomplishments through the composition of Standard Operating Procedures (SOPs), reports, and manuscripts.

Permitting:

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

Biosecurity:

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

Husbandry Pilot Studies:

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

Mark spring runs and upwelling – The refugia will use a highly sensitive GPS unit to mark spring upwellings and openings to an accuracy within a few centimeters. These locations will be checked and remeasured during routine sampling events to track the movement and/or closure of spring upwellings and opening.

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

Task 2. Research

The Research Plan for 2023 will involve a series of projects designed to improve propagation of captive populations, genetic assessment of wild populations, and development of reintroduction plans. To inform refugia collections and reintroduction plans, the EARP will conduct a population genetic analysis of Comal Springs riffle beetle and Peck's cave amphipod and build on 2022 research by doing a mark-recapture study on San Marcos salamanders. Collaborative research will focus on dryopid beetle propagation, tagging Comal Springs riffle beetles for tracking individual survival in the refugia, and future collection and reintroduction strategies.

The total cost for proposed 2023 research, given the following projects, is approximately$685,100. The following section describes the basic components of each of these proposed 2023 activities. FWS salary and support for all research is budgeted at $180,000 for the 2023 Work Plan. FWS salary is incorporated into each of the research projects as FWS leads or co-leads each project. Unfortunately, end of year 2022 partnered research efforts with BIO-WEST did not clear until 2023 and is reflected under Task 2 Partnered Research as "BIO-WEST: CSRB Propagation 2022 Rollover" in the sum of $1,518. Previously unspent Task 2 funds in the approximate sum of $212,780 are budgeted to fund 2023 partnered research projects.

The EARP is asking for an additional $304,502 to be added to the 2023 Task 1 budget. This will come from previously unspent funds for system improvements, the purchase of automated Controller/Monitoring units, expanding live food production systems to reduce dependency on external sources, and to transition FWS staff to permanent positions within the EARP. refugia.Table 6. Updated table showing the level of knowledge for each covered species. Knowledge score is a gradient from 0 to 5, where 0 is complete lack of knowledge and 5 indicates the existence of documented procedures for that species. Species with knowledge scores of 5 in each category indicate the species is in complete refugia.

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

Edwards Aquifer diving beetle	1	0	0	0	0

Project 1:

Title: Continuation of genetic assessment of Comal Springs riffle beetle
Species: *Heterelmis comalensis*
Principal: USFWS
Overview: A population wide assessment through fine sampling can provide population metrics to inform future conservation and refugia needs. FWS will work to collect Comal Springs riffle beetles across their range. FWS staff will use high-throughput sequencing to make population measurements at the genetic level.
Budget: $99,856
Benefit to the Refugia: A genetic assessment of the Comal Springs riffle beetle population at Landa Lake will provide valuable information on genetic variation and distribution of that variation in the wild. We do not yet know the extent individuals move between spring openings, thus genetic material (migration). Unique variation at specific spring openings would require different levels of representation in the refugia to reflect wild populations. Better understanding the variation in the wild would inform the minimum number of individuals needed in refugia to maintain wild variation in captivity.
Expected Results: A report will be presented to the EAA and a peer review publication will be generated, if appropriate.

Project 2:

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

- BIO-WEST support: **Year 1: $125,000**, Year 2: $125,000
- FWS support: $30,000
- Total: $155,000

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

Project 3:

Title: Tagging aquatic invertebrates

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

- Auburn University Support: **Year 1: $64,240**, Year 2: $52,080, Total: $116,320
- FWS Support: $30,000
- Total: $94,240

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

Project 4:

Title: Genetic assessment of wild Peck's cave amphipod
Species: *Stygobromus pecki*
Principal/Co-PI: Texas State University / USFWS
Overview: The refugia can reliably collect, house, and propagate Peck's cave amphipod, but little is known about their genetic diversity or population structure. This study will assess the genetic diversity of Peck's cave amphipod in the wild and the refugia populations. This will be a two-year project where tissues are collected, DNA process, and methods optimized the first year. The second year will be sequencing and data analysis.
Budget: Two-year study

- Texas State Support: **Year 1: $32,900**, Year 2: $98,822, Total: $131,722
- FWS Support: $30,000
- Total: $62,900

Benefit to the Refugia: This study will assess the population structure and genetic

diversity of wild Peck's cave amphipod. This study will also determine how well the captive refugia population reflects the wild population and the inform reintroduction plan.
Expected Results: Interim report will be presented to the EAA and a peer review publication will be generated, if appropriate.

Project 5:

Title: Mark recapture of wild San Marcos salamanders
Species: *Eurycea nana*
Principal/Co-PI: USFWS
Overview: A successful reintroduction requires individuals to survive after reintroduction. To determine if individual survive reintroduction events, the same individuals need to be recaptured through repeated surveys. To fully assess reintroduction success, a mark recapture study must occur first to determine baseline expectation for recapture rates of uniquely identified individuals. Once this baseline expectation is determined, future reintroduction success rates can be more accurately measured. This research will inform the future reintroduction strategies by assessing how often individuals are recaptured after being marked. Additionally, this research will inform how often salamanders stay in the same location or move between locations, helping the Refugia determine key locations that will increase successful reintroduction of San Marcos salamanders, in the event reintroduction is necessary.
Budget: $33285
Benefit to the Refugia: Inform reintroduction plans and add to the knowledge matrix
Expected Results: Report will be presented to the EAA and a peer review publication will be generated, if appropriate

Project 6:

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

Budget: Two-year study

- **University of Texas Support: Year 1: $84,759**, Year 2: $112,719
- **FWS Support: $30,000**
- **Total: 114,759**

Benefit to the Refugia: Assess the optimal timing for captive propagation of San Marcos salamanders and identify potential reproduction triggers to inform further research.
Expected Results: Interim report will be presented to the EAA and a peer review publication will be generated, if appropriate.

Task 3. Species Propagation and Husbandry

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

Task 4. Species Reintroduction

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

Reintroduction Plan for 2023: None

Any anticipated triggers being prepared for: Given current weather predictions, spring flows, and the Edwards Aquifer water level, no anticipated triggers are anticipated during the 2022 performance period.

Task 5. Reporting

5.1 Species specific Propagation plans (SOPs): Refine throughout year as needed
5.2 Species specific Genetic Management plans: Texas wild-rice, Texas blind salamander, San Marcos salamander, Peck's cave amphipod; contingent on when genetic study results are finished.
5.3 Species specific reintroduction plans: Refine as needed
5.4 2023 EAHCP Annual Program reporting– A year-end report of 2023 activities will be provided to the EAA no later than 1/31/2024.
5.5 Program reporting as required by ITP and TPWD. TPWD Scientific Research Permit Report will be filed July 31, 2023.
5.6 Descriptions and photographs of procedures from collections to restocking – Photographs and documentation of collection and restocking will be included in the monthly report to the EAA CSO along with the year-end report.
5.7 Summaries of any data analyses, research, or genetic analyses – Research projects and results of collection efforts will be provided to the EAA in the monthly reports, year-end documentation, and stand-alone documents (agreed upon by Center director and HCP

CSO).

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

5.9 Monthly electronic reports to HCP CSO: A monthly report of all activities will be provided to the HCP CSO. We anticipate providing the report by the 10th of each month for the previous month's activities.

Task 6. Meetings and Presentations

Planning or coordination meetings:

- Yearly planning meeting with SMARC and UNFH staff

- Public meetings
  - EAA Board
    - End of year report
    - Present research results
  - Implementing Committee
    - End of year summary
  - Stakeholder Committee
    - End of year summary
  - Science Committee
    - Methods for research projects
    - Present research results
  - Professional Scientific Meetings

Monitoring:

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

Budget:

U.S. Fish and Wildlife Service 2023		Task Budget Amount	Total Task Budget Amount
TASK 1	**Refugia Operations**		**$960,750**
	SMARC Refugia & Quarantine Bldgs.		
	Equipment & Building Maintenance	$10,000	
	Utilities	$10,000	
	UNFH Refugia & Quarantine Bldgs.		
	Equipment & Building Maintenance	$10,000	
	Utilities	$20,000	

	U.S. Fish and Wildlife Service 2023	Task Budget Amount	Total Task Budget Amount
	SMARC Species Husbandry and Collection Salaries	$190,000	
	UNFH Species Husbandry and Collection Salaries	$290,000	
	Water Quality System	$10,000	
	Divers Salaries	$3,500	
	Fish Health	$8,000	
	SMARC Reimbursable	$80,000	
	UNFH Reimbursable	$160,000	
	Subtotal	*$787,500*	
	Admin Cost Subtotal	*$173,250*	
TASK 2	**Research**		$685,101.36
	BIO-WEST: CSRB Propagation 2022 Rollover	$1,518.36	
	BIO-WEST: Dryopid	$125,000	
	Texas State University: PCA Genetics	$32,900	
	University of Texas: Salamander Gene Expression	$84,759	
	Auburn University: Invertebrate Tagging	$64,240	
	FWS Salary	$180,000	
	FWS Materials	$73,141	
	Subtotal	*$561,558.36*	
	Admin costs for Task 2	*$123,543*	
TASK 3	**Species Propagation and Husbandry**		$0
	Subtotal		
TASK 4	**Species Reintroduction**		$0
	Subtotal		
TASK 5	**Reporting**		$84,421
	SMARC Staff	$53,197	
	UNFH Staff	$16,000	
	Subtotal	*$69,197*	
	Admin costs for Task 5	*$15,224*	
TA	**Meetings and Presentations**		$20,488

U.S. Fish and Wildlife Service 2023		Task Budget Amount	Total Task Budget Amount
	SMARC Staff	$12,000	
	UNFH Staff	$4,793	
	Subtotal	*$16,793*	
	Admin costs for Task 6	*$3,695*	
	TOTAL	**$1,750,760.36**	

*Agreement with Texas State is pending.

<u>Projected (2023) Budget Summarized by Task:</u>

Task 1: $960,750
Task 2: $685,101.41
Task 3: $0
Task 4: $0
Task 5: $84,421
Task 6: $20,488

<u>Projected (2023) Subcontractor Expenses Summarized by Task</u>

Task 1: $0
Task 2: BIO-WEST CSRB Propagation Rollover ($1,518.36)
Task 2: BIO-WEST ($125,000)
Task 2: Texas State ($32,900)
Task 2: University of Texas ($84,759)
Task 2: USGS Auburn University Co-op ($64,240)
Task 3: $0
Task 4: $0
Task 5: $0
Task 6: $0

Timeline of 2023 Milestones

January	Subcontracted research awards executed
	2023 Specific Research Study Plans finalized
July	Submit and renew TPWD permit
September	Draft Research Reports
December	Draft Annual report

Literature Cited

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

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

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

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

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

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

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

2023 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Desiree M. Moore and Dr. Katie Bockrath

San Marcos Aquatic Resources Center
U.S. Fish and Wildlife Service

Table of Contents

Background

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

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

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

Objectives

Our objective is to examine the recapture rates associated with wild San Marcos salamanders tagged with p-Chip microtransponder tags.

Benefits to the Refugia

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

Methods

Pilot Study

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

Field Study

San Marcos salamanders were collected from three sites across Spring Lake and the headwaters of the San Marcos River. The three sites were near the Meadows Center (Hotel), surrounding the diversion pipe (Diversion), and in the San Marcos River just below the eastern spillway (Eastern Spillway). Divers collected salamanders for tagging from the floor of Spring Lake at the Diversion site once monthly in May and June 2023. SMARC staff snorkeled to collect salamanders for tagging from the Hotel and Eastern Spillway sites twice each in May and once each in June. Additionally, divers joined snorkelers to collect salamanders for tagging at the deeper areas of the Hotel site at the first May collection and the June collection (Table 1). A tagging station was set up on the bank near each site.

Table 1. The number of tagged and recaptured San Marcos salamanders from each site each field day of the San Marcos salamander mark-recapture study in 2023. The number of untagged salamanders that were collected and released without tagging due to size restrictions or because tagging was completed is also reported.

Date	Site	# tagged	# recaptured	# untagged	total catch
9-May-23	Eastern Spillway	82	0	5	87
10-May-23	Diversion	33	0	0	33
11-May-23	Hotel	53	0	8	61
30-May-23	Eastern Spillway	53	0	16	69
31-May-23	Hotel	22	0	0	22
12-Jun-23	Eastern Spillway	75	6	20	101
14-Jun-23	Hotel	74	6	25	105
20-Jun-23	Diversion	62	2	8	72
26-Jun-23	Hotel	0	9	21	30
27-Jun-23	Eastern Spillway	0	4	90	94
10-Jul-23	Hotel	0	3	19	22
12-Jul-23	Diversion	0	2	78	80
13-Jul-23	Eastern Spillway	0	4	53	57
8-Aug-23	Eastern Spillway	0	2	95	97
10-Aug-23	Hotel	0	3	54	57
22-Aug-23	Hotel	0	1	101	102
24-Aug-23	Eastern Spillway	0	0	108	108
6-Sep-23	Diversion	0	5	79	84
13-Sep-23	Hotel	0	3	23	26
14-Sep-23	Eastern Spillway	0	1	59	60
25-Sep-23	Hotel	0	0	51	51
27-Sep-23	Eastern Spillway	0	1	94	95
10-Oct-23	Eastern Spillway	0	3	145	148
11-Oct-23	Diversion	0	5	87	92
12-Oct-23	Hotel	0	1	43	44
23-Oct-23	Hotel	0	0	60	60
24-Oct-23	Eastern Spillway	0	1	104	105
8-Nov-23	Diversion	0	4	95	99
14-Nov-23	Eastern Spillway	0	2	90	92
16-Nov-23	Hotel	0	0	14	14

SMARC staff tagged wild San Marcos salamanders with p-Chips to individually identify the salamanders upon recapture. First, staff anesthetized salamanders by immersion in tricaine methanesulphonate (MS-222, 0.5 g/L) buffered with sodium bicarbonate. Staff then examined salamanders and rejected any with visible injuries to prevent harming them further through tagging. Each salamander was measured to obtain the snout-to-vent length (SVL), sexed if possible, and injected with a p-Chip subcutaneously at the base of the tail near the left hindlimb. Tail clips were also collected from each salamander and will be used for eventual population genetic analyses. Salamanders were placed in a container of fresh river water to recover from anesthesia and tagging. Staff released the salamanders after they began swimming normally again. The divers and snorkelers returned the salamanders to the interstitial spaces among rocks in the general area where they were collected to provide cover for optimal healing and protection from predators.

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

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

To analyze these preliminary data, SMARC staff developed summary statistics to analyze salamander recapture rates and sizes across sites. Because salamander collectors targeted salamanders ≥20mm SVL, no salamanders under 20mm SVL were used in analyses. A one-way ANOVA and post-hoc pairwise t-tests were used to determine differences in salamander size among sites. Net movement directionality was not examined due to no movements being recorded. The amount of time spent searching for salamanders will be used for modeling purposes in year two of the study but has not yet been analyzed.

Results

A total of 453 San Marcos salamanders were tagged across sites, with 46% tagged at Eastern Spillway, 33% tagged at Hotel, and 21% tagged at Diversion (Table 2). The recapture rate across sites was 13%, with the highest rate occurring at the Diversion site (17%). The recapture rate at Hotel was 15%, and the lowest recapture rate was 10% at the Eastern Spillway (Table 2). Eleven of the tagged salamanders were recaptured twice, Five at Diversion, three at Eastern Spillway, and three at Hotel. Additionally, one salamander was recaptured four times at Diversion, which was every collection since tagging until December.

There were 2,013 San Marcos salamanders collected for this study in 2023. Collections varied by site and month (Figure 1). The average number of salamanders collected per collection event was 91 at Eastern Spillway, 78 at Diversion, and 46 at Hotel.

The lengths of all salamanders ≥20 mm SVL collected in 2023 (Table 3) were different among sites ($F_{2,\,834.14} = 40.32$, $P < 0.001$), and post-hoc pairwise t-tests were used to determine that salamanders at the Eastern Spillway site were larger than salamanders at the Hotel (P = < 0.001) and Diversion (P = < 0.001) sites. The Hotel and Diversion sites were not statistically different (P = 0.13).

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

	Tagged				Recaptures			
Site	#	Mean SVL (mm) ± SD	Min SVL (mm)	Max SVL (mm)	#	Mean SVL (mm) ± SD	Min SVL (mm)	Max SVL (mm)
Eastern spillway	209	28.7 ± 4.3	20	40	20	26.8 ± 4.4	21	33
Diversion area	96	27.2 ± 3.2	20	32	16	27.8 ± 2.9	22	31
Hotel area	148	27.0 ± 3.1	20	35	23	26.9 ± 3.8	20	35
Total	453	27.8 ± 3.8	20	40	59	27.1 ± 3.7	20	35

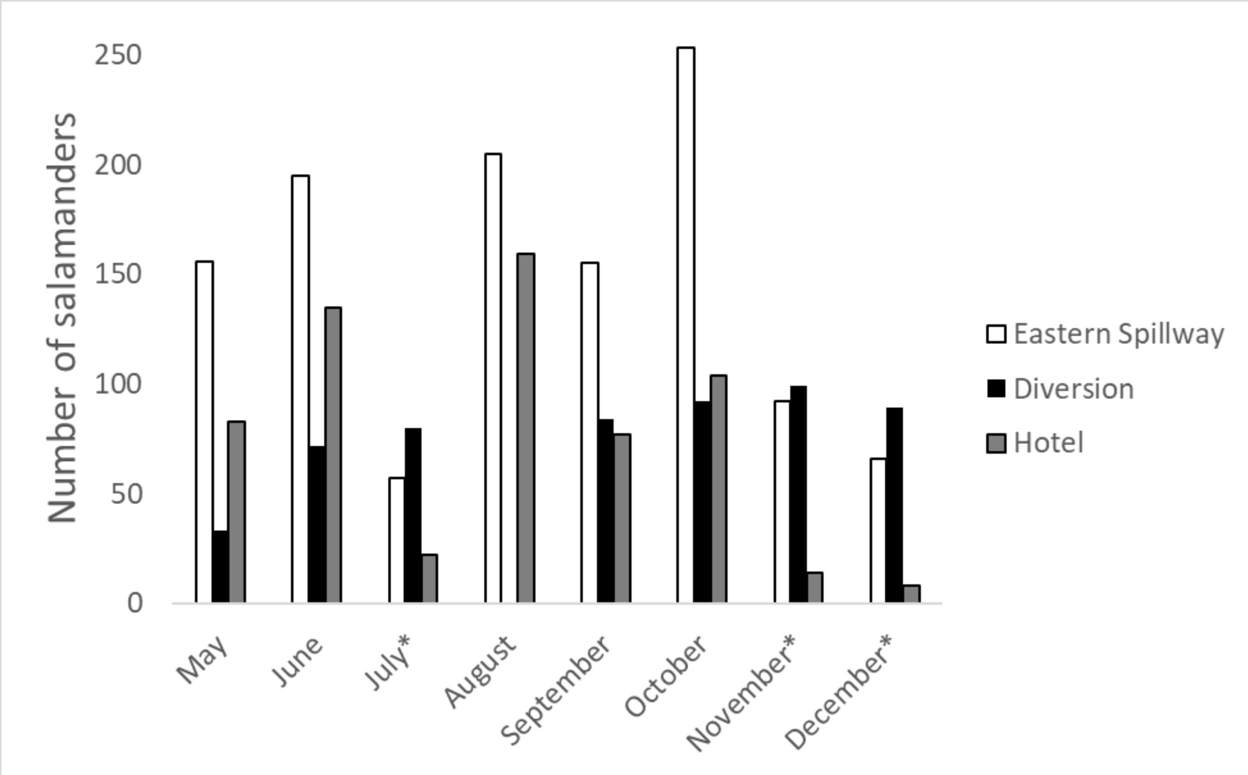


Figure 1. The number of San Marcos salamanders collected each month at each site in 2023. Eastern Spillway and Hotel sites were sampled twice monthly except for months with an asterisk (*), in which they were sampled once. Diversion was sampled once monthly except for August, when it could not be sampled.

Table 3. The snout-vent lengths of all salamanders ≥20 mm SVL collected for this project in 2023, where the mean and standard deviation (Mean ± SD), minimum (Min), and maximum (Max) of the lengths are reported. Additionally, the length data for all salamanders collected are reported. Salamanders <20 mm SVL are most likely underrepresented due to collection goals.

	Salamanders ≥20 mm SVL				All salamanders collected			
Site	#	Mean SVL (mm) ± SD	Min	Max	#	Mean SVL (mm) ± SD	Min	Max
Eastern spillway	964	28.0 ± 4.1	20	40	1019	27.4 ± 4.8	8	40
Diversion area	425	26.5 ± 3.6	20	35	507	24.8 ± 5.1	8	35
Hotel area	397	26.2. ± 3.4	20	35	489	24.1 ± 5.4	8	35
Total	1785	27.2 ± 3.9	20	40	2013	25.9 ± 5.3	8	40

References

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

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

Comal Springs dryopid beetle (*Stygoparnus comalensis*) research: improving our understanding of their life-cycle and refugia requirements

Interim Report
2023

Background

The Comal Springs dryopid beetle, *Stygoparnus comalensis* Barr and Spangler, 1992 (Coleoptera: Dryopidae) is a troglobitic beetle known primarily from Comal Springs, Comal County, Texas, USA; it has also been collected from several springs in Hays County, Texas (Gibson et al., 2008). It is protected by the US Fish and Wildlife Service (USFWS) and has 22 ha of designated critical habitat (USFWS, 1997, 2013). Like many other species in the Edwards Aquifer, *S. comalensis* faces numerous threats to its habitat with respect to water quality and water quantity and is presently a Covered Species in the Edwards Aquifer Habitat Conservation Plan (EAHCP). A self-propagating captive refugia population of *S. comalensis* is a goal of the EAHCP, and a better understanding of the habitat, ecology, and life history of this species is essential for meeting that goal.

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

Previous limited efforts to develop a system to maintain *S. comalensis* in captivity did not produce a setup that was distinctly better for this species than any other (BIO-WEST, 2022). For example, the use of live *Platanus* in one of the most recent captive housing chamber designs may be unnecessarily unwieldy for long-term maintenance if other conditions are just as suitable without requiring maintenance of live plants. Field-based observations of this plant species have largely been anecdotal or coincidental (e.g., occurrence of *S. comalensis* near *Platanus* roots in Spring Lake). Clearly, more information on the habitats and biology of this species is needed, while assessment with rigorous experimental and statistical tests will help to better determine preference and performance of *S. comalensis* in a variety of different conditions.

Original Proposal and Objectives

The original proposal was primarily developed by Dr. Ely Kosnicki (former BIO-WEST staff) in collaboration with USFWS based on prior research in the system. The original objectives of this proposal were:

1. Obtain more definitive estimates of larval growth
2. Design and implement the design of an aquaria for maintaining all life stages
3. Expand the collection range

Initial progress and lessons learned in 2023

Dr. Matthew Pintar joined BIO-WEST in 2023 and gained accessed to the SMARC refugia in April. At that time, the aquarium holding previously collected *S. comalensis* had not been monitored in a year. When this system was checked in April it contained no adult *S. comalensis* and fewer than 20 larvae. Because of the unknown origin and age of these mixed larvae, they had no utility for the original objective of estimating larval growth. These larvae were allowed to remain in the original housing chamber, and their numbers dwindled over the following months as efforts focused on collecting new adults.

During much of spring and summer 2023, work focused on Objective 3 (expanding the collection range) and searching for adults in the Comal Springs system. Prior observations that *S. comalensis* occurred on or around roots and on submerged wood served as the basis for collecting efforts. In addition to manually searching around existing wood, roots, and rocks in springs, conditioned wood (found submerged in unfavorable locations elsewhere in Landa Lake, such as not directly on springs) was placed in or on springs at several dozen individual sites in Spring Runs 1, 2, and 3, with a much greater focus around Spring Island. Each site was checked approximately weekly until the end of July when the substantial drop in flows left most of the sites in the spring runs dry. Furthermore, no beetles were found at any of the sites in the spring runs, so collection efforts subsequently focused around Spring Island. Expanding the collection range of this species has been a tertiary objective of this and previous projects, but because of the scarcity of beetles in 2023 and the lack of records on locations of specific sites that were checked in the previous study (including the presence, absence, and/or abundance of beetles at those sites), initial efforts in 2023 focused on gaining an understanding of the system while building the framework for potential future *in-situ* habitat studies and methods for biological monitoring.

By early August, fewer than five adults had been obtained and were alive at SMARC. The lack of adults, combined with the known low oviposition rate and long larval stage, meant that over the remaining timeframe of this specific project (just over 1 year), obtaining an adequate sample size of larval growth over the entire larval stage (per original objective 1) was unlikely. Furthermore, while producing estimates of larval growth and the number of instars that this species passes through could provide novel information on this species, the results of prior work (BIO-WEST, 2022) indicate that the difficulty in obtaining accurate larval size measurements may not produce substantially more useful information than we already have on this species. Additionally, the expansiveness of a setup required for individually housing both breeding pairs of adults and rearing larvae to accurately track individual growth could inhibit use of space for other studies of this species. Gaining a marginally better understanding of growth and development rates also does not necessarily progress substantially towards understanding the optimal conditions that are needed for survival of *S. comalensis* in both captivity and the wild. Therefore, research efforts under this project shifted away from the initial objective of understanding development across the lifecycle of *S. comalensis* towards determining responses to, and preferences for, various environmental conditions.

Collecting efforts continued throughout 2023, and from August through October 2023, one or two adults were typically found each week. Most individuals collected have been from two sites north of Spring Island. A few adults have been found at three other sites near Spring Island, with one adult found on a lure for Comal Springs riffle beetle low-flow monitoring along the Western Shoreline, where few dryopids have ever been found. A few dryopid larvae were collected for the studies outlined below, but their burrowing habitats mean they are difficult to collect. Their visual similarity to the many other larvae typically found on wood makes them difficult to coarsely identify when quickly searching wood before they often burrow. Larvae were collected in August and early September but have not been found since. As of November 2023, seemingly no larvae remain in captivity (although some may be burrowed and not detected). A few larvae were observed to have died during the course of experiments, but most loss (disappearance, burrowing, or unobserved mortality) of larvae has occurred when they were housed in the captive housing chamber from the previous study.

During the time between habitat choice experiments described below, adults are housed communally. What appear to be breeding attempts have been observed when searching the aquarium and sorting adults, but no eggs or larvae have yet to be observed in the adult housing chamber.

Design of habitat choice experiments

A series of ongoing habitat choice experiments were initiated at SMARC in late July 2023. The general idea is that by providing a contrast between two or more habitat conditions we will be able to statistically assess whether dryopids prefer one condition over the other through their presence or activity in that habitat.

In most of these experiments, dryopid adults and/or larvae were assessed simultaneously with adults and/or larvae of *Stenelmis sexlineata* Sanderson, 1938 (Coleoptera: Elmidae). *Stenelmis sexlineata* is an aquatic riffle beetle that reaches approximately the same size as adult dryopids and commonly co-occurs as both adults and larvae with *S. comalensis*, especially at the sites near Spring Island where dryopids are found most often. While *S. sexlineata* has eyes and is a widespread species across the central United States (Schmude, 1992), the populations in Comal and San Marcos springs are among the southernmost populations of this species and exhibit unique coloration that potentially suggests this species may warrant further study. The inclusion of *S. sexlineata* primarily serves to provide a contrast to habitat preferences of dryopids since they are in different families and one has fully developed eyes while the other does not. If initial work suggests similar habitat preferences among the two species, then *S. sexlineata* may serve as an imperfect surrogate to study habitat preferences with greater statistical replication since it is a non-threatened species.

The first series of experiments served to work out the design of the experimental chamber(s) and ensure that these setups could effectively be used to answer the intended questions. Both initial chambers were constructed from clear plastic, but due to their presumed subterranean habitat use, the entire setup was covered in black plastic that shields the beetles from any light. However, after initial studies, the sides of the chambers, in addition to the top, have been blacked out to enable responses to light (see below), and further modification of this setup will depend on the outcome of those studies. Ultimately, two setups have been used for all studies to this point.

- Setup 1 (Fig. 1) is the primary design for paired habitat choice experiments. This is an elongated housing chamber (12” x 3” x 3”) with water flowing into the center of the chamber and out each end. An object is placed at each end of the chamber and the proportional occurrence of beetles on either side can be used to determine habitat preference. This setup is used to assess habitat use on a daily basis, providing beetles time to explore the chamber and choose a preferred side.

- Setup 2 (Fig. 2) is a 5.75" x 5.75" x 6" chamber that can be used to compare more than two habitat types, assess short-term (across minutes or hours) changes to habitat use, or assess relative changes to the habitat itself (such as relative consumption rates of different leaves).

Between different experiments, the study chambers are cleaned to remove any debris, environmental cues, or any other buildup within the chambers. Many aspects of the experiment are randomly assigned as is feasible – individual beetles, positions of objects, arrangement of housing chambers, etc, which reduces the possibility of bias in the experiments. Some of the initial experiments used multiple beetles of the same type within each housing chamber, but recent experiments have included only one beetle of each type per chamber –each chamber has at most one dryopid adult, one *Stenelmis* adult, and one larva. This enables individual beetles to be tracked across multiple days and to accurately statistically account for individual-level changes within an experiment.

Preliminary results of habitat choice experiments

Below is a summary of the primary questions that have been investigated with habitat choice experiments and their preliminary results. These results are meant to provide an overview of patterns observed but do not represent final results. Statistical results are not presented as most of these questions have further replication, additional experiments, or in-depth analysis pending.

Responses to flow

Initial experiments have not shown a clear response to flow among either species or life stage. Two experimental setups were tested. The first design had inflow at one end and outflow only at the opposite end. The second design had inflow at the center, outflow at one end, and the other end blocked to prevent flow. In both experiments, the proportion of adults of both species on either side of the housing chamber was approximately 50:50, indicating no clear preference for inflow vs outflow (first experiment) or outflow vs no flow (second experiment). However, when no objects were placed inside the housing chamber, dryopids have been occasionally (~30% of the time) observed clinging to the mesh on the outflow. This could indicate some response to flow that is not being otherwise detected, or it could indicate that the beetles seek out some more complex structure to cling to. No beetles have ever been observed on the inflow, likely because it is suspended above the middle of the chamber and thus inaccessible. In both experiments, the inflow and outflow have been ~4 cm above the bottom of the chamber, which may restrict the ability of the beetles to respond to it since it may be difficult for adults to climb the smooth walls of the chamber (and impossible for the larvae to do so). Further experiments with different designs to better elucidate responses to flow will be planned in the future.

Habitat type preference

Following observations that both life stages of both beetle species tended to associate with various objects inside housing chambers, a series of experiments were conducted to determine relative preferences between three general types of objects: rocks, wood, and leaves. This also builds on the observation that dryopids are often observed on wood in Landa Lake.

The first experiment tested whether there were relative preferences between a small limestone rock and a similar-sized piece of conditioned sycamore wood. Initial results indicated a strong preference for wood by dryopid adults (86% on wood versus 14% on rocks), a similar preference by *Stenelmis* adults (78% on wood), and a less clear preference by *Stenelmis* larvae (58% on wood). Limited replication with dryopid larvae (N=5) had only one beetle under wood, with the other four beetles on the open bottom of the housing chamber (i.e., not in association with any object type).

The second experiment tested whether there were relative preferences between wood (preferred by adults during the previous experiment) and a similar-sized clump of conditioned sycamore leaves. To hold the leaves in place, a small limestone rock was placed on top, while a rock was also placed on top of the wood for a symmetrical design. In this experiment, 62% of dryopid adults were on wood while *Stenelmis* adults were split 50:50 between wood and leaves. Among the larvae, 75% of *Stenelmis* larvae were within the leaf clump and 25% were under the wood. Among the dryopid larvae, 50% were in the leaf clump, 0 were on or under the wood, while 50% were in the open on the bottom of the housing chamber.

Tree leaf species preference and consumption

Several experiments have been performed to assess whether different species of dead, dried, sterilized, and then conditioned tree leaves are preferred as either habitat and/or food by the beetles. The primary focus of these studies has been on larvae and have ranged in duration from one- or two-day habitat preference studies to a month-long study of relative leaf consumption rates. Overall, these studies require further analysis to fully assess their outcomes, along with follow-up studies to elucidate finer preferences. Generally, there have not been clear preferences for habitat use (one species versus another) while relative consumption rates have not yet been determined. An example of a leaf consumption experiment setup is shown in Fig. 3.

Use of interstitial space

In most experiments of habitat preferences that have been conducted this year, microhabitat use has been documented in addition to the primary habitat contrast of interest tested in the experiment. While formally documenting the use of microhabitats will require aggregating and standardizing the data across all of these different studies, there have been some very clear patterns that have emerged. *Stenelmis* larvae are almost always found within interstitial spaces – usually clinging to a piece of wood/rock between the underside of that object and the bottom of the chamber – or within cracks on the wood/rock, or between leaves in leaf clumps. *Stenelmis* adults are also almost always clinging to an object, typically wood or rocks, and are usually underneath, on the sides, or in cracks. This contrasts to dryopid adults, which while they are also often clinging to various objects, are commonly (>50% of the time) on the top or sides of wood/rocks and clearly moving around. Data on dryopid larvae is much more limited but they were clearly more commonly observed being active and away from any objects than *Stenelmis* larvae.

Responses to light

As a species with vestigial eyes and presumed subterranean habitat use, any responses by dryopids to light could inform design of housing chambers in refugia and inform us of basic biological responses to environmental cues. The ability of insects to detect various wavelengths of light varies among taxa, but species with compound eyes are typically able to detect ultraviolet (UV) and various human-visible wavelengths (typically blue and green, but also sometimes red). Ongoing experiments are assessing the responses of dryopid adults, along with *S. sexlineata* adults and larvae, to full visible spectrum (white) light, red light, blue light, green light, UV light (Fig. 4), and no light (control).

Year 1 Summary

During year one of this project, availability of dryopids limited initial progress on the two primary objectives outlined in the original scope of work. However, during that time progress was made towards becoming acquainted with the system, finding sites where beetles could be collected, and developing methods for potential future *in-situ* studies of dryopids in the Comal Springs system.

Once adult dryopids were obtained, preliminary experiments at SMARC helped to fine-tune a system for testing habitat and other environmental preferences of dryopids (both adults and larvae) and enabled comparison to a co-occurring species, the elmid *S. sexlineata*. These experiments have shown that adult dryopids have a clear preference for occupying organic material, and there is a slight preference for wood among adults. This contrasts with no clear habitat preferences among larval dryopids and a clear preference for leaves among larval elmids. Although experiments assessing dryopid use of leaves requires more analysis to determine results, the greater use of wood than leaves potentially suggests that further work assessing preferences for wood species, conditioning, and other characteristics could better elucidate differences among this potentially preferred habitat.

Despite being a spring-endemic species and typically found at spring openings, there has not been evidence that *S. comalensis* is attracted to flowing water, although this will be a subject of further study. Further, preliminary results suggest that *S. comalensis* is much more active than the similarly sized, co-occuring *S. sexlineata*. Larval dryopids, but especially adults, have been commonly observed actively moving throughout their experimental and holding chambers, including in areas directly exposed to light. This potentially suggests that their vestigial eyes do not restrict their behavioral periods of greatest activity to dark hours, which is common among many insects. Further experiments are necessary to determine additional environmental conditions that are preferred by dryopids and any results may show which conditions are most preferred and potentially most useful for successfully maintaining this species in captivity.

Year 2 Scope of Work

The proposed scope of work for year 2 is built upon the limited amount of available literature for this species and the first-hand knowledge obtained during studies performed in 2023. The primary objective of the second year of this study will be continued experimentation to elucidate preferred habitats of *S. comalensis* and conditions that contribute to survival in captivity. In addition to fine-tuning responses to wood, leaves, and flow that were explored through experiments during year 1, there remain a multitude of conditions that could contribute to an optimal habitat that need to be examined. These factors include things such as substrate size, biofilm coverage, and presence of other organic material, particularly plant roots. Additionally, conspecific and heterospecific attraction and interactions will be explored to assess if beetle movement is influenced by other animals in the system.

A secondary goal during year 2 will be to study the development of any larvae that are produced by the current captive adult population, breeding habits of adults, and conditions that result in successful reproduction. Given the current small number of adults, lack of larvae, and long larval period, efforts will be focused on individual-level short-term changes. This will be accomplished through methods such as assessing biomass changes among larvae and adults based on quality of food provided, as well as assessing time to successful reproduction when provided different food resources.

The availability of beetles has been the major obstacle to continued study of this species with sufficient statistical replication. The final, tertiary, goal of the second year of this study will be to continue to search for locations where beetles are present and persistent while also developing methods that can be used to reliably assess presence, act as potential attractants, and collect beetles.

References

Barr, C.B., and P.J. Spangler. 1992. A new genus and species of stygobiontic dryopid beetle, *Stygoparnus comalensis* (Coleoptera: Dryopidae), from Comal Springs, Texas. Proceedings of the Biological Society of Washington 105: 40–54.

BIO-WEST. 2022. Life-history aspects of *Stygoparnus comalensis* Barr and Spangler, 1992 (Coleoptera: Dryopidae): Comal Springs dryopid beetle research 2021. Final Report. Prepared for the Edwards Aquifer Authority. 24 pp.

Bowles, E.B., and T.L. Arsuffi. 1993. Karst aquatic ecosystems of the Edwards Plateau region of central Texas, USA: a consideration of their importance, threats to their existence, and efforts for their conservation. Aquatic Conservation: Marine and Freshwater Ecosystems 3: 317–329.

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

Schmude, K.L. 1992. Revision of the riffle beetle genus *Stenelmis* (Coleoptera: Elmidae) in North America, with notes on bionomics. PhD thesis. University of Wisconsin. 388 pp.

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

United States Fish and Wildlife Service. 2013. Endangered and threatened wildlife and plants; revised critical habitat for the Comal Springs dryopid beetle, Comal Springs riffle beetle, and Peck's cave amphipod. Federal Register 78: 63100–63127.

Figures to be incorporated later

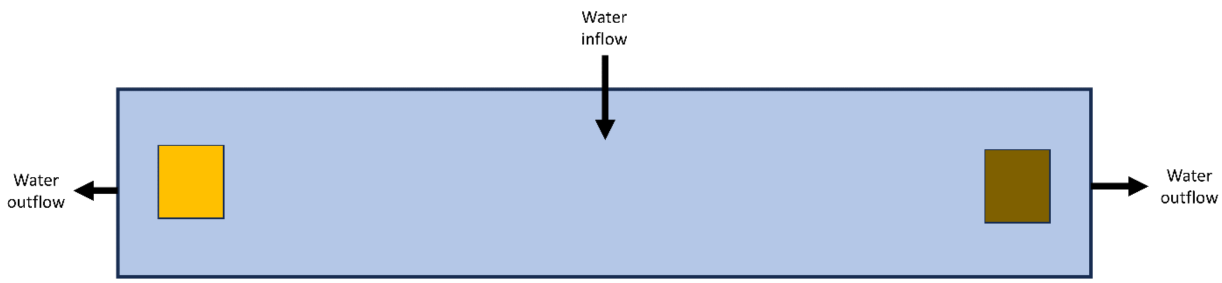


Figure 1. Illustration of the layout of the primary design for paired habitat choice experiments. Figure is from the top-down perspective on a 12×3×3-inch chamber with water inflow at center and outflow at both ends. Colored squares represent two different objects on opposite side of the chamber (e.g., two different species of leaves, wood and rocks, etc.). Figure is not to scale.

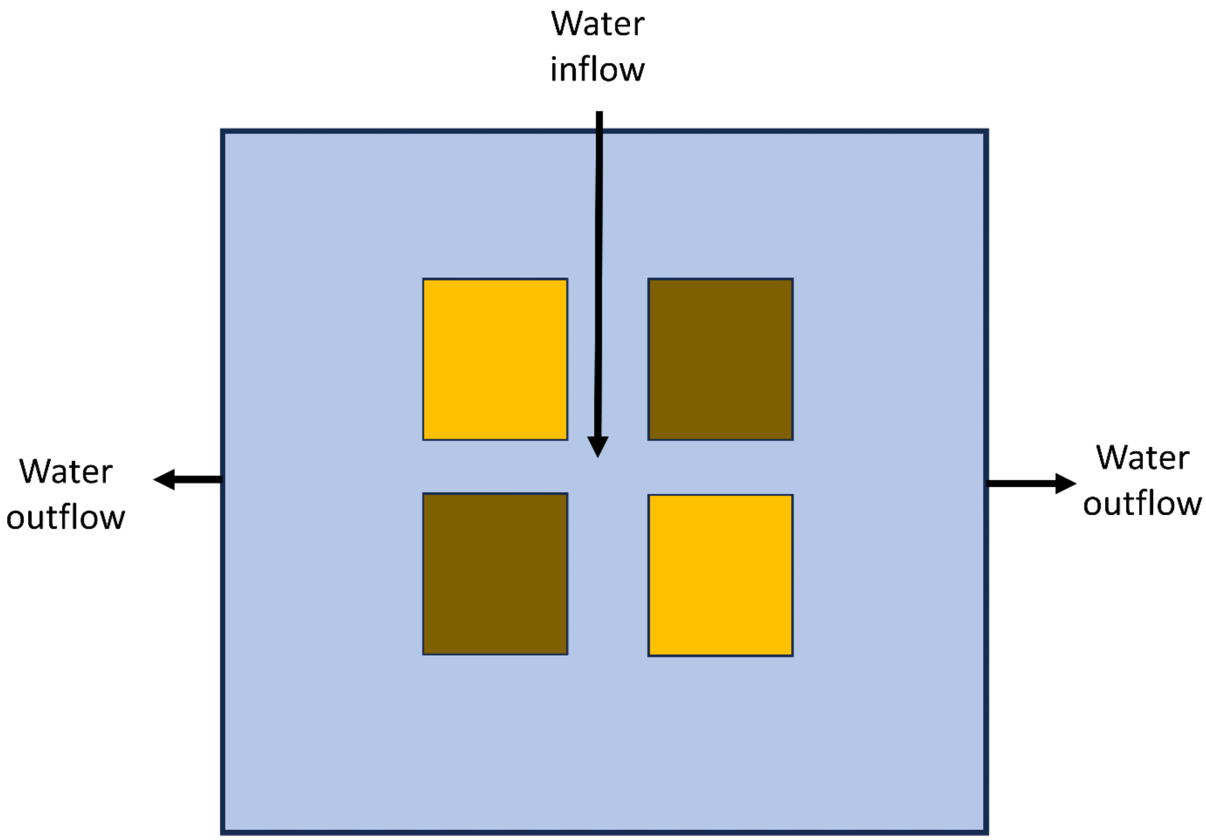


Figure 2. Illustration of the layout of the secondary design for habitat choice experiments. Figure is from the top-down perspective on a 5.75×5.75×6-inch chamber with water inflow at center and outflow on two sides. Colored squares can represent two different species of leaves used to assess habitat use by larval beetles. Figure is not to scale.

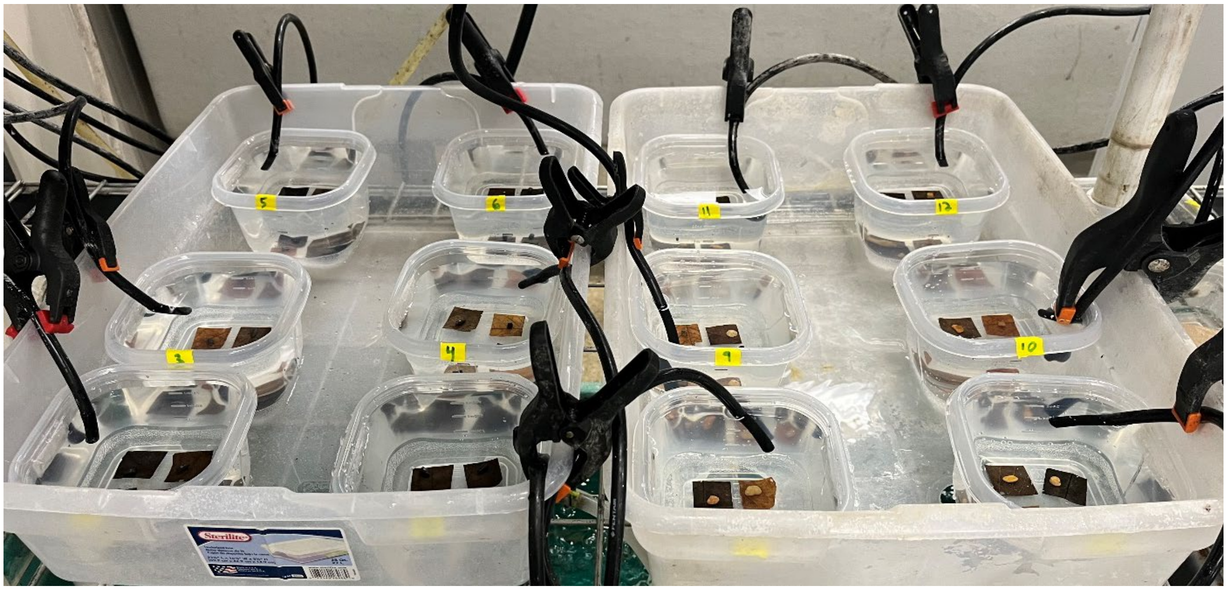


Figure 3. Photo of an experiment using the design in Fig. 2 to assess consumption rates of different species of leaves. This photo shows conditions during setup of the experiment before beetles and lids were added.

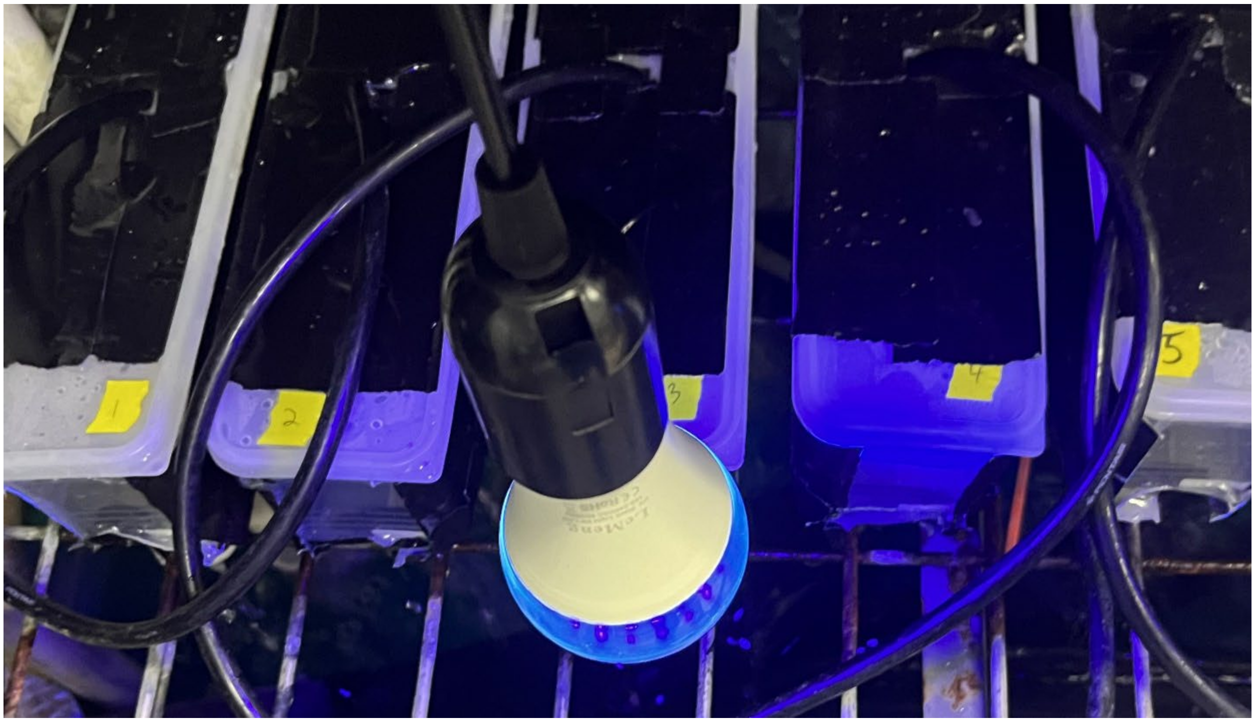


Figure 4. Photo showing experimental setup using chambers illustrated in Fig. 1 during an experiment testing beetle responses to ultraviolet (UV) light. In this experiment, the chamber was blacked out to prevent light from entered from all sides except the end with the lights above, while the entire setup was covered in black plastic to prevent other light sources in the room from influencing activity in the chambers.

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

Investigator: Shannon K. Brewer; U.S. Geological Survey, Alabama Cooperative Fish and Wildlife Research Unit; 334-750-5632; skb0064@aubum.edu

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

Proposal Draft: September 27,202

Project period: January 1, 2023-June 30, 2025

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

Justification:

Peck's Cave Amphipod is a diminutive (< 11-mm, USFWS, 2013), federally endangered species that occurs in the Edwards Aquifer and is the focus of ongoing monitoring efforts. The amphipod is endemic in groundwater springs and nearby habitats of the Edwards Aquifer. Peck's Cave Amphipod is uniquely adapted to groundwater ecosystems where it tends to occur in the highest densities. The amphipod is adapted to these habitats via a laterally flattened body, and they lack eyes and pigment; however, much of the rest of the species life history in wild populations is unknown.

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

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

As tags have become smaller over time, their use has increased where individual or batch identification is needed. Passive integrated transponders (PIT), for example, have several characteristics that increased the accuracy of mark-recapture studies (Gibbons and Andrews 2004; Hewitt et al. 2010). Recaptures of small animals have been used for a variety of purposes including estimating sampling efficiency (Price and Peterson 2010), estimating population size (Pine et al. 2013), estimating survival (Moore and Brewer 2021) and growth (Walters et al. 2012), evaluating movement (Steffensmeier et al. 2022) and habitat use (Teixeira and Cortes 2007), and even studying animal behavior (McCormick and Smith 2004). The technological advancements have been impressive in recent years (Musselman et al. 2017). P-Chips are a relatively new tagging technology that have been used on small, endangered fish with success (Moore and Brewer 2021). Additionally, p-Chips have been successfully used on insects like the Western Honeybee *Apis mellifera* (Tenczar et al. 2014) and Rock Ant *Temnothorax albipennis* (Robinson et al. 2014) by external adhesion. P-Chips are micro-transponder tags (500 x 500 x 100 μm) that are powered by a handheld laser wand that is connected to a computer. They are lightweight and have high retention in small fishes. The p-Chip could be attached externally using a non-toxic adhesive or internally on larger amphipods to allow individual identification.

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

Objectives

1) Our first objective is to evaluate attachment of p-Chips and short-term tag retention on CSRB and Peck's Cave Amphipod. There are multiple ways to attach tags- both internal and external. An excellent starting point to determine the appropriate location and material used to attach the tag.
2) Our second objective is to tag Peck's Cave Amphipod to determine longer-term retention of the tag and survival of the tagged animal. We will use amphipods held at the San Marcos Aquatic Resources Center for this evaluation so we can best simulate their holding environment.

Approach

Objective 1: The first objective on Amphipods will be completed in year 1. The CSRB portion will be set up and running in year 1 but not completed until year 2.

Laboratory studies will be conducted at the San Marcos Aquatic Resources Center in conjunction with USFWS biologists because the source water is ideal for the species. Because Peck's Cave Amphipod is anticipated to molt every ~50 days, we will first evaluate relatively short-term tag retention of externally placed and internal tags through a single molt. Although little is known about their biology, these amphipods are assumed to reach adulthood in a year going through several instars; thus, examining tags through a molting cycle is critical to determine if the tags could only be used through one molt or longer. For this objective, p-Chips will either be affixed on the dorsal side of the amphipod (or suitable surrogate species) using two different types of glue (non-toxic, cyanoacrylate-free superglue such as Loctite or Hopson and dental cement) or internally tagged (see below). Internal tagging is preferred because the tag might be retained after molting, but external tagging would be the next best option. External tags would be useful for short-term tracking of larger adults because they do not molt as frequently as smaller individuals. We will first evaluate whether tags can be internally implanted between the walking legs without complete mortality or impeding their ability to swim. Our initial evaluation will only be completed on about 5 individuals to ensure immediate survival and successful swimming (assuming this is successful, we will move to the experiment below).

We will externally tag some amphipods (two treatment groups with different glue types) and keep two groups of controls. The tagged individuals will be externally tagged along the midline of the dorsal using one of two types of glue. Individuals will be randomly selected to determine treatment group (control, 2 negative controls: glue type 1, or glue type 2). In each trial, we will include 5 controls, 5 of each glue type (negative controls), and 10 of each treatment ill amphipods per trial). Tags will be affixed using a tiny drop of glue and the p-Chip will be placed flat against the glue. Control amphipods will be handled but no glue or tag affixed (5 individuals), whereas the negative control groups will have a drop of glue attached (one of two types, 5 individuals each). This will allow us to separate any issues related to either the glue type or holding conditions. Only the largest individuals available will be used for the study because they would be most likely to accommodate a tag. Tagged and control amphipods will be checked daily to determine tag retention and survival. The trial will end after 50 days or after all amphipods have molted. We realize the externally placed tag will be shed when molting, but we want to ensure that the amphipods successfully molt. We will complete 3-6 trials depending on variability among trials. Each trial will last approximately 50 days and multiple trials may be conducted at the same time.

If our initial trial for internal tagging Amphipods is successful, we will proceed with an experiment to determine long-term retention and survival of the marked animal through molting. We will internally place the tag on the lower ventral side between two pair of walking legs. The tag will be placed parallel but offset the midline to avoid the digestive tract and reproductive organs of the amphipod. Like the above experiment, we will have a treatment and control groups, where one control is simply handled and held with the others, and another has the tag needle gently inserted but no tag placed. We estimate holding 10 amphipods together per trail (6 tagged, 2 control, and 2 negative control) and completing 5-8 trails (depending on variability among trials, ~30 tagged amphipods). Each trial will last approximately 50 days and multiple trials may be conducted at the same time. If internal tagging is not successful, the funds will be pushed to the beetle objectives.

We will also tag CSRB using two tag types- one that would be useful for more passive batch tagging (e.g., elastomer) and a tag that will provide individual identification (e.g., p- Chips). Adult *M pusillus* and/or *M vulnerata* (400-600 beetles) will be tagged at the aquatic center because the source water is ideal for the species. Beetles will be assigned randomly to either a control (no tag) or treatment (tag) group. Control beetles will be subjected to the same handling as treatment beetles but without tagging. Experiments will last a minimum of 90 days and treatment beetle survival will be monitored using stationary readers and an active reader. Survival of control beetles will be evaluated daily. Kaplan-Meier curves (Goel et al. 2010) will be used to visualize survival over time. We will test that the survival curves did not differ by calculating a log rank test comparing survival curves within each experiment. We will use the "survival" package in Program R (Therneau 2020).

Visible implant elastomer (VIE) and p-Chips will both be used. VIE tags are typically injected into tissue but could be sprayed in a fine mist across the back of the beetle. It remains flexible and visible after it dries, but the retention using this approach is unknown. P-Chips are a relatively new tagging technique that have been used on small, endangered fish (Moore and Brewer 2021) with success. Additionally, p-Chips have been successfully used on insects like the Western Honeybee *Apis mellifera* (Tenczar et al. 2014) and Rock Ant *Temnothorax albipennis* (Robinson et al. 2014) by external adhesion. P-Chips are micro-transponder tags (500 x 500 x 100 µm) that are powered by a handheld laser wand that is connected to a computer. They are lightweight and have high retention in small fishes. The p-Chip could be attached externally using a non-toxic adhesive to beetles to allow individual identification.

Objective 2: The second objective will be completed by the end of year 2. Laboratory studies will again be conducted at the San Marcos Aquatic Resources Center (hereafter aquatic center) in conjunction with USFWS biologists. We will tag the amphipods using p-Chips. We will tag amphipods at the San Marcos Aquatic Resources Center because the source water is ideal for the species. Amphipods will be assigned randomly to either a control (no tag), negative control (injection but no tag) or treatment (tag) group. Experiments will last for 3-6 months, and treatment amphipod survival will be monitored using stationary readers (where tags are scanned when the amphipods pass through a reader location) and active scans. Survival of control beetles will be evaluated daily. The goal is to examine whether amphipods can successfully reproduce (deposit eggs in their brood pouch that successfully hatch) so larger individuals will be selected for these trials. We will complete 5-8 trials of this experiment where each trail consists of 10 control, 10 negative control, and 10 tagged individuals (i.e., 30 per trial).

Analysis- Kaplan-Meier curves (Goel et al. 2010) will be used to visualize survival over time. We will test that the survival curves did not differ by calculating a log rank test comparing survival curves within each experiment. We will use the "survival" package in

Program R (Therneau 2020).

Deliverables: Quarterly reports will be provided. An interim report will be provided at the end of year 1. A final report will be submitted to the EAA by December 31, 2024.

Budget: Justification provided on last page.

Schedule:

Month	Activity
January – March 2023	Order supplies
February – April 2023	Short-term retention
May – July 2023	Set up longer-term experiment
May – October 2023	Retention & survival through molting (50-day trials)
September 2023 – January 2024	Set up/conduct longer-term trials (3-6 months)
October – December 2023	Draft interim report
November 2023 – February 2024	Analyze amphipod data
November 2023 – April 2024	Set up CSRB experiment (2 tag types)
April – October 2024	Conduct CSRB experiment (90-day trials)
September – November 2024	Analyze CSRB data
October – December 2024	Draft final report

References:

EA Recovery Implementation Program, 2021

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

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

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

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

Mollenhauer, R. and S. K. Brewer. 2017. Multinomial N-mixture models improve the applicability of electrofishing for developing population estimates of stream-dwelling

Smallmouth Bass. North American Journal of Fisheries Management 37:211-224.

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

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

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

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

Robinson, E. J. H., 0. Feinerman, and N. R. Franks. 2014. How collective comparisons emerge without individual comparisons of the options. Proceedings of the Royal Society B: Biological Sciences 281:20140737.

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

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

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

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

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

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

Budget	Year 1 2023	Year 2 2024	Totals
Supplies and Materials	$ 13,780.00	$ 1,400.00	$ 15,180.00
Equipment	$ 10,300.00	$ 2,600.00	
Aburn Travel	$ 6,678.00	$ 4,452.00	$ 11,130.00
Auburn Salary	$ 23,914.00	$ 35,870.43	$ 59,784.43
Sub Total	$ 54,672.00	$ 44,322.43	$ 98,994.43
Indirect costs (17.5%)	$ 9,568.00	$ 7,756.43	$ 17,324.43
Total	$ 64,240.00	$ 52,078.86	$ 116,318.86

Genomic diversity of wild and refuge Peck's Cave amphipod (*Stygobromus pecki*) populations

2023 Interim Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program

Prepared by Dr. Katie Bockrath and Dr. Chris Nice

San Marcos Aquatic Resources Center
U.S. Fish and Wildlife Service

Table of Contents

Background

Peck's cave amphipod, *Stygobromus pecki*, occupy spring opening habitats in a limited range including Comal Springs, Landa Lake and nearby Hueco Springs in central Texas. These blind, subterranean amphipods are listed as endangered and face a number of threats including reductions of spring flows and loss of habitat due to climate change and increased human demand for ground water resources in central Texas. In response, the San Marcos Aquatic Resources Center (SMARC) has established a captive assurance population, or Refugia, as a buffer against declines or extinctions of wild populations. Effective captive holding and propagation requires careful consideration of the structure of genetic variation in wild and captive populations to maximize conservation of genetic diversity and minimize inbreeding or outbreeding depression. This project is specifically designed to meet this management need.

The goal for 2023 efforts was to conduct collections of Peck's cave amphipods (PCA) at individual spring openings across the Comal Springs system and to extract DNA from collected individuals.

Objectives

Assess the genetic diversity of the PCA across the Comal Springs system to inform Refugia collections, captive propagation and reintroduction strategies.

Methods

Field Collections

Pecks cave amphipods are often found on poly cotton lures used to collect Comal Springs riffle beetles; thus, PCA were first collected as bycatch from Comal Springs riffle beetle collection efforts. PCA were collected from lures placed at spring openings in Spring Run 1, Spring Run 2, Spring Run 3, Upper Spring Run (Spring Run 4), Western Shoreline, and Spring Island (Figure 1). A total of 100 poly cotton lures were placed in the Comal Springs system in 2023. Lures were placed in spring openings in April, July, and October. Lures were retrieved four weeks post placement in May, August, and November, respectively. Additional collections were conducted using small dip nets following EARP PCA collection SOPs at spring upwellings at Western Shore and Spring Run 1. A target of thirty individuals from each sampling location was set to ensure a representative sample for each location was included in the study. All individuals were preserved in 95-100% ethanol and stored in a -80º C freezer until processing. The San Marcos Aquatic Resources Center (SMARC) collected GPS locations of all spring openings where a lure was set.

DNA Extractions

Extractions of genomic DNA from PCA samples is planned for November to December of 2023. Extractions will be performed using the Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Alameda, CA, USA) which has proven effective for generating high quality genomic DNA from PCA and other *Stygobromus* samples (Nice, C.C, unpublished). DNA concentrations will be quantified using a Qubit fluorometer (Fisher Scientific) in preparation for genomic library preparation.

Figure 1. General locations for each spring run in the Comal Springs system in Comal County, Texas, where the Comal Springs riffle beetle have been collected (Bosse, Tuff & Brown 1988; Barr 1993; Coleman, Gibson, and Norris, 2022, pers. comm.).

Results

Field Collections

A total of 129 PCA were collected and preserved for analyses. Three of the six locations produced at least N=30 individuals for analysis. Only two individuals were collected from Spring Run 1. No PCA were observed at Spring Run 2 or Upper Spring Run. Collections from all locations will continue into 2024. All lab work and analyses are scheduled to occur in 2024.

Table 1.Peck's cave amphipod (PCA) collection and take information for 2023. Sampling location, number of poly cotton lures set at each location, number of poly cotton lures with PCA, the number of PCA hand collected at each site, and the total number of PCA collected at each location are reported.

Location	Number of Lures Set	Number of Lures with PCA	Number of PCA Collected from Lures	Number of PCA Hand Collected	Total Number of PCA Collected
Spring Run 1	30	1	1	1	2
Spring Run 2	10	0	0	0	0
Spring Run 3	40	17	47	0	47
Western Shore	40	11	26	8	34
Spring Island	40	12	46	0	46
Upper Spring Run	20	0	0	0	0
Totals	180	41	120	9	129

Interim report for 2023

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

Ruben U. Tovar and David M. Hillis

Narrative of 2023 accomplishments

Successful reproduction is contingent on a number of both endogenous and exogenous mechanisms. Environmental cues (e.g., circadian rhythm, change in seasonal temperature, etc.) are perceived by an organism's sensory organs (eyes—phototransduction; olfactory bulb—chemosensory; skin—temperature), and are part of the initial pathways that indicate the ideal reproduction times for the salamanders. The difference in sensory organs associated with underground living (e.g., eye and pigment reduction, dorsal-ventrally compressed heads, elaborated lateral line and chemosensory, etc.) play a role in how these subterranean species perceive their environment relative to their surface cousins. Having a fundamental grasp of these comparative sensory systems may allow us to pinpoint the evolutionary differences between surface and subterranean species. Importantly, this will also give us insight into which sensory modalities are important and favored for breeding in each respective species given their environment (*E. rathbuni*-subterranean vs. *E. nana*-surface).

US Fish and Wildlife (FWS) staff at the San Marcos Aquatic Resources Center (SMARC) have tried to induce oviposition with hormones, separation, and light ques for both *E. nana* and *E. rathbuni* to induce reproductive activity and oviposition. Hormones were successful for *E. rathbuni*, but nothing has reliably worked for San Marcos salamander (*E. nana*). For FWS, the ultimate goal of this study is to track the development of sensory organs responsible for communicating environmental ques that initiate reproduction, and to compare gene expression between different tissues during reproduction. Accomplishing this will inform the FWS about both organ development and the genetic underpinnings contributing to reproduction. To do this we have employed a novel microCT scanning protocol that allows us to both scan soft tissue and extract DNA post scanning. During 2023, we have managed to collect a developmental series for *E. rathbuni* and *E. nana*, microCT scanned them, and tested the DNA extraction protocol on previously preserved and scanned embryos as proof of concept for utilizing the specimens collected for this study. We are now well positioned to move forward with sequencing and initiating a RNA-based reference for future comparative studies (e.g. comparing tissues from reproductive vs. non-reproductive salamanders). This future sequencing work will help the FWS identify genes associated with reproduction in *E. rathbuni* and *E. nana*.

Summary of accomplishments

- In 2023 oviposition's were observed and we collected twelve embryonic stages for both *E. nana*, and *E. rathbuni* (n=24).
- We targeted and fixed four stages for *E. rathbuni* and *E. nana* representing embryonic development days 21, 31, 34, 40, as described by (Tovar et al. 2021).
- Importantly, the proprietary fixative allows downstream molecular work to be accomplished. We have evidence of this with adult salamander tissue, however this was the first time embryonic/developmental tissue was being used.
- Each stage was contrast enhanced using iodine and microCT scanned (diceCT).
- From these scans, we have started to establish the first developmental atlas of these endangered species (*E. rathbuni* and *E. nana*). Although a good amount of developmental progression is diagnosed by external morphology (head/tail bud, somite's, etc.), we have started to explore further into the tissue resulting in the segmentation of the underlying soft tissue (neural tube, optic stalk, etc.).
- Tissues from the same species that represent stages later in development (part of an NSF grant) have been sent for sequencing as a pilot that will inform us on the best practices moving forward with embryonic tissue. These later stage tissues have shown great results.

Interim report for 2023

DiceCT scans

Here our goal was to use an embryonic series to track and describe developmental progression and target sensory organ development. Importantly, this fixation method allows us to isolate RNA for downstream sequencing (see next section). We were able to intercept oviposition's by female of both *E. rathbuni* and *E. nana* to obtain three individuals for every stage. One was used for diceCT and for sequencing. Below is a gross overview of the developmental series for *E. rathbuni* and *E. nana*, please see full descriptive data in Figure 3.

Results

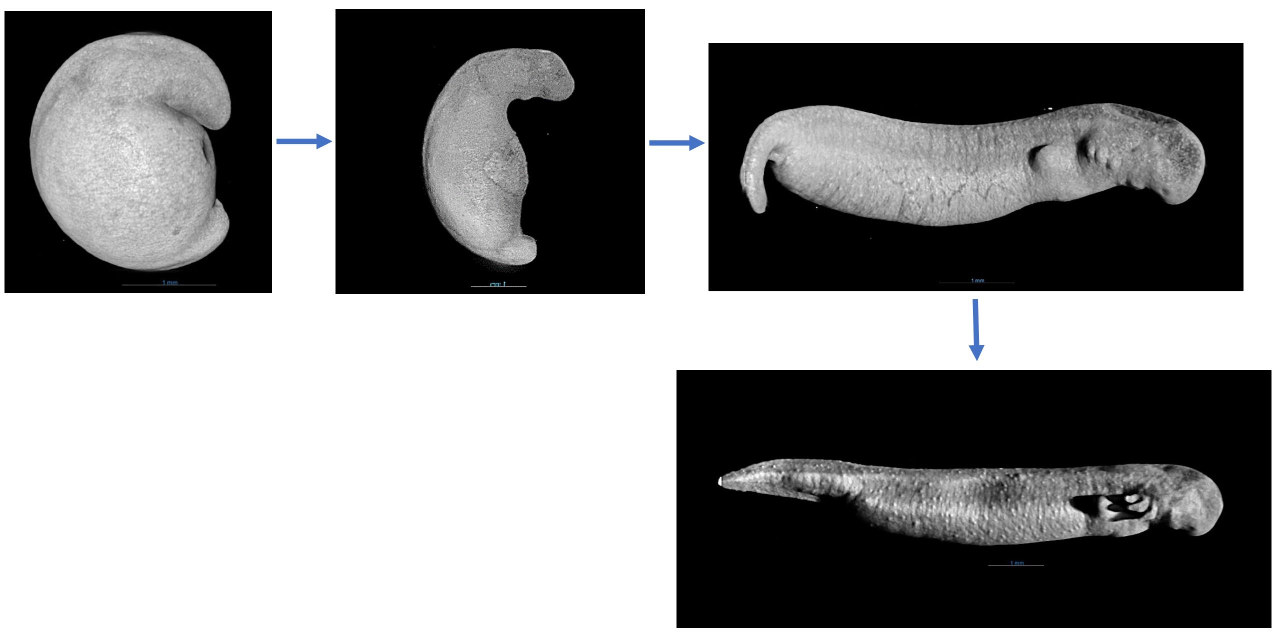


Figure 1. *E. nana* flow chart of developmental series from stage 21-40.

***E. rathbuni* stages 21-40**

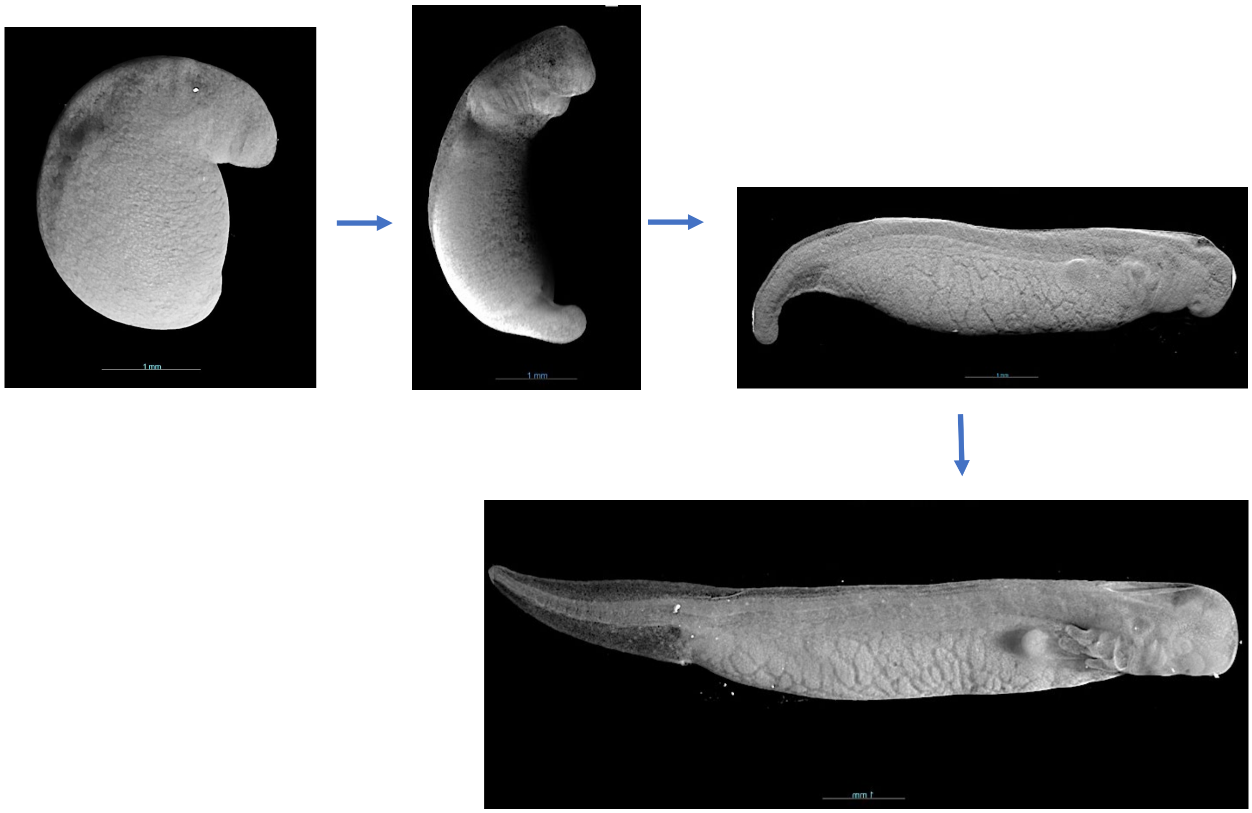


Figure 2. *E. rathbuni* flow chart of developmental series from stage 21-40.

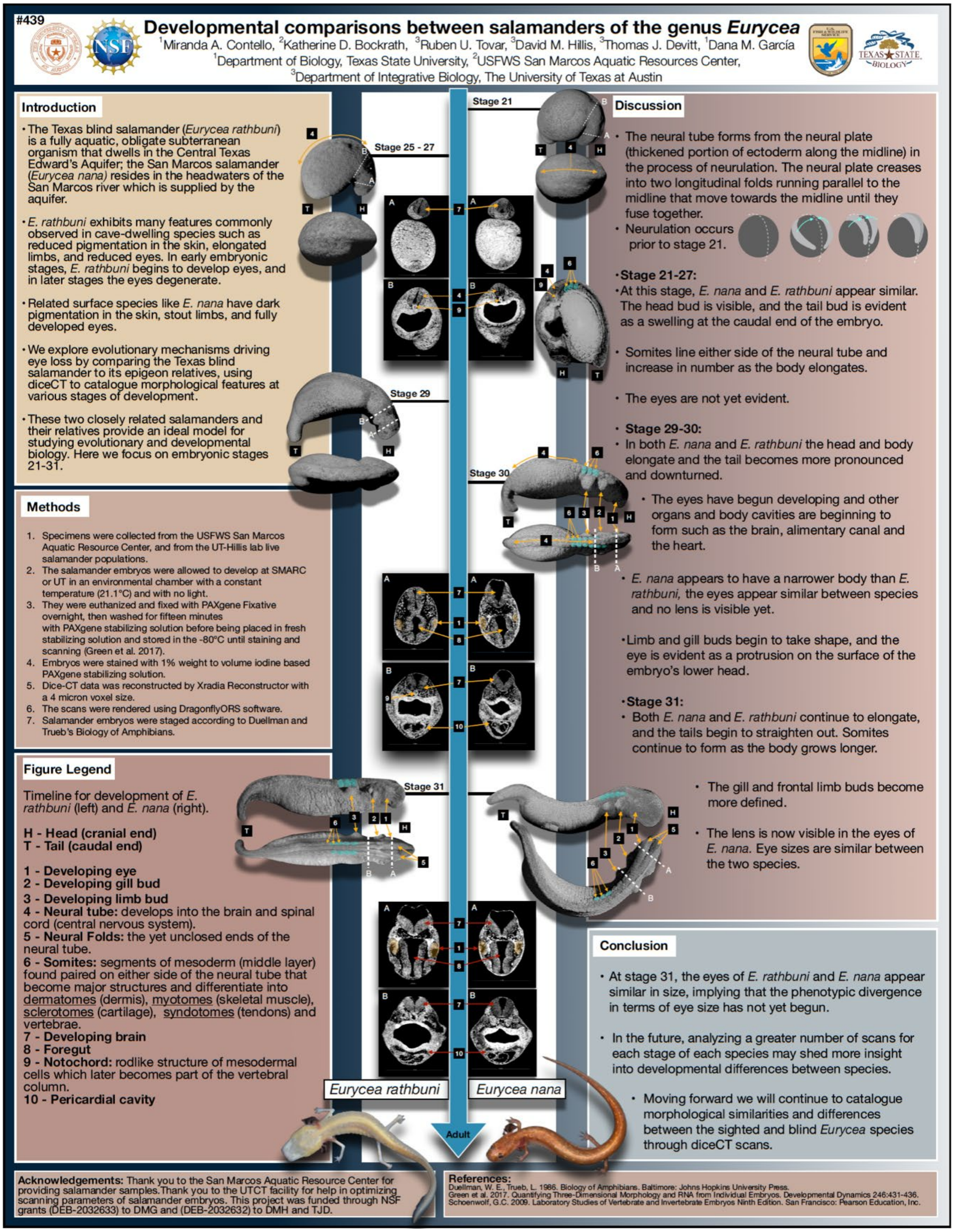


Figure 3. The culmination of the developmental atlas description. We are working on drafting a manuscript of this work.

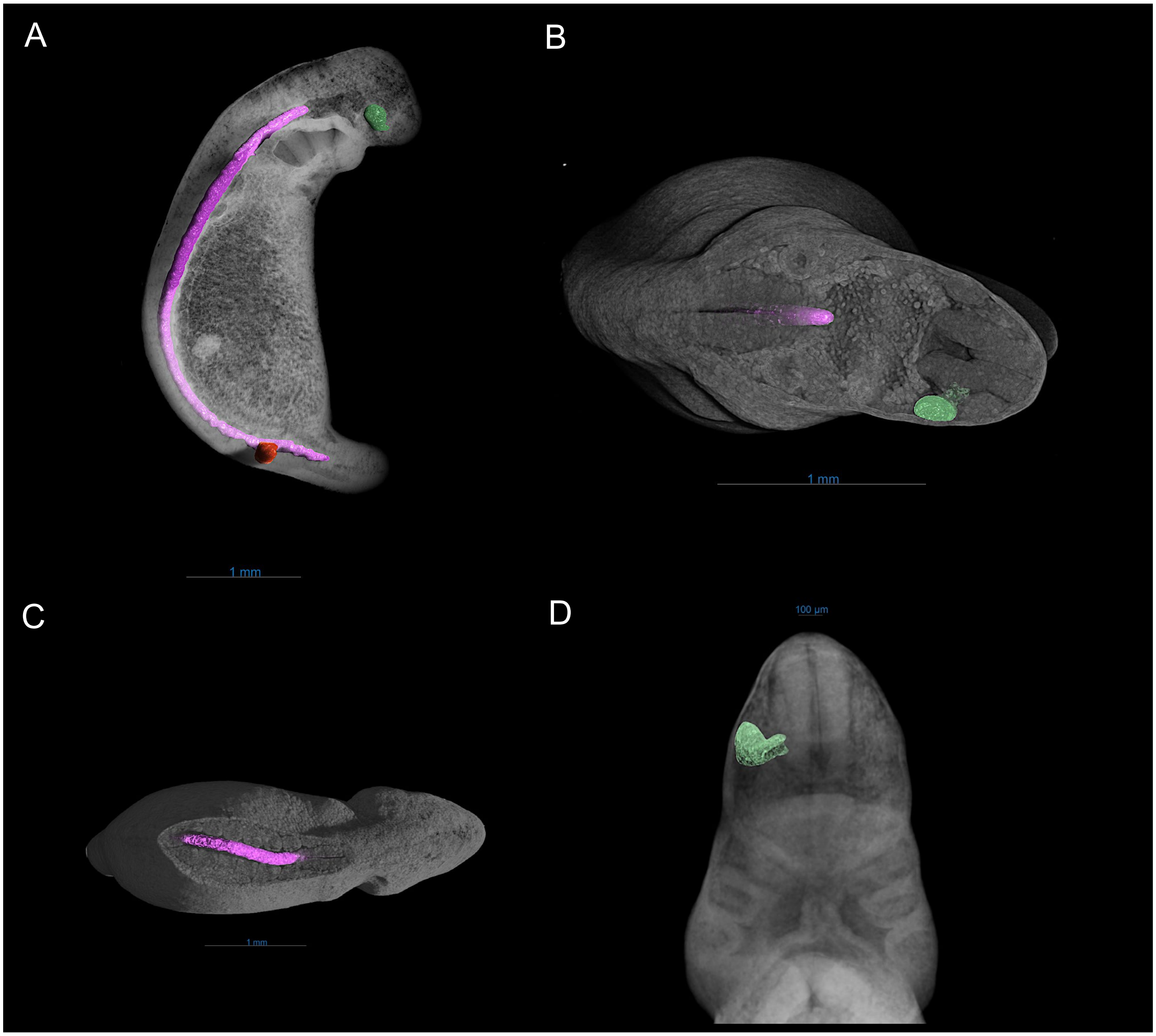


Figure 4. Segmented soft tissue of *E. rathbuni* at stage 31. Segmented components of the central nervous system can be identified here including, the neural tube (purple), somite (A, red), and optic stalk and cup (green). At stage 31 in *E. rathbuni* several cutaway angles are provided including transverse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

Preliminary sequencing

Ground truthing RNA-isolations, Quality Checks and RNA sequencing using diceCT scanned tissues. This is an important initial step to assure the success of RNA sequencing and downstream construction of a respective transcriptome.

Tissues from previously diceCT scanned individuals were RNA-isolated over the summer and several quantification approaches were used to confirm RNA quantity and quality. These tissues consisted of eyes, olfactory epithelium, and skin from around the head. Importantly, these tissues comprise different species, developmental stages, and tissue types respectively. Extra care was given to represent as much variation found in our samples as possible. Interestingly, one of these individuals represents one of the first collected stages for *E. rathbuni* one month post oviposition in 2021. By running through the protocol with this subset of 12 individuals first gives us confidence for the success of embryos associated with the developmental atlas and descriptive expression as part of this grant.

Results

MultiQC scores allow us to analyze the quality of the sequences (e.g. over represented sequences, and nucleotide analysis). Please see the below MultiQC scores for RNA sequences associated with different tissues, developmental stage, and species. I pushed ahead with sequencing even though there seemed to be some inconsistency of Qubit readings and RIN scores. I am happy to show the small batch of twelve tissues submitted for Tag-sequencing (a form of RNA-seq) were successfully sequenced except for one (*E. rathbuni*_1_1_Skin) (Fig. 5). The *E. rathbuni* one month post oviposition skin tissue sample failing is not surprising given the lack of DNA fragment distribution. It is more likely that this tissue sample experienced a momentary environment that depleted the RNA within the tissue (e.g. RNases etc.). Having said that the overall success of sequencing this test batch is a positive indicator that one can receive quality RNA sequences using the diceCT/PAXGene protocol, and I will move forward with RNA-isolations for the already diceCT scanned embryonic series representing the developmental atlas for the respective species (*E. rathbuni*, *E. nana*, and *E. sosorum*).

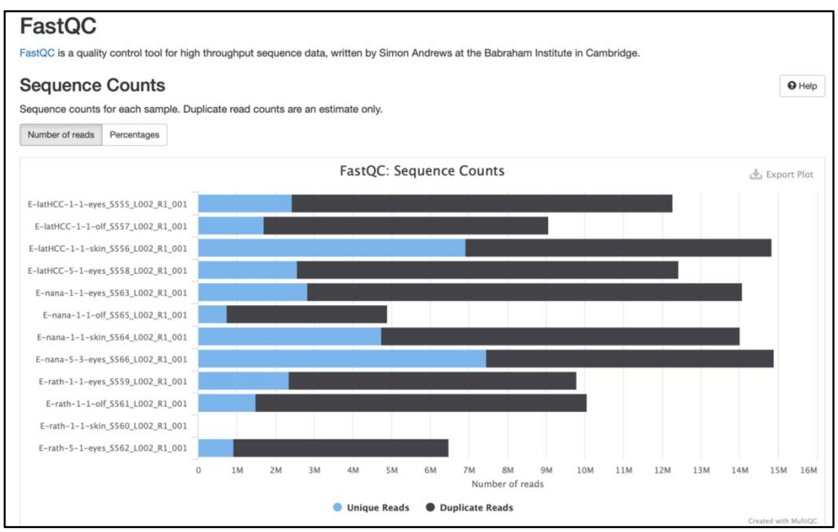
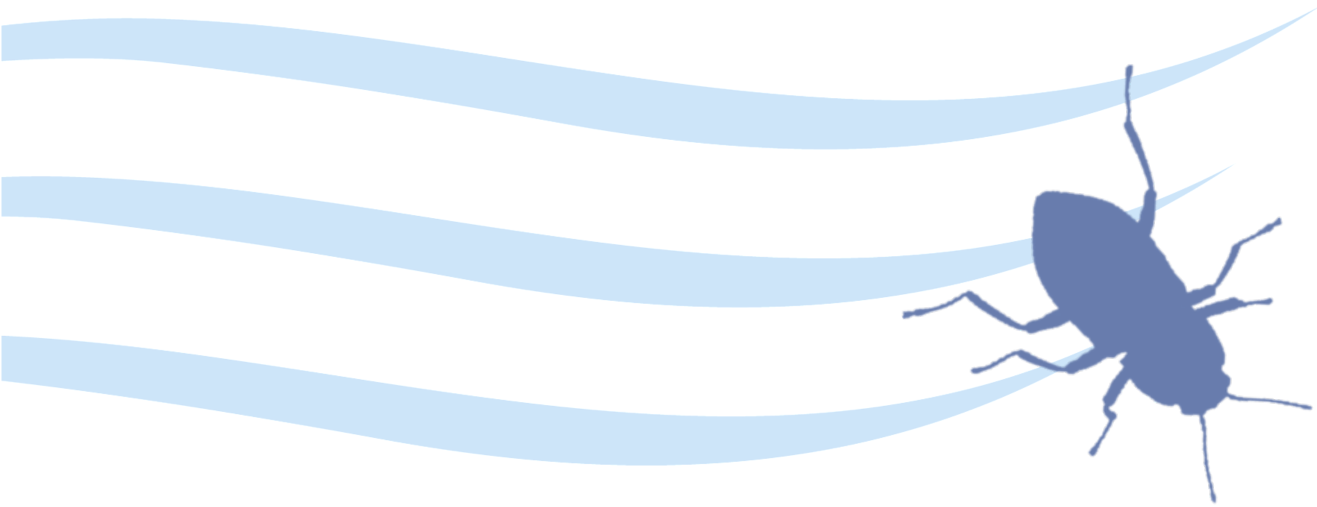


Figure 5. A parameter of unique vs. duplicate reads in the sequence data. Unique reads are favored over duplicates in that they are typically more informative in downstream analysis.

Genetic Assessment of the Comal Springs Riffle Beetle the Comal System – Interim Report

2023 Research Report for the Edwards Aquifer Authority

From the Edwards Aquifer Refugia Program



Prepared by Dr. Katie Bockrath and Desiree M. Moore

San Marcos Aquatic Resources Center
U.S. Fish and Wildlife Service

Table of Contents

Background

The Comal Springs system in New Braunfels, Texas is the prominent recreational feature of Landa Park. It is surrounded by residential housing and is heavily modified with the addition of paved river edges. Despite its recreational use, endemic groundwater species persist at the spring upwellings located across the lake. In addition to habitat destruction and heavy recreational usage, groundwater species are sensitive to fluctuations in environmental conditions. Drought events and water usage put immense pressure on ground water availability in the Edwards Aquifer and can lead to low flow and high temperature conditions. The Edwards Aquifer Refugia Program (EARP) serves to develop functional refugia for endemic species dependent on flow from the Edwards Aquifer. In the event of a catastrophe, these endemic species will be brought into the EARP Refugia until they can be reintroduced. To ensure the population is accurately reflected in the Refugia, it is critical to understand how populations are structured across a species range and where individuals should be sampled to capture a representative collection of their genetic diversity. Here, we aim to assess the genetic diversity of the Comal Springs riffle beetle found in spring upwellings across the Comal Springs system.

Gonzales (2008) and Colman (2021) found distinct genetic clustering among riffle beetle species across central Texas, as expected. Their data also showed genetic separation between the Comal Springs and San Marcos Springs populations of Comal Springs riffle beetle. When assessed at a finer scale, both Gonzales (2008) and Colman (2021) showed distinct clustering of subpopulations across the Comal Springs system with one of the studies suggesting distinct genetic lineages among Spring Runs (Colman 2021). Together, these results suggest that the species is dispersal–limited and several populations harbor unique genetic diversity.

The goal of this study was to collect Comal Springs riffle beetles at individual spring openings across the Comal Springs System and collect a genome-wide genetic dataset to generate a population genetic survey and identify evolutionarily significant units. The genetic data gathered will inform future Refugia collection needs by ensuring the total genetic diversity of this population is reflected in the Refugia. The same dataset will be used to inform reintroduction strategies if a salvage event were to occur.

2023 research efforts focused on collecting a sufficient number of individuals from locations in Comal Springs system.

Objectives

Assess the genetic diversity of the Comal Springs riffle beetle across the Comal Springs system to inform Refugia collections reintroduction strategies.

Methods

Field Collections

Poly Cotton lures were used to collect Comal Springs riffle beetles in Landa lake and Spring Runs. A collection plan for Comal Springs riffle beetle was established with the EAA and BIO-WEST prior to performing the study. A target of 30 adult individuals from each sampling location was set to ensure a representative sample for each location was included in the study. Criteria were established to determine the number of adults that could be taken each time lures were retrieved from 80 BIO-WEST biomonitoring and research lures in Spring Run 1, Spring Run 3, Upper Spring Run (Spring Run 4), Western Shoreline, and Spring Island (Figure 1). BIO-WEST was conducting an occupancy study in tandem with this genetic study. BIO-WEST placed poly-cotton lures in spring openings in April, July, and October. To ensure sufficient beetles were retained in the population to inform the occupancy study and to ensure sufficient beetles were collected for the genetics study, a graded collection strategy was set. If greater than eight beetles were collected from a lure, up to 4 beetles were retained for this project. If between five and eight beetles were collected, two could be retained. If fewer than five were collected, one was retained. Larvae were collected from the lures to supplement adult collections. Additionally, EARP staff set 10 lures in April and collected beetles from Spring Run 2 to ensure full coverage of the Comal Springs area. Lures were retrieved after four weeks to check for beetles. All beetles collected from EARP lures set in Spring Run 2 were retained for this project. Retained individuals were preserved in 95-100% ethanol and stored in a -80º C freezer until processing. The San Marcos Aquatic Resources Center (SMARC) collected GPS locations of all spring openings from which beetles were collected.

DNA Extractions

DNA extractions were carried out using a Qiagen DNeasy Blood and Tissue DNA extraction kit. A disposable plastic pestle was used to break the exoskeleton to allow the DNA extraction buffers to reach the internal tissues. DNA extractions were quantified using a Qubit Fluorometer and stored at -80 ºC until further analysis.

Figure 1. General locations for each spring run in the Comal Springs system in Comal County, Texas, where the Comal Springs riffle beetle have been collected (Bosse, Tuff & Brown 1988; Barr 1993; Coleman, Gibson, and Norris, 2022, pers. comm.).

Results

Field Collections

A total of 90 adult Comal Springs riffle beetles were preserved and retained for analyses (Table 1). Additionally, 73 larvae were preserved and retained. A target number of adult individuals (N=30) were collected at Spring Island. A near target number of adults were collected from Spring Run 3 and Western Shore. When adults and larvae are combined, a sufficient number of individuals were collected from Spring Run 3, Western Shore, and Spring Island. An insufficient number of individuals were collected form Spring Runs 1 and 2, and Upper Spring Run. Collections from Spring Runs 1 and 2 and Upper Spring Run will continue into 2024. All lab work and analyses are scheduled to occur in 2024.

DNA Extractions

DNA was successfully extracted from all collected Comal Springs riffle beetles; 95 adults and 118 larvae.

Table 1. Comal Springs riffle beetle (CSRB) collection and take information for 2023. Adult and larval CSRB take are reported separately, and the total CSRB take is given.

Location	Adult CSRB Observed	Adult CSRB take	Larval CSRB take	Total CSRB take
Spring Run 1	1	1	0	1
Spring Run 2	1	1	6	7
Spring Run 3	90	30	23	53
Western Shore	92	24	37	61
Spring Island	228	39	58	97
Upper Spring Run	0	0	0	0
Totals	412	95	118	219

Works Cited

Colman, W. (2021). Genetic Diversity of the Comal springs riffle beetle (Heterelmis comalensis). T. T. G. I. Forum. The 2021 Texas Groundwater Invertebrate Forum, The 2021 Texas Groundwater Invertebrate Forum.

Gonzales, T. K. (2008). Conservation genetics of the Comal springs riffle beetle (Heterelmis comalensis) populations in Central Texas, with examination of molecular and morphological variation in Heterelmis sp. throughout Texas. https://digital.library.txstate.edu/handle/10877/3118, Texas State University. Master of Science.

Nunziata, S. O., Weisrock, David W. (2018). "Estimation of contemporary effective population size and population declines using RAD sequencing data." Heredity (120): 196-207.

January 2023 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dr. Katie Bockrath and Adam Daw

With contributions from

Desirée Moore, Shawn Moore, Ben Thomas, Dominique Alvear, and Braden West

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staffing

Hiring actions continue to progress to bring on permanent positions at both SMARC and UNFH. Currently, 5 of the 7 positions have been filled. The status of each position is listed below.

Location	Position	Status
San Marcos	Research Biologist (GS 9/11)	Desirée Moore has filled this position.
San Marcos	Biologist (GS 7/9)	Braden West has filled this position.
San Marcos	Biological Technician (GS 5/7)	Shawn Moore has Filled this position.
San Marcos	Biological Technician (GS 5)	Position announcement to be posted to USAJobs.gov February 3, 2023
Uvalde	Biologist (GS 7/9)	Dominique Alvear has filled this position.
Uvalde	Biological Technician (GS 5/7)	Ben Thomas has filled this position.
Uvalde	Biological Technician (GS 5)	Position announcement posted to USAJobs.gov January 25, 2023.

Species Collection

On January 19, Thomas, Daw and Alvear collected 36 Comal Springs fountain darters from Landa Lake, New Braunfels. Twenty-two were kept for the UNFH refugia (Figure 1).

Husbandry

Uvalde

Daw continued training Alvear on invertebrate husbandry. Daw built the first of the electrical control panels for the new control/monitoring systems and installed it on one of the tank systems in the refugia (Figure 2,3).

SMARC

West began training Shawn Moore on general husbandry tasks and captive food culture maintenance. West completed the first draft for a daily care and duties standard operating procedure (SOP). Desirée Moore updated information on all tank labels in the SMARC refugia and quarantine.

Animal Health

EARP staff received Bd/Bsal testing results from the San Diego Zoo for salamanders kept in quarantine. No wild caught Texas blind salamanders in quarantine were positive for *Batrachochytrium dendrobatidis* (Bd). One of two wild caught Comal Springs salamanders was positive for *Batrachochytrium dendrobatidis* (Bd). Sixteen of thirty-eight San Marcos salamanders tested were positive for *Batrachochytrium dendrobatidis* (Bd). No salamanders were positive for *Batrachochytrium salamandrivorans* (Bsal).

Task 2 Research

Dryopid Life History

Funding was awarded through GrantSolutions. Dr. Bockrath and Desiree Moore scheduled a meeting with Matt Pintar (BIO-WEST) for February 1 to discuss the project and timelines.

San Marcos Salamander Mark Recapture

Desiree Moore conducted a pilot study tagging San Marcos salamanders with p-chips in preparation for the mark recapture study. The salamanders were scanned weekly. Thus far, no tag loss has occurred and there have been no mortalities due to tagging. The start of mark recapture portion of this project was postponed a few months due to the recent gasification event in the refugia. The project was slated to start February 2023 but will now start in April or May.

Reproductive Gene Expression in San Marcos Salamanders

Ruben Tovar (University of Texas, Austin) collected samples for RNA analysis and CT scanning. Many of the mortalities that resulted from the recent gasification event were preserved and will be used in this study.

Comal Springs Riffle Beetle Population Genetics

A field schedule was established for launching and checking poly cotton lures. Two-hundred poly-cotton lures were created and individually barcoded with multi-colored zip-ties (Figure 4) to ensure unique identification back to a specific GPS marked spring opening, even if the lure were to shift locations while in the field. Below are the proposed poly-cotton lure launch and check/collection date ranges:

Launch: Week of February 13

Check 1: Week of March 13

Check 2: Week of April 10

Check 3: Week of May 8

Tagging Aquatic Invertebrates

Funding was awarded through GrantSolutions. Previously preserved Peck's cave amphipod and Comal Springs riffle beetle mortalities have been identified as initial tagging test subjects. The captive bred Comal Springs riffle beetles from the 2022 Density/Biofilm study will be used for live organism tag testing. P-chips and a p-chip reader were purchased.

Genetic Assessment of Peck's Cave Amphipod

Funding was awarded through GrantSolutions. Dr. Bockrath met with Dr. Chris Nice and Dr. Kate Bell (Texas State University) to discuss the collections schedule and general timeline for the project.

Additional Accomplishments

EARP staff gave a tour of the EARP building to Erica Mize (FWS Data Management Science Advisor).

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

The 2022 Draft Annual EARP Report and revised 2022 Research Reports were sent to the EAA for review.

Task 6 Meetings and Presentations

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

Desiree Moore presented the 2022 small-bodied salamander tagging study at the 2023 Texas Conservation Symposium

Summary of January Activities

- Shawn Moore filled the open Biological Sciences Technician position at the San Marcos Aquatic Resources Center
- The Uvalde National Fish Hatchery Biological Sciences Technician position is open for applications
- The first electrical panel for the automated monitoring and control system was constructed
- Bd/Bsal disease results came back from San Diego Zoo
- Externally partnered research funds were awarded in GrantSolutions
- San Marcos salamanders were tagged with p-chips in anticipation for the full-scale mark recapture study
- Salamander mortalities were preserved and retained for the comparative gene expression study
- Poly-cotton lures were constructed for the Comal Springs riffle beetle genetic assessment study
- Dr. Bockrath met with Dr. Chris Nice (Texas State University) and Matt Pintar (BIO-WEST) to discuss research projects

Tables and Figures

Table 1. January's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for January 2023. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	--	--	0	0	15	11	294	446
Fountain darter: Comal	NT	22	14	36	0	27	191	4	122	204
Comal Springs riffle beetle	NT	NT	--	--	0	0	NA	46	36	2
Comal Springs dryopid beetle	NT	NT	--	--	0	0	NA	6	2	4
Peck's cave amphipod	NT	NT	--	--	0	0	25	75	114	157
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	0	4	82	0	90	66
San Marcos salamander	NT	NT	--	--	0	7	28	2	75	166
Comal Springs salamander	NT	NT	--	--	0	2	51	2	59	91
Texas wild rice plants	NT	NT	--	--	0	0	0	0	205	207

Figure 1. Dominique Alvear collecting Comal Springs fountain darters in Landa Lake, New Braunfels.

Figure 2. Adam Daw wiring the electrical panel for the new control/monitoring system.

Figure 3. The electrical panel installed on a Walchem, Inc control/monitoring system on a tank in the UNFH refugia.

Figure 4. Poly-Cotton lures with color coded barcodes for unique idntification in the field. Barcode starting posistion is designated by the black zip-tie.

February 2023 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Dr. Katie Bockrath and Adam Daw

With contributions from

Desirée Moore, Shawn Moore, Ben Thomas, Dominique Alvear, and Braden West

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

On February 6, Dominique Alvear, Adam Daw, and Braden West collected 32 Comal Springs riffle beetles and nine Comal Springs dryopid beetles (CSDB) from Spring Island, New Braunfels. One juvenile CSDB was released, and the rest of the beetles were brought to the Uvalde National Fish Hatchery (UNFH) for the Refugia.

On February 14, Shawn Moore and West collected 30 San Marcos salamanders from the San Marcos River below the Spring Lake Dam (Figure 1). Three salamanders were released and 27 were retained for quarantine at the San Marcos Aquatic Resources Center (SMARC).

West purchased materials for and completed a refreshment of the Diversion Springs net to be placed in Spring Lake, San Marcos, TX. Highly visible red and white floats and new rigging rope were added.

Husbandry

Uvalde

Alvear began quarterly inventory of Peck's cave amphipod numbers. Alvear also conducted two 24-hour trials of the Peck's cave amphipod movement pilot study to begin optimizing boxes for breeding. Daw began updating plumbing on two tank systems in the refugia.

Alvear and Daw met with West at the UNFH to plan further standardization of animal feeding practices between the UNFH and the SMARC.

SMARC

Eleven clutches of salamander eggs were laid by salamander species held in refugia. Shawn Moore and West moved each clutch to separate culture systems to monitor development and hatch success. Shawn Moore and West worked to troubleshoot juvenile salamander escape and modify the box design to increase survival and retention.

Shawn Moore and West worked with Desiree Moore to tag 27 Texas blind salamanders in the refugia to begin tracking individual information (Figure 2).

West trained Shawn Moore and volunteer Holly Wood on the inventory process of Comal Springs riffle beetle and Peck's cave amphipod (Figure 3).

Task 2 Research

Dryopid Life History

Dr. Bockrath and Desiree Moore met with Matt Pintar (BIO-WEST) on February 1 to discuss the project and timelines.

San Marcos Salamander Mark Recapture

Desiree Moore continued the pilot study tagging San Marcos salamanders with p-chips in preparation for the mark-recapture study. The salamanders were scanned weekly. Thus far, no tag loss occurred and there were no mortalities due to tagging. The field tagging portion of this project was rescheduled for April 2023 due to the recent gasification event in the refugia.

Reproductive Gene Expression in San Marcos Salamanders

Dr. Bockrath and Ruben Tovar (University of Texas, Austin) met to discuss the research schedule, sampling, and needs of the project. Dr. Tovar worked on generating a reference gene expression database by sequencing samples.

Comal Springs Riffle Beetle Population Genetics

Due to the potential effects of lure setting on biomonitoring events, a plan was created to obtain beetles for this project from lures set by BIO-WEST. On February 23, Dr. Bockrath, Adam Daw, and Desiree Moore met with Dr. Chad Furl (EAA), Kristy Smith (EAA), Edmund Oborny (BIO-WEST), and Matt Pintar to discuss the new plan.

Tagging Aquatic Invertebrates

Desiree Moore began testing tagging on previously preserved Peck's cave amphipod and Comal Springs riffle beetle mortalities. Although the Peck's cave amphipods were too brittle to tag due to ethanol preservation, Desiree Moore successfully tagged the Comal Springs riffle beetle (Figure 4). Desiree Moore met with Randy Gibson (SMARC) to discuss the collection of a common amphipod species to test tagging on non-preserved specimens.

Genetic Assessment of Peck's Cave Amphipod

Dr. Bockrath and Dr. Chris Nice (Texas State University) met to discuss the research schedule, sampling, and needs of the project. It was determined that Peck's cave amphipod sampling will occur with the Comal Springs riffle beetle population genetics sampling and will be supplemented with additional sampling where needed.

Additional Accomplishments

EARP staff gave a tour of the EARP buildings to Jade Florence (FWS Wildlife Biologist Ecological Services).
All EARP staff met with representatives from Texian Geospatial to learn how to operate the new fine-scale GPS system (Figure 5).

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

The revised 2022 Annual EARP Report was sent to the EAA for review.

Task 6 Meetings and Presentations

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

EARP staff attended the Edwards Aquifer Habitat Conservation Plan biological goals meeting.

Desiree Moore, Shawn Moore, and Braden West presented their work at the USFWS Fish and Aquatic Conservation Region 2 Science Symposium.

Summary of February Activities

- The EARP collected 32 Comal Springs riffle beetles and nine Comal Springs dryopid beetles (CSDB) from Spring Island, New Braunfels
- The EARP collected 30 San Marcos salamanders from the San Marcos River below the Spring Lake Dam
- The process to tag refugia Texas blind salamanders with p-Chips began
- The first Comal Springs riffle beetle was tagged with a p-Chip
- Dr. Bockrath met with Dr. Chris Nice (Texas State University) and Ruben Tovar (University of Texas, Austin) to discuss research projects

Tables and Figures

Table 1. February's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for February 2023. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	--	--	0	0	39	18	255	428
Fountain darter: Comal	NT	NT	--	--	0	0	4	2	118	202
Comal Springs riffle beetle	NT	32	0	32	0	0	0	0	36	2
Comal Springs dryopid beetle	NT	8	1	9	0	0	0	NA	2	4
Peck's cave amphipod	NT	NT	--	--	0	0	4	13	110	155
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	0	0	1	0	89	66
San Marcos salamander	27	NT	3	30	0	48	3	4	72	210
Comal Springs salamander	NT	NT	--	--	0	0	0	0	59	91
Texas wild rice plants	NT	NT	--	--	0	0	0	0	205	207


Figure 1. Shawn Moore and Braden West in the San Marcos River for San Marcos salamander collection.

Figure 2. Braden West and Shawn Moore tagging Texas blind salamanders.

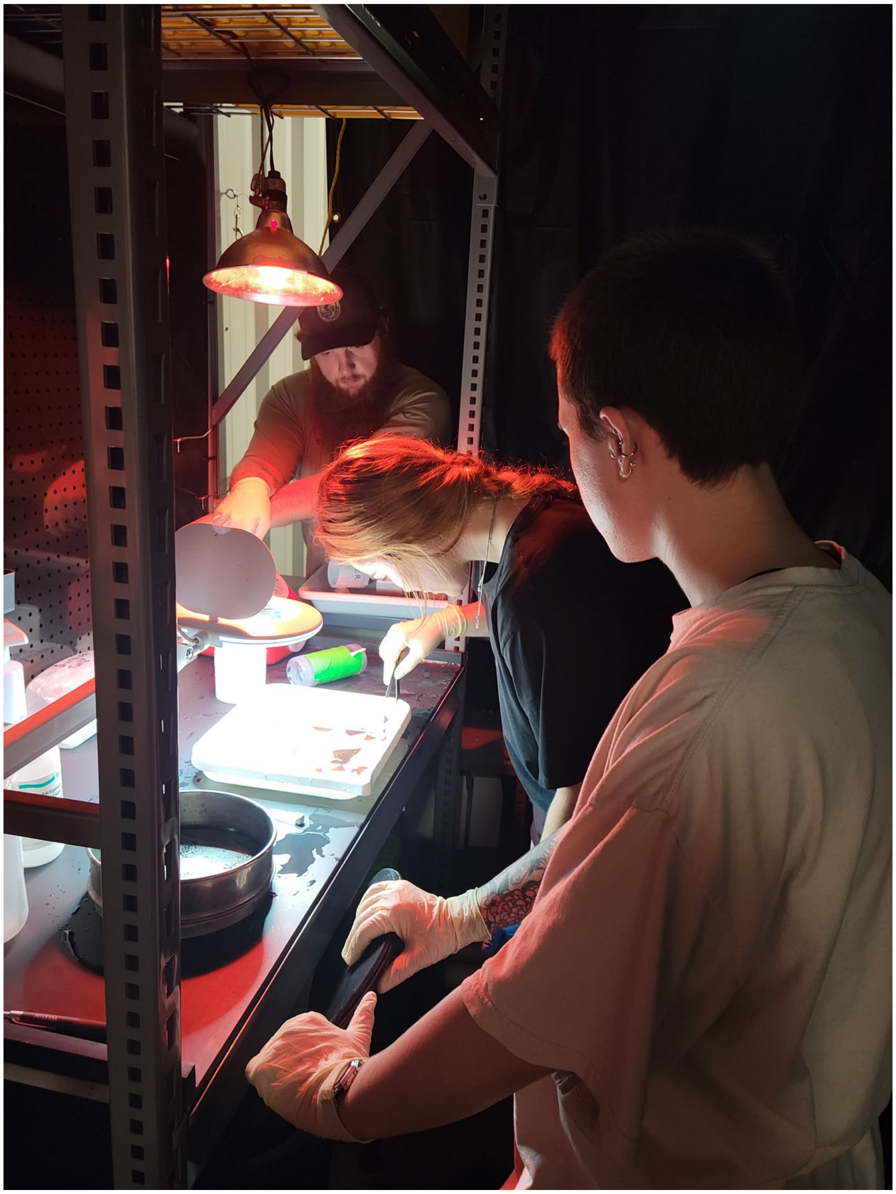


Figure 3. Braden West instructing Shawn Moore and volunteer Holly Wood on proper invertebrate inventory procedures.

Figure 4. A Comal Springs riffle beetle tagged with a p-Chip.

Figure 5. Representatives from Texian Geospatial training EARP staff to operate the new EOS Gold GPS system.

March 2023 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desirée Moore, Dominique Alvear, and Braden West

With contributions from

Dr. Katie Bockrath, Adam Daw, Shawn Moore, and Nicholas Yvon

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staffing

Benjamin Thomas's last day was March 21. A candidate tentatively accepted the GS-5 Biological Sciences Technician position at the Uvalde National Fish Hatchery (UNFH) and began the onboarding process with a potential start date of May 7. In the interim, Nicholas Yvon is stepping in to assist Dominique Alvear in the daily husbandry until further staff is hired at the UNFH.

Species Collection

On March 9, Desiree Moore, Shawn Moore, Journey Moreno (Student Conservation Association (SCA), SMARC), David Thomasson (SCA, SMARC), and Braden West collected 61 Fountain darters from the San Marcos River below Rio Vista Dam (Figure 1).

On March 23, Alvear, Adam Daw, S. Moore, Celeste Palmquist (SCA, SMARC), Thomasson, and West traveled to Spring Island, New Braunfels to collect Comal Springs dryopid beetles and Peck's cave amphipods. No Comal Springs dryopid beetles were observed. A total of 23 juvenile and 108 adult Peck's cave amphipods were collected and brought to the UNFH for the refugia.

On March 29, S. Moore and West collected San Marcos salamanders from Spring Lake, San Marcos. Fifty-four adult individuals were collected for refugia, and 21 juveniles were caught and released.

Husbandry

Uvalde

Seventeen salamander eggs were laid by San Marcos salamanders held in refugia. Thomas and Yvon moved the clutch to a separate culture system to monitor development and hatch success.

Alvear and Daw met to discuss modifications to the chambers for the Peck's cave amphipod juvenile exclusion pilot study.

Daw began updating plumbing on a tank system in quarantine.

SMARC

Ten distinct clutches totaling 127 eggs were laid by salamanders held in refugia. Eggs were collected and transferred to separate culture systems to monitor growth and development.

S. Moore developed and implemented a new tank-labelling strategy at the SMARC. New tank labeling will allow for increased robustness of data collection and further quality control when animals are moved between systems and buildings.

S. Moore and Moreno learned to repot Texas wild rice plants (Figure 2)

Daw and West discussed future construction and design of partially recirculating systems for animals held in refugia.

Animal Health

On March 14, Thomas and Daw collected 10 San Marcos fountain darters from the San Marcos River and 10 Comal Springs fountain darters from the Comal River. The darters were sent for parasite analysis at the USFWS Southwestern Fish Health Unit, Dexter NM.

On March 30, S. Moore and West collected 27 skin swabs from San Marcos salamanders held in quarantine to be analyzed for *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal).

Task 2 Research

Dryopid Life History

Matt Pintar and Israel Prewitt (BIO-WEST) inventoried the Comal Springs dryopid beetle system and repaired leaks on the system. One adult and 22 larval dryopid beetles were found in the system.

San Marcos Salamander Mark Recapture

D. Moore continued the pilot study tagging San Marcos salamanders with p-chips in preparation for the mark-recapture study. The salamanders were scanned weekly. Thus far, no tag loss occurred and there were no mortalities due to tagging. The field tagging portion of this project was rescheduled for May 2023 to prioritize collections for the refugia standing stock.

Reproductive Gene Expression in San Marcos Salamanders

Ruben Tovar (University of Texas, Austin) has all microCT scans for Texas blind salamander but is missing a developmental stage for San Marcos salamander. Tovar collected RNA from a San Marcos salamander and began working on sequencing the RNA to generate the reference transcriptome.

Comal Springs Riffle Beetle Population Genetics

A collection plan for Comal Springs riffle beetle was established with the EAA and BIO-WEST. Criteria were established to determine the number of adults collected from BIO-WEST biomonitoring lures. Larvae will be collected from the lures to supplement adult collections.

Tagging Aquatic Invertebrates

Dr. Bockrath and D. Moore met with Dr. Shannon Brewer (Auburn University) to discuss the successful p-Chip tagging of live beetles and to set up dates for Dr. Brewer to conduct tagging at the SMARC.

D. Moore tested p-Chip tagging on freshly collected *Stygobromus flagellatus* collected by Victor Castillo (Edwards Aquifer Research and Data Center) from the Texas State University Artesian Well. Although these surrogate amphipods were too small to inject a tag, the practice aided in developing a technique. Four tagged Comal Springs riffle beetles had 100% survival and retention after 10 days and were able to move against the current in their holding tube (Figure 3).

Genetic Assessment of Peck's Cave Amphipod

Dr. Bockrath met with Dr. Chris Nice (Texas State University) to discuss field sampling plans. A plan was developed to take Peck's cave amphipod bycatch from BIO-WEST biomonitoring lures and supplement with additional collections outside of the biomonitoring effort.

Additional Accomplishments

All authors of the Comal Springs riffle beetle handbook met to discuss publishing the document. West was selected to be the lead author and began developing a timeline for revision and publication.
All authors of the San Marcos salamander handbook discussed publishing the document. D. Moore was selected to be the lead author. The authors began the revision process.

Dr. Bockrath and D. Moore discussed the publication of other applicable 2021 and 2022 research.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Dr. Bockrath and Daw drafted and submitted the 2024 EARP Work Plan.

Dr. Bockrath and Daw amended the 2023 Budget and Work Plan for approval by the implementation committee.

Task 6 Meetings and Presentations

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

On March 21, D. Moore led the first part of a two-part workshop teaching participants to tag aquatic species at the SMARC (Figure 4). Alvear, Dr. Bockrath, and Daw attended the workshop to be able to contribute to EARP tagging efforts in the future.

On March 23, Dr. Bockrath and Dr. David Britton attended the EAHCP Implementation Committee Meeting.

On March 28, Dr. Bockrath attended the EAHCP Biological Objectives Subcommittee (Texas wild rice and Fountain Darter) meeting.

On March 30, Dr. Bockrath, Daw, and West attended the EARP first quarterly research meeting for 2023.

Summary of March Activities

- The EARP collected 61 San Marcos fountain darters from the San Marcos River, San Marcos
- The EARP collected 10 San Marcos fountain darters from the San Marcos River and 10 Comal Springs fountain darters from the Comal River for analysis at the Southwestern Fish Health Unit
- The EARP collected 23 juvenile and 108 adult Peck's cave amphipods from Spring Island, New Braunfels
- Several EARP staff attended a P-Chip workshop to learn how to use p-Chips for EARP tagging efforts
- Dr. Bockrath and Dr. Britton attended the EAHCP Implementation Committee Meeting.
- Dr. Bockrath attended the EAHCP Biological Objectives Subcommittee (Texas wild rice and Fountain Darter) meeting

Tables and Figures

Table 1. March's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for March 2023. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	61	10	4	75	0	0	26	5	229	426
Fountain darter: Comal	NT	10	0	10	0	0	2	43	116	150
Comal Springs riffle beetle	NT	NT	0	0	0	17	0	2	36	17
Comal Springs dryopid beetle	NT	0	0	0	0	6	NA	0	2	10
Peck's cave amphipod	NT	131	7	138	0	0	NA	0	110	197
Edwards Aquifer diving beetle	NT	NT	--	--	0	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	0	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	0	0	0	1	89	65
San Marcos salamander	54	NT	21	75	26	0	1	7	97	205
Comal Springs salamander	NT	NT	--	--	0	0	0	0	59	91
Texas wild rice plants	NT	NT	--	--	0	0	4	13	201	196

Figure 1. Shawn Moore and Braden West collecting San Marcos fountain darters from the San Marcos River below Rio Vista Dam.

Figure 2. Shawn Moore with a freshly repotted Texas wild rice plant.

Figure 3. A p-Chip-tagged Comal Springs riffle beetle walking along a piece of cloth.

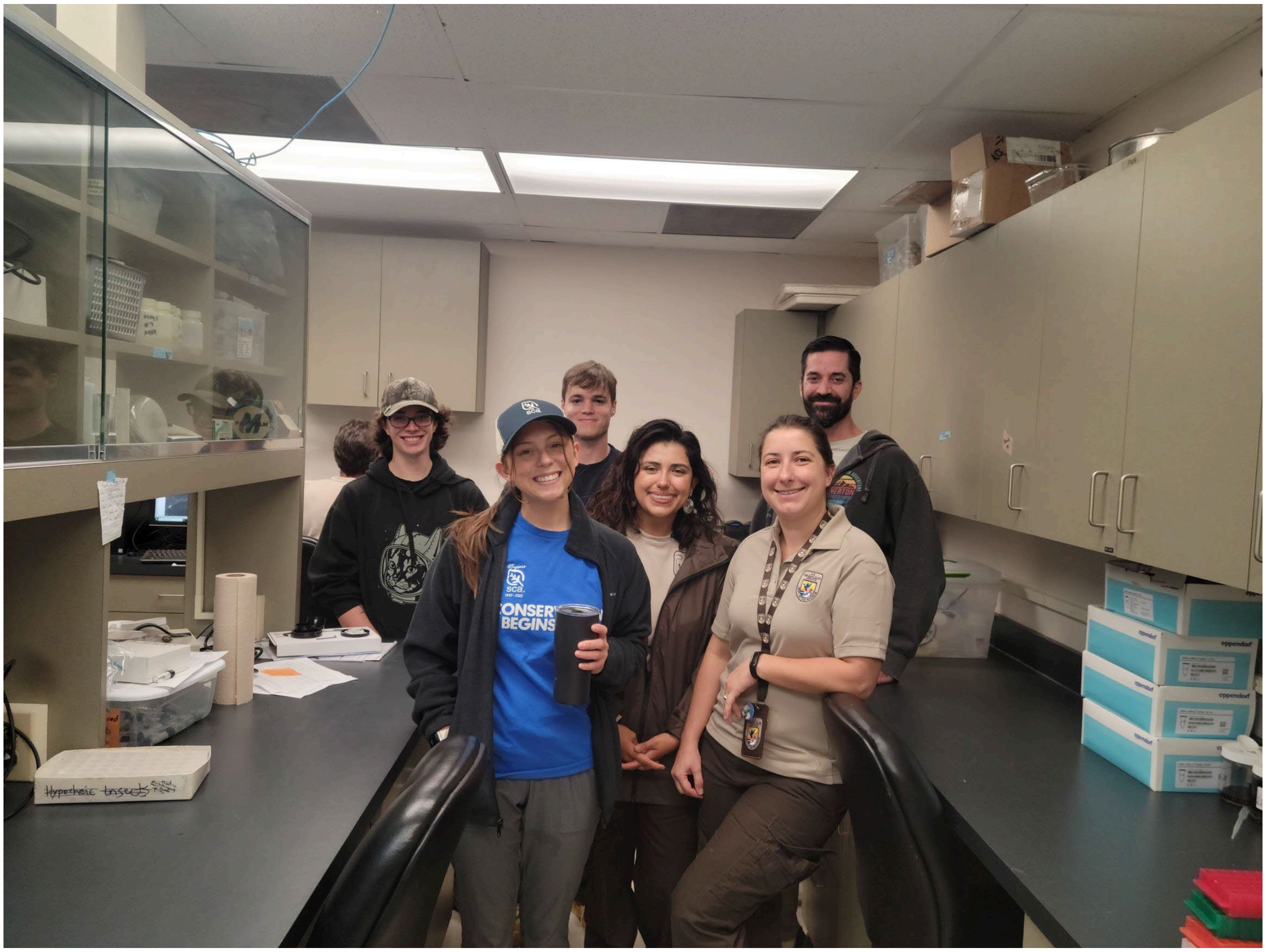


Figure 4. Celeste Palmquist, Journey Moreno, David Thomasson, Dominique Alvear, Dr. Katie Bockrath, and Adam Daw at the p-Chip tagging workshop at the SMARC.

April 2023 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desirée Moore, Dominique Alvear, and Braden West

With contributions from

Dr. Katie Bockrath, Adam Daw, Shawn Moore, and Nicholas Yvon

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

On April 4, Shawn Moore, Desiree Moore, and Braden West collected 34 San Marcos salamanders from below Spring Lake Dam in the San Marcos River, San Marcos, Texas (Figure 1).

On April 17, Justin Crow (SMARC), Somerley Swarm (SMARC), and West attached the drift net to Diversion Spring in Spring Lake, San Marcos, Texas (Figure 2).

On April 18, 19, and 20, S. Moore and West transferred 182 fountain darters collected by BIO-WEST biomonitoring from the San Marcos River to the San Marcos Aquatic Resources Center (SMARC).

Husbandry

<u>Uvalde</u>

Dominique Alvear administered a three-day 1% Sea Salt treatment to Comal Springs fountain darters due to a high mortality rate in QB01. Mortalities continued and the fish were moved to smaller holding tanks to be monitored and treated with a one-hour formalin dip. Mortalities decreased following the formalin treatment.

Alvear constructed the updated chambers for the Peck's cave amphipod juvenile exclusion pilot study. Three trials were conducted to ensure the chamber was functional and Peck's cave amphipods would not be lost during the study.

Alvear began semi-annual inventories of the Texas blind salamander, Comal Springs salamander, and San Marcos salamander.

Nicholas Yvon and Alvear repotted and transferred 63 containers of Texas wild rice.

Two clutches of eggs were laid by San Marcos salamanders held in refugia tanks RE05 and R07, with 22 eggs and 26 eggs, respectively. Yvon moved the clutches to a separate culture system to monitor development and hatch success.

Adam Daw set up a larger *Daphnia* culture tank to increase production at the Uvalde National

Fish Hatchery (UNFH) and evaluate for future expansion of the *Daphnia* cultures at the UNFH and SMARC.

<u>SMARC</u>

S. Moore designed and built a novel method for culturing red worms as a captive food source. The new 'worm tower' design minimizes escape and promotes reproduction of red worms.

S. Moore built and tested experimental cultures of *Hyallela* amphipods collected from SMARC ponds.

Four clutches of eggs were laid by Texas blind salamanders totaling 57 eggs. S. Moore moved the clutches to a separate culture system to monitor development and hatch success.

West began construction and testing of prototype partially recirculating systems for refugia tanks. The new systems incorporated a chiller and a 90-watt UV filter in addition to discharge, pH, and temperature monitoring capabilities.

Alvear, Daw, and West began revising the standard operating procedure for Peck's cave amphipod culture.

Task 2 Research

Dryopid Life History

D. Katie Bockrath and Dr. Matt Pintar (BIO-WEST) met to discuss the current dryopid set up and needed research. They discussed setting up challenge studies to determine dryopid habitat preferences to improve captive housing design.

Dr. Pintar checked the dryopid system to determine the number of adults, status of the system, and status of the conditioning leaves and wood. There were no adults found in the system.

San Marcos Salamander Mark Recapture

D. Moore continued the pilot study tagging San Marcos salamanders with p-chips in preparation for the mark-recapture study. The salamanders were scanned weekly. Thus far, no tag loss occurred and there were no mortalities due to tagging. D. Moore met with divers Justin Crow

(SMARC) and Somerley Swarm (SMARC) to create a plan for the diving in Spring Lake to tag San Marcos salamanders. Additionally, D. Moore scheduled snorkeling tagging trips with all SMARC staff helping with this project.

D. Moore wrote up a standard operating procedures document for p-Chip tagging in the field for this project.

Reproductive Gene Expression in San Marcos Salamanders

Dr. Bockrath met with Ruben Tovar (University of Texas, Austin) to discuss RNA sequencing data quality. The data quality was as expected and suitable for transcriptome assembly to establish a gene expression reference to compare all planned reproductive and developmental stages.

Comal Springs Riffle Beetle Population Genetics

BIO-WEST set out 80 lures for their projects, which will also provide individuals for genetic analyses. Dr. Bockrath created a plan with Dr. Pintar to work together on the collection of these lures. Dr. Bockrath, D. Moore, and West set 13 lures in Spring Run 2 to ensure all the spring runs are represented in the genetic analyses.

Tagging Aquatic Invertebrates

D. Moore met with Dr. Shannon Brewer (Auburn University) to discuss Dr. Brewer's trip to the SMARC to conduct tagging.

Two of the four Comal Springs riffle beetles tagged by D. Moore survived and retained their tags after one month. The other two beetles were not located during the inventory, which could indicate mortality or burrowing behavior.

Genetic Assessment of Peck's Cave Amphipod

Dr. Bockrath coordinated with BIO-WEST staff to collect Peck's cave amphipods from their biomonitoring lures.

Additional Accomplishments

Dr. Bockrath taught D. Moore and West how to properly extract DNA and conduct PCR in preparation for upcoming research (Figure 3).

EARP staff gave a tour of the EARP buildings to several City of Austin employees.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Dr. Bockrath reviewed progress reports for partnered research.

Dr. Bockrath met regularly with EARP staff to discuss publishing 2021-2022 research.

Task 6 Meetings and Presentations

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

EARP staff attended all the Edwards Aquifer Habitat Conservation Plan biological goals meetings.

Summary of April Activities

- The EARP collected 34 San Marcos salamanders from below Spring Lake Dam in the San Marcos River
- The EARP attached the drift net to Diversion Spring in Spring Lake

- The EARP received 182 fountain darters collected by BIO-WEST from the San Marcos River during biomonitoring
- The EARP set 13 lures in Spring Run 2 for the Comal Springs riffle beetle genetics research project
- EARP staff attended all the Edwards Aquifer Habitat Conservation Plan biological goals meetings.

Tables and Figures

Table 1. April's new collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for April 2023. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	177	NT	5	182	--	0	12	5	217	416
Fountain darter: Comal	NT	NT	--	--	--	0	12	43	104	104
Comal Springs riffle beetle	NT	NT	--	--	--	0	NA	2	36	17
Comal Springs dryopid beetle	NT	NT	--	--	--	0	0	0	2	10
Peck's cave amphipod	NT	NT	--	--	--	0	17	0	97	183
Edwards Aquifer diving beetle	NT	NT	--	--	--	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	--	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	--	0	0	1	89	65
San Marcos salamander	39	NT	14	53	54	0	2	7	149	183
Comal Springs salamander	NT	NT	--	--	--	0	0	0	59	91
Texas wild rice plants	NT	NT	--	--	--	0	0	13	201	188

Figure 1. Shawn Moore collecting San Marcos salamanders from below the Spring Lake Dam in the San Marcos River.

Figure 2. Somerley Swarm and Justin Crow diving in Spring Lake to attach the drift net to Diversion Spring.

Figure 3. Braden West, Desiree Moore, and Justin Crow learning from Dr. Katie Bockrath how to perform PCR in the genetics lab at the SMARC.

May 2023 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desirée Moore, Dominique Alvear, and Braden West

With contributions from

Dr. Katie Bockrath, Adam Daw, Jonathan Donahey, and Shawn Moore

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staffing

Jonathan Donahey began working at the Uvalde National Fish Hatchery (UNFH) as a Biological Science Technician on May 8. Donahey attended Northern Michigan University where he obtained a Bachelor of Science in Fisheries Management. He volunteered as a technician working on lake trout diet analysis and stable isotope analysis. Donahey enjoys Detroit sports and being outdoors in his spare time.

Species Collection

On May 3, Shawn Moore and Braden West received 501 Comal fountain darters from BIO-WEST staff as part of biomonitoring efforts in Landa Lake, New Braunfels, Texas. All darters were kept for quarantine at the San Marcos Aquatic Resources Center (SMARC).

On May 15, S. Moore and West set traps for Texas blind salamanders in Primer's Fissure and Johnson's Well (Figure 1). Fifteen salamanders were observed in total and four were kept for quarantine at the SMARC. Seven Texas blind salamanders were injected with p-Chip microtransponder tags and released. Four of the seven tagged salamanders were recaptured one time. An additional salamander was recaptured three times. No salamanders were observed moving between sample sites.

On May 31, Adam Daw, Dominique Alvear and Donahey collected 88 San Marcos fountain darters from the San Marcos River in the area below the eastern spillway of Spring Lake Dam. All darters were kept for the UNFH.

On May 31, S. Moore and West collected 12 Texas wild rice plants from Section A of the San Marcos River. All plants were kept for the SMARC.

Husbandry

<u>*Uvalde*</u>

Donahey began training with Alvear on the daily tasks for the fountain darter and salamander husbandry. Alvear and Donahey finished the yearly task of repotting Texas wild rice (Figure 2).

Alvear finished the quarterly inventories of the Peck's cave amphipod, where six females were found with full brooding pouches. These females were separated into individual boxes to monitor juvenile hatch and survival rates due to the cannibalistic nature of the Peck's cave amphipod.

Alvear conducted quarterly inventories of the Comal Springs riffle beetles and the Comal Springs dryopid beetles.

Daw began modifications on tank RE01 in preparation for a new controller. A new controller was added to tank RE11. Daw updated Rack System 2 in the invertebrate room to allow the addition of a CO_2 injector and UV sterilizer to further improve water quality.

Alvear, Daw, and Donahey began building new shelving rack systems to organize storage in the refugia and invertebrate room.

SMARC

West completed construction of a second recirculating quarantine rack system at the SMARC.

West began construction of recirculating systems for use in the refugia space. Each system consisted of two raceway tanks, UV sterilizer, CO_2 injector, and a chiller/heater, providing more security and stability than previous designs.

S. Moore made improvements to captive red worm cultures, testing the addition of cardboard to control the buildup of moisture in the system.

S. Moore continued working with captive amphipod cultures, observing substrate decomposition in culture tanks and replacing unsuitable substrate.

Animal Health

On May 2, the Southwest Fish Health Center conducted their yearly site inspection at the UNFH. Thirty San Marcos fountain darters and five Comal Springs fountain darters were taken, and abdominal swabs were obtained from the salamanders.

Task 2 Research

Dryopid Life History

Dr. Katie Bockrath and Desiree Moore met with Dr. Matt Pintar (BIO-WEST) to discuss the use of choice chambers in Comal Springs dryopid beetle research. Dr. Pintar set up dryopid choice chambers at the SMARC.

BIO-WEST staff searched for dryopids in the wild and set out wood lures in several springs to attract beetles. Dr. Bockrath and Dr. Pintar collected 11 dryopids and transported them to the SMARC for this project.

San Marcos Salamander Mark Recapture

D. Moore continued the pilot study tagging San Marcos salamanders with p-chips in preparation for the mark-recapture study. The salamanders were scanned weekly. Thus far, no tag loss occurred and there were no mortalities due to tagging.

Several members of the SMARC staff, interns, and volunteers contributed to the collection, processing, and release of San Marcos salamanders in the San Marcos River and Spring Lake (Figure 3). Salamanders were collected from the San Marcos River below the eastern spillway of the Spring Lake Dam May 9 and 30 (Table 2). Salamanders were collected from Spring Lake near the Diversion pipe May 10 and near the Hotel site May 11 and 31. All salamanders were released back to the area they were captured after they fully recovered from anesthesia.

Reproductive Gene Expression in San Marcos Salamanders

Ruben Tovar (University of Texas, Austin) began the process of ordering kits and reagents for RNA isolation of previously microCT-scanned embryos of Texas blind and San Marcos salamanders.

Comal Springs Riffle Beetle Population Genetics

Dr. Bockrath and West joined BIO-WEST staff in the field to retrieve biomonitoring and EARP lures across the Comal system. Comal Springs riffle beetles were preserved for genetic analysis according to the criteria developed for this project (Table 3). EARP lures in Spring Run 2 were redeployed.

Tagging Aquatic Invertebrates

Dr. Shannon Brewer (Auburn University) traveled to the SMARC to view the housing of SMARC invertebrates and test different glues and refine tagging methods for the Comal Springs riffle beetle (Figure 4).

Genetic Assessment of Peck's Cave Amphipod

Dr. Bockrath and West coordinated with BIO-WEST staff to collect Peck's cave amphipods from their biomonitoring lures across the Comal system and the EARP lures in Spring Run 2 (Table 3). All Peck's cave amphipods collected were preserved for genetic analysis.

Additional Accomplishments

EARP staff gave a tour of the EARP buildings to the City of San Marcos Conservation Crew. EARP staff gave a tour of the EARP buildings to several New Braunfels Utilities employees.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

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

Dr. Bockrath attended all Edwards Aquifer Habitat Conservation Plan Biological Objectives meetings possible.

Summary of May Activities

- Jonathan Donahey began working as a Biological Science Technician at the UNFH
- The EARP received 501 Comal fountain darters from BIO-WEST
- The EARP collected four Texas blind salamanders from Primer's Fissure and Johnson's Well.
- The EARP collected 88 San Marcos fountain darters from the middle San Marcos River
- The EARP collected 12 Texas wild rice plants from Section A of the San Marcos River
- The Southwest Fish Health Center conducted their yearly site inspection at the UNFH
- The EARP tagged and released 243 San Marcos salamanders for the mark-recapture study
- The EARP and BIO-WEST collected 93 Comal Springs riffle beetle larvae (41) and adults (52) and 48 Peck's cave amphipods for genetic analysis

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for May 2023. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	88	0	88	111	0	24	37	304	380
Fountain darter: Comal	501	NT	0	501	--	0	22	19	82	85
Comal Springs riffle beetle	NT	NT	--	--	--	0	NA	0	36	17
Comal Springs dryopid beetle	NT	NT	--	--	--	0	0	2	2	8
Peck's cave amphipod	NT	NT	--	--	--	50	0	22	97	212
Edwards Aquifer diving beetle	NT	NT	--	--	--	0	0	--	0	0
Texas troglobitic water slater	NT	NT	--	--	--	0	0	--	0	0
Texas blind salamander	4	NT	11	15	--	0	0	1	89	64
San Marcos salamander	NT	NT	--	--	38	0	0	5	187	178
Comal Springs salamander	NT	NT	--	--	--	0	1	0	61	91
Texas wild rice plants	12	NT	0	12	--	0	8	5	193	181

Table 2. The number of tagged and recaptured San Marcos salamanders from each site each field day of the San Marcos salamander mark-recapture study.

Date	Site	# tagged	# recaptured
9-May-23	eastern spillway	82	0
10-May-23	diversion area	33	0
11-May-23	hotel area	53	0
30-May-23	diversion area	53	0
31-May-23	hotel area	22	0

Table 3. Comal Springs riffle beetle (CSRB) and Peck's cave amphipod (PCA) collection information from EARP lures at Spring Run 2 and BIO-WEST biomonitoring lures at all other locations. Adult and larval CSRB take are reported separately, and the percentage of adult CSRB take out of all adult CSRB encountered from lures is given.

Location	Adult CSRB encountered	Adult CSRB take	% Adult CSRB take	Larval CSRB take	Total CSRB take	PCA take
Spring Run 1	1	1	100%	0	1	1
Spring Run 2	1	1	100%	6	7	0
Spring Run 3	37	15	41%	8	23	30
Western Shore	42	16	38%	13	29	4
Spring Island	90	19	21%	14	33	13
Upper Spring Run	0	0	0%	0	0	0
Totals	171	52	30%	41	93	48

Figure 1. Braden West and Shawn Moore placing traps into Primer's Fissure for Texas blind salamander collection.

Figure 2. Jonathan Donahey placing a pump into a Texas wild rice tank at the UNFH after rice repotting.

Figure 3. Somerley Swarm, Justin Crow, Aaron Wallendorf, Braden West, Desiree Moore, and Daniela Cortez on the barge near the diversion pipe in Spring Lake.

Figure 4. Dr. Shannon Brewer testing different glues for tagging Comal Springs riffle beetles at the SMARC.

June 2023 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desirée Moore, Dominique Alvear, and Braden West

With contributions from

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

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staffing

Heidi Meador began working at the Uvalde National Fish Hatchery (UNFH) as a Biological Science Technician on June 5. Heidi finished her B.S. degree in Zoology, and some graduate work in entomology at the University of Wyoming. She started her career in biology as a technician with Wyoming Game and Fish and then moved into federal service at Saratoga National Fish Hatchery (NFH). While at Saratoga NFH, Meador worked primarily with Wyoming toads and the culture of their feeder insects. Meador also worked with lake trout, brown trout, rainbow trout, cutthroat trout, and Kokanee salmon. Heidi enjoys outdoor activities, competitive long-range shooting, and scuba diving.

Species Collection

On June 28, Adam Daw, Johnathan Donahey, Meador, Shawn Moore, and Braden West collected Peck's cave amphipods (PCA) and Comal River fountain darters from Spring Island, New Braunfels, Texas. Seventy-six PCA were collected, of which 73 were retained for quarantine at the San Marcos Aquatic Resources Center (SMARC). One hundred sixty darters were collected, of which 154 were retained for quarantine at the UNFH. Submerged wood was examined for Comal Springs dryopid beetles and larvae, but none were found.

Husbandry

<u>*Uvalde*</u>

Meador began training with Alvear on the daily tasks for the fountain darter, salamander and Texas wild rice husbandry.

Alvear conducted an inventory of the female Peck's cave amphipods that were separated into individual boxes in May. Most of the females no longer had juveniles in their brooding pouches and no juveniles were recovered in the box. For the Peck's cave amphipods that were seen with juveniles still attached, Alvear opted to remove them from the female and place them into a box and monitor them for long-term survival.

Alvear began making a Peck's cave amphipod egg incubator tumbler to collect eggs at earlier stages.

Alvear, Daw, and Donahey got two quarantine racks operational again in preparation for potential species salvage in the summer.

Alvear, Donahey, and Meador conducted the biannual inventory of all San Marcos fountain darters in the refugia (Figure 1).

Daw continued constructing new controller boxes for both the UNFH and SMARC.

Daw and Donahey replaced a broken chiller on quarantine rack QB7.

Daw installed a new type of water circulation pump in a rice tank at the UNFH to see if the new pump provides better water flow than the current circulation pumps.

West visited the UNFH. While on site, Alvear, Daw, and West continued drafting an updated Peck's cave amphipod propagation and maintenance SOP.

S. Moore visited UNFH. Daw provided a tour and an overview of advanced system monitoring. Alvear, Donahey, and S. Moore discussed advancements in captive reproduction, animal feeding, and further efforts in maintaining mortality preservation.

SMARC

West finished construction of a new prototype partially recirculating system for use in the SMARC refugia space.

West constructed a new stainless-steel gear-washing and disinfection station behind the SMARC refugia building.

S. Moore transferred three clutches of Texas blind salamander and one clutch of San Marcos salamander eggs to incubation tanks in the SMARC quarantine on June 26.

Task 2 Research

Dryopid Life History

Because efforts to collect Comal Springs dryopid beetles for choice chamber experiments were unsuccessful, logs were placed at Spring Island to provide additional collection sources. Dryopid beetle larvae were found on the logs, providing evidence that adults were in the area. Dr. Katie Bockrath, Desiree Moore, and Dr. Matt Pintar (BIO-WEST) met to discuss the potential of additional research examining captive housing set ups to hold larvae and test housing preferences for larvae. Because Comal Springs drypoid and riffle beetles are often observed together, dryopid beetles are housed under very similar conditions as Comal Springs riffle beetles in the refugia, despite empirical evidence that these housing configurations are suitable for drypoid beetles. While we continue to attempt to collect adult dryopid beetles, Dr. Bockrath and Dr. Pintar will test these assumptions though observing larval preference by comparing current Comal Springs riffle beetle captive holding conditions to differing light, flow, housing, and biofilm conditions.

San Marcos Salamander Mark Recapture

D. Moore continued the pilot study tagging San Marcos salamanders with p-chips for the mark-recapture study. The salamanders were scanned weekly. Thus far, no tag loss occurred and there were no mortalities due to tagging.

Several members of the SMARC staff and interns contributed to the collection, processing, and release of San Marcos salamanders in the San Marcos River and Spring Lake (Figure 2). Salamanders were collected from the San Marcos River below the eastern spillway of the Spring Lake Dam June 12 and 27 (Table 2). Salamanders were collected from Spring Lake near the Diversion pipe June 20 and near the Hotel site June 14 and 26. All salamanders were released back to the area they were captured after they fully recovered. Across all sites, 27 salamander recaptures occurred in June (Table 2).

Reproductive Gene Expression in San Marcos Salamanders

The preliminary RNA sequencing data were as expected and suitable for transcriptome assembly. Therefore, Ruben Tover (University of Texas, Austin) began ordering kits and reagents for RNA isolation of previously diceCT-scanned embryos of Texas blind and San Marcos salamanders. Tovar also began isolating RNA for sequencing and developed a plan to process the anatomical data from diceCT scans while waiting for RNA sequencing completion.

Comal Springs Riffle Beetle Population Genetics

Dr. Bockrath began preparing Comal Springs riffle beetles for DNA extraction by placing individuals in separate DNA extraction tubes.

Dr. Bockrath, S. Moore, and West retrieved lures from Spring Run 2 and collected three adult and six larval Comal Springs riffle beetles for genetic analysis (Figure 3).

Tagging Aquatic Invertebrates

Dr. Shannon Brewer (Auburn University) selected a candidate to fill the master's student position working on this project. Dr. Brewer began purchasing the selected tagging supplies needed to begin trials.

Genetic Assessment of Peck's Cave Amphipod

Dr. Bockrath began preparing Peck's cave amphipods for DNA extraction by placing individuals in separate DNA extraction tubes.

Dr. Bockrath, S. Moore, and West collected four *Stygobromus* sp. from Spring Run 2 for genetic analysis (Figure 3). One of four specimens was confirmed by West and Randy Gibson (SMARC) to be *Stygobromus pecki*. The remaining three specimens are suspected to be *S. pecki* but could not be identified using physical characteristics. All individual identifications will be confirmed using genetics.

Additional Accomplishments

The paperwork and approval process for filling the last Biological Sciences Technician position at the SMARC was completed. Richelle Jackson, a former Student Conservation Association intern, was selected to fill the position starting July 17.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Dr. Bockrath conducted grants management reporting for partnered research.

Task 6 Meetings and Presentations

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

EARP staff met with Edwards Aquifer Authority staff at the Education Center for the quarterly research meeting and a tour (Figure 4).

Summary of June Activities

- The EARP collected 73 PCA from Spring Island for the Refugia
- The EARP collected 154 Comal River Fountain darters from Spring Island for the Refugia
- The EARP tagged and released 211 San Marcos salamanders for the mark-recapture study
- The EARP recaptured 27 tagged San Marcos salamanders in the mark-recapture study
- West and S. Moore visited UNFH to discuss upcoming work and homogenization of procedures
- Heidi Meador began working at UNFH
- EARP staff collected 3 adult and 6 larval Comal Springs riffle beetles from Spring Run 2 for Research

- EARP staff collected 1 confirmed and 3 suspected Peck's cave amphipods from Spring Run 2 for Research
- A master's student was selected to continue the invertebrate tagging research at Auburn University
- EARP and EAA staff met at the Education Center for the quarterly research meeting

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for June 2023. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	0	--	--	0	89	32	214	338
Fountain darter: Comal	NT	154	6	160	188	0	47	5	223	80
Comal Springs riffle beetle	NT	NT	--	--	--	0	NA	NA	36	17
Comal Springs dryopid beetle	NT	NT	--	--	--	0	0	NA	2	8
Peck's cave amphipod	73	NT	3	76	--	0	0	25	97	187
Edwards Aquifer diving beetle	NT	NT	--	--	--	0	0	--	0	0
Texas troglobitic water slater	NT	NT	--	--	--	0	0	--	0	0
Texas blind salamander	NT	NT	--	--	4	0	0	0	93	64
San Marcos salamander	NT	NT	--	--	--	0	1	4	186	174
Comal Springs salamander	NT	NT	--	--	--	0	1	1	60	90
Texas wild rice plants	NT	NT	--	--	12	0	0	0	205	181

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

Date	Site	# Tagged	# Recaptured	# Untagged	Total Capture
9-May-23	eastern spillway	82	0	5	87
10-May-23	diversion area	33	0	0	33
11-May-23	hotel area	53	0	8	61
30-May-23	eastern spillway	53	0	16	69
31-May-23	hotel area	22	0	0	22
12-Jun-23	eastern spillway	75	6	20	101
14-Jun-23	hotel area	74	6	25	105
20-Jun-23	diversion area	62	2	8	72
26-Jun-23	hotel area	0	9	21	30
27-Jun-23	eastern spillway	0	4	90	94

Figure 1. Jonathan Donahey and Heidi Meador removing tank substrate in preparation for San Marcos fountain darter inventory.

Figure 2. Justin Crow (SMARC), Shawn Moore, Randy Gibson (SMARC), and David Thomasson (Student Conservation Association intern, SMARC) preparing to collect San Marcos salamanders at the Hotel site in Spring Lake.

Figure 3. Dr. Katie Bockrath instructing Shawn Moore on identification of Comal Springs riffle beetle and cotton lure processing procedure at Spring Run 2, Landa Park, New Braunfels, Texas.

Figure 4. Edwards Aquifer Refugia Program staff attended the quarterly meeting at the Edwards Aquifer Authority's Education Outreach Center at Morgan's Wonderland Camp, San Antonio, Texas. From left to right: Braden West, Adam Daw, Dominique Alvear, Shawn Moore, Desiree Moore, Dr. Katherine Bockrath.

July 2023 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desirée Moore, Dominique Alvear, and Braden West

With contributions from

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

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Staffing

Richelle Jackson began working at the San Marcos Aquatic Resources Center (SMARC) as a Biological Science Technician on July 17. Jackson earned a bachelor's degree in Natural Resource Management and Environmental Studies from Texas State University. Jackson returns to us after working as a Student Conservation Association intern for the EARP last year. Outside of work, Jackson enjoys snorkeling, SCUBA diving, Muy Thai, and cats.

Species Collection

On July 6, Dominique Alvear and Adam Daw collected 28 San Marcos fountain darters from the San Marcos River below the Rio Vista Dam, which were taken back to the Uvalde National Fish Hatchery (UNFH).

On July 6, S. Moore and West collected 179 San Marcos fountain darters from the San Marcos River upstream of Sessom Creek. One hundred seventy-seven were retained for quarantine at the SMARC. One San Marcos salamander was captured while dip-netting for San Marcos fountain darters. The individual was visually inspected to determine its involvement in the San Marcos salamander mark-recapture study and was returned to its initial collection location.

Jackson, S. Moore, and West checked the Diversion net (Spring Lake, San Marcos, Texas) on Mondays, Wednesdays, and Fridays beginning on July 14 (Figure 1). Five juvenile and two adult San Marcos salamanders were collected from the net in July. All adult animals were visually inspected for their involvement in the San Marcos salamander mark-recapture study and returned to Spring Lake.

From July 25-27, Jackson and S. Moore transferred 466 Comal River fountain darters collected during BIO-WEST biomonitoring conducted at the Old Channel on July 25, Landa Lake on July 26, and the New Channel on July 27 to the SMARC. Daw transferred 455 of the darters to the UNFH on July 31.

Husbandry

<u>*Uvalde*</u>

Jonathan Donahey and Heidi Meador conducted bi-annual inventories of all San Marcos fountain darters in the refugia.

Meador adjusted the amphipod egg incubator Alvear made for potential use to reduce salamander egg loss due to fungus. Meador transferred 26 Texas blind salamander eggs to the egg incubator, but the eggs were later deemed unfertilized. On July 31, four clutches of salamander eggs were discovered and transferred to individual holding tanks with eggs split between egg incubators and egg hammocks to test methods to improve water flow. Two clutches of Texas blind salamanders, one clutch of San Marcos salamanders, and one clutch of Comal Springs salamanders were included in the pilot.

Meador began culturing white worms for potential future use in feeding juvenile salamanders.

Alvear conducted five Peck's cave amphipod juvenile exclusion trials.

Alvear checked on the Peck's cave amphipods juveniles that were manually removed from the female's brood pouch last month to monitor grow and survival. Six of the nine juveniles were found and seemed to be normal.

Alvear began training Donahey and Meador on the use of a microscope and fish dissections.

Alvear did a literature review of potential treatments available for treating the observed internal parasites in the Comal River fountain darters from the June 2023 collection. A preliminary trial using a Praziquantel bath for 24 hours was conducted. No fish died during the trial or the following days.

Staff transferred Texas wild rice between refugia tanks to take inventory and clean the old tank. Rice that was replanted in two new types of pots in the winter/spring were visually evaluated for differences in health between them and rice repotted in the standard pots. The rice in the new pots both had better root formation and potentially better leaf growth compared to the rice in the standard pots (Figure 2). The new pots are thought to improve water flow through the pot substrate and therefore improve root health.

Daw finished re-plumbing tank RE01. Tank RE09 was acid washed and is next in queue for re-plumbing. Daw installed a controller to monitor overall well water pressure, temperature, flow rate, and water dissolved gas. Daw installed an electric ball valve on the main refugia well water line that will automatically shut off the water to the refugia during gas supersaturation events. Daw added a chiller to the system to help acclimate future rice.

<u>SMARC</u>

Ten clutches of salamander eggs were produced in July. One clutch of San Marcos salamander eggs and nine clutches of Texas blind salamander eggs were transferred to incubation racks in the SMARC quarantine.

West pressure-tested new recirculating systems in the SMARC refugia. Prototype recirculating systems were made ready to hold animals. A redundant invertebrate system was brought online.

West gave a seminar to Jackson and S. Moore covering commonly used hand and power tools at the SMARC. West covered material handling, basic tool safety, personal protective equipment, and hazardous materials safety.

S. Moore trained Jackson on daily and weekly refugia tasks such as feeding, captive food culture maintenance, biosecurity, and field work.

Animal Health

On June 28, 154 Comal Springs fountain darters were collected and taken to the UNFH. Within 20 days in quarantine, 78 mortalities were recorded. Although it is normal to have higher mortality rates in the Comal Springs fountain darters, this high mortality rate was still concerning as most seemed to be hemorrhaging from the gills. Alvear performed necropsies and found various abnormalities. Many fish had extremely swollen gill filaments with white cysts varying in size and shape. Patches along the base caudal fin were inflamed and these fish died with their mouths open. Upon internal examination, juvenile *Leptorlynchoides* were found in the peritoneal cavity. Dr. Huseyin Kucuktas of the Southwestern Native Aquatic Resources and Recovery Center (SNARRC) was contacted to discuss the use of Praziquantel, an anthelmintic, as a treatment. Alvear met with Dr. David Huffman from Texas State University to further identify the parasites that were photographed. Dr. Huffman identified the parasites as *Leptorlynchoides* sp., *Centrocestus* sp., and *Haplorchis* sp. As the parasitic life cycle of these use fish as intermediate host, it was determined that it may not be possible to kill parasites already established in the darters, but the use of Praziquantel may interrupt the parasite reproduction and overall lessen the parasitic load the fish carry. Further investigation of the varying densities of parasites throughout the Comal River was suggested to potentially increase survival rates in captivity, and treatment trials were suggested to reduce parasite reproduction and improve fountain darter survival.

Staff received Bd case reports from the San Diego Zoo Wildlife Alliance on July 24. Of the 44 individuals tested, 12 returned as positive for Bd and 32 were either negative or inconclusive. All animals tested negative for Bsal.

Task 2 Research

Dryopid Life History

Dr. Katie Bockrath and Dr. Matt Pintar (BIO-WEST) met to discuss larval habitat choice experiments including biofilm/food choice, where different species of leaves are sterilized and conditioned and offered to the larvae to determine if they show preference. Additionally, choice experiments examining the effect of light on larval behavior were discussed.

Dr. Pintar collected eight Comal Springs dryopid beetles for the adult habitat choice trials. A light sensitivity trial is ongoing.

San Marcos Salamander Mark Recapture

Several members of the SMARC staff and interns contributed to the collection, processing, and release of San Marcos salamanders in the San Marcos River and Spring Lake (Figure 3). Salamanders were collected from the San Marcos River below the eastern spillway of the Spring Lake Dam July 13 (Table 2). Salamanders were collected from Spring Lake near the Diversion pipe June 12 and near the Hotel site June 10. All salamanders were released back to the area they were captured after they fully recovered. Across all sites, nine salamander recaptures occurred in July (Table 2).

Reproductive Gene Expression in San Marcos Salamanders

Ruben Tover (University of Texas, Austin) continued RNA isolation of previously diceCT-scanned embryos of Texas blind and San Marcos salamanders.

Comal Springs Riffle Beetle Population Genetics

Dr. Bockrath continued preparing Comal Springs riffle beetles for DNA extraction by placing individuals in separate DNA extraction tubes.

S. Moore and West retrieved all remaining lures in Spring Run 2 (Landa Park, New Braunfels, Texas) on July 20. Each of the remaining lures were left dry due to the lower water line. No target species remained on the dry lures. Lures were not returned to the spring run following collection.

Tagging Aquatic Invertebrates

Brian De La Torre (Auburn University) filled the master's student position for this project. Dr. Shannon Brewer (Auburn University) received the tagging supplies needed to begin trials and worked with students to develop a housing system that provides easy monitoring of tagged and control individuals without harming beetles.

Genetic Assessment of Peck's Cave Amphipod

Dr. Bockrath continued preparing Peck's cave amphipods for DNA extraction by placing individuals in separate DNA extraction tubes.

Additional Accomplishments

EARP staff gave a tour of the EARP buildings to a Directorate Fellowship Program intern.

EARP staff gave a tour of the SMARC facility to Damon Childs and two EAHCP interns.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

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

Summary of July Activities

- The EARP collected 28 San Marcos fountain darters from the San Marcos River below the Rio Vista Dam
- The EARP collected 179 San Marcos fountain darters from the San Marcos River upstream of Sessom Creek
- The EARP checked the Diversion net (Spring Lake, San Marcos, Texas) on Mondays, Wednesdays, and Fridays beginning on July 14
- Richelle Jackson began working at the San Marcos Aquatic Resources Center (SMARC) as a Biological Science Technician
- The EARP transferred 466 Comal River fountain darters collected during BIO-WEST biomonitoring

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for July 2023. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	205	28	2	207	--	0	88	26	126	375
Fountain darter: Comal	466	NT	0	466	--	0	38	5	185	152
Comal Springs riffle beetle	NT	NT	--	--	--	0	NA	NA	36	17
Comal Springs dryopid beetle	NT	NT	--	--	--	0	NA	NA	2	8
Peck's cave amphipod	NT	NT	--	--	--	0	NA	NA	97	187
Edwards Aquifer diving beetle	NT	NT	--	--	--	0	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	--	0	0	0	0	0
Texas blind salamander	NT	NT	--	--	--	0	0	0	93	64
San Marcos salamander	0	NT	8	8	--	0	2	4	184	170
Comal Springs salamander	NT	NT	--	--	--	0	2	0	58	90
Texas wild rice plants	NT	NT	--	--	--	0	9	0	196	181

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

Date	Site	# Tagged	# Recaptured	# Untagged	Total Capture
9-May-23	eastern spillway	82	0	5	87
10-May-23	diversion area	33	0	0	33
11-May-23	hotel area	53	0	8	61
30-May-23	eastern spillway	53	0	16	69
31-May-23	hotel area	22	0	0	22
12-Jun-23	eastern spillway	75	6	20	101
14-Jun-23	hotel area	74	6	25	105
20-Jun-23	diversion area	62	2	8	72
26-Jun-23	hotel area	0	9	21	30
27-Jun-23	eastern spillway	0	4	90	94
10-Jul-23	hotel area	0	3	19	22
12-Jul-23	diversion area	0	2	78	80
13-Jul-23	eastern spillway	0	4	53	57

Figure 1. Shawn Moore returning the Diversion net to the water after emptying its contents into a cooler.

Figure 2. Heidi Meador holding up one of the experimental Texas wild rice pots, which has roots extending past the bottom of the pot.

Figure 3. Braden West, Dr. Katie Bockrath, Shawn Moore, Somerley Swarm, and Justin Crow at the Diversion collection for the San Marcos salamander mark-recapture study in Spring Lake.

August 2023 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desirée Moore, Dominique Alvear, and Braden West

With contributions from

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

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

On August 7, Richelle Jackson, Shawn Moore, and Braden West set traps at Johnson's Well, Purgatory Creek Natural Area, San Marcos, Texas for Texas blind salamander. No traps were set in Primer's Fissure due to low water levels. Traps were checked on August 9, 11, 14, 16, 18 and 21. Four individuals were collected, and one was retained for refugia at the San Marcos Aquatic Resources Center (SMARC; Figure 1).

On August 17, Dominique Alvear, Adam Daw, Jonathan Donahey, Jackson, Heidi Meador, S. Moore, and West collected 72 San Marcos fountain darters along with 10 Texas wild rice plants below the Spring Lake Dam. All the fountain darters and Texas wild rice were transported to the Uvalde National Fish Hatchery (UNFH).

On August 31, Dr. Katie Bockrath, Daw, Donahey, Jackson, Meador, S. Moore, and West collected 118 Comal Springs fountain darters, 98 Peck's cave amphipods, and 12 Comal Springs salamanders. The fountain darters and amphipods were transported to the UNFH, and the salamanders were transported to the San Marcos Aquatic Resource Center.

SMARC staff checked the drift net placed over Diversion Springs, Spring Lake, San Marcos, Texas on Mondays and Thursdays for the entirety of the month of August. Seven San Marcos salamanders were collected in the net in August, including one tagged with a p-Chip as part of ongoing mark-recapture research. No animals were retained for refugia purposes, and all animals were released in a manner consistent with husbandry protocol.

Husbandry

<u>*Uvalde*</u>

On August 8, Meador discovered a clutch of San Marcos salamander eggs and transferred them to a holding tank for closer monitoring.

Alvear began quarterly inventories of the Comal Springs riffle beetle and Comal Springs dryopid beetles.

Refugia staff painted refugia and invertebrate room walkways with non-slip paint.

Alvear monitored quarantine rack 6 daily as it housed the Comal Springs fountain darters being used for the preliminary trial for the use of Praziquantel.

Daw installed two monitor/controllers in the UNFH invert room and started teaching the UNFH refugia staff how to make the electrical boxes that connect to the monitor/controllers.

Daw installed the main CO_2 distribution line in the refugia room.

Daw picked up the new *Daphnia* culture tanks for the SMARC and UNFH and installed the tanks at the UNFH (Figure 2).

Refugia staff started installing new epoxy tabletops and sinks for new live food area and work benches.

SMARC

Refugia staff demarcated a new area for live food production. Staff assembled shelves and moved old equipment to storage. Staff installed flow-control valves and drains onto *Daphnia* culture tanks.

Jackson and S. Moore transferred San Marcos salamanders, San Marcos fountain darters, and Texas blind salamanders from quarantine to refugia. Sixteen Texas blind salamanders were tagged with p-Chip tags prior to the transfer to allow individual tracking.

One clutch of second-generation captive-bred Texas blind salamander eggs was produced. Eggs were transferred to the nursery system for further monitoring.

S. Moore continued training Jackson on invertebrate inventory procedures.

West continued monitoring water quality on newly constructed recirculating systems. S. Moore and West moved Texas blind salamanders into the system.

West began construction on a second recirculating system in the refugia space.

West constructed mounting points for controllers in the refugia and made plans for future recirculating system construction.

Animal Health

West submitted 20 San Marcos fountain darters and 20 Comal fountain darters to the Southwestern Fish Health Unit.

In the Comal fish, *Centrocestus formosanus* was observed in 9 of 10 fish. Monogenetic trematodes were observed on the gills from 9 of 10 fish examined.

In the San Marcos fish, *Centrocestus formosanus* was observed in 1 of 10 fish. Moderate to high numbers of monogenetic trematodes were also observed on gills from all 10 fish examined. Large numbers of myxozoan parasites, possibly *Myxobolus* sp., were found on one fish. Furthermore, large numbers of *Ichthybodo* sp. parasites were also observed on another fish.

Task 2 Research

Dryopid Life History

Dr. Matt Pintar (BIO-WEST) regularly looked for adult Comal Springs dryopid beetles in the wild with no success. The low spring flows resulted in silty conditions, and it was difficult to find adults and larvae at regular observation locations.

Dr. Pintar completed several short-term (1-4 days) microhabitat preference trials for larvae and the few adults available. Additionally, *Stenelmis sexlineata* adults were included for comparison due to cooccurrence. These paired choice experiments examined whether beetles prefer things like different leaf species, leaf packs vs wood, and light vs shaded while also recording where the beetles are relative to other objects to assess their use of interstitial space. Dr. Pintar also began preparing a longer-duration experiment examining relative consumption rates of different leaf species by larvae.

San Marcos Salamander Mark Recapture

Several members of the SMARC staff and interns contributed to the collection, processing, and release of San Marcos salamanders in the San Marcos River and Spring Lake (Figure 3). Salamanders were collected from the San Marcos River below the eastern spillway of the Spring

Lake Dam August 8 and 24 (Table 2). Salamanders were collected from Spring Lake near the Hotel site August 10 and 22. The collection from near the Diversion pipe was canceled this month due to a lack of divers available. All salamanders were released back to the area they were captured after they fully recovered. Across all sites, six salamander recaptures occurred in August (Table 2). Additionally, one salamander was recaptured in the net over Diversion Springs during husbandry collections.

Reproductive Gene Expression in San Marcos Salamanders

Ruben Tover (University of Texas, Austin) continued RNA isolation of previously diceCT-scanned embryos of Texas blind and San Marcos salamanders.

Comal Springs Riffle Beetle Population Genetics

Dr. Bockrath collected 38 adult and 32 larval Comal Springs riffle beetles from the lures set and retrieved by BIO-WEST the week of August 7. Combined with the April collection, 163 Comal Springs riffle beetles were retained for genetic analysis (Table 3).

Tagging Aquatic Invertebrates

Brian De La Torre (Auburn University) reviewed alternative tag types to p-Chips as back-up options while Dr. Shannon Brewer (Auburn University) continued to work with students to develop a housing system that provides easy monitoring of tagged and control individuals without harming beetles.

Genetic Assessment of Peck's Cave Amphipod

Dr. Bockrath collected 38 Peck's cave amphipods as bycatch from the Comal Springs riffle beetle lures set and retrieved by BIO-WEST the week of August 7. Combined with the April collection, 86 Peck's cave amphipods were retained for genetic analysis (Table 3).

Additional Accomplishments

August 30, Dr. Bockrath and West gave an interview to KSAT about the EARP and drought conditions.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

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

On August 4, D. Moore, S. Moore, and West presented EARP research at the Southwestern Association of Naturalist conference in San Antonio, Texas (Figure 4).

Summary of August Activities

- The EARP collected four Texas blind salamanders from Johnson's Well and retained one for the SMARC
- The EARP collected 72 San Marcos fountain darters and 10 Texas wild rice plants for the UNFH
- The EARP collected 118 Comal Springs fountain darters and 98 Peck's cave amphipods for the UNFH and 12 Comal Springs salamanders for the SMARC
- The EARP collected and released seven San Marcos salamanders from Diversion Springs
- The EARP recaptured seven tagged San Marcos salamanders in the mark-recapture study

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for August 2023. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	72	1	73	105	81	56	22	204	436
Fountain darter: Comal	NT	118	0	118	--	0	64	9	228	143
Comal Springs riffle beetle	NT	NT	--	--	--	--	NA	NA	36	17
Comal Springs dryopid beetle	NT	NT	--	--	--	--	NA	NA	2	8
Peck's cave amphipod	NT	98	10	108	--	--	3	NA	101	187
Edwards Aquifer diving beetle	NT	NT	--	--	--	--	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	--	--	0	0	0	0
Texas blind salamander	1	NT	3	4	--	--	0	1	87	63
San Marcos salamander	0	NT	7	7	--	--	6	0	179	168
Comal Springs salamander	12	NT	6	18	--	--	1	0	58	90
Texas wild rice plants	NT	10	0	10	--	--	9	5	187	176

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

Date	Site	# Tagged	# Recaptured	# Untagged	Total Capture
9-May-23	eastern spillway	82	0	5	87
10-May-23	diversion area	33	0	0	33
11-May-23	hotel area	53	0	8	61
30-May-23	eastern spillway	53	0	16	69
31-May-23	hotel area	22	0	0	22
12-Jun-23	eastern spillway	75	6	20	101
14-Jun-23	hotel area	74	6	25	105
20-Jun-23	diversion area	62	2	8	72
26-Jun-23	hotel area	0	9	21	30
27-Jun-23	eastern spillway	0	4	90	94
10-Jul-23	hotel area	0	3	19	22
12-Jul-23	diversion area	0	2	78	80
13-Jul-23	eastern spillway	0	4	53	57
8-Aug-23	eastern spillway	0	2	95	97
10-Aug-23	hotel area	0	3	54	57
22-Aug-23	hotel area	0	1	101	102
24-Aug-23	eastern spillway	0	0	108	108

Table 3. Comal Springs riffle beetle (CSRB) and Peck's cave amphipod (PCA) collection information for August. Adult and larval CSRB take are reported separately, and the total CSRB and PCA take for 2023 is given.

Location	Adult CSRB encountered	Adult CSRB take	Larval CSRB take	Total August CSRB take	Total 2023 CSRB take	August PCA take	Total 2023 PCA take
Spring Run 1	0	0	0	0	1	0	1
Spring Run 2	0	0	0	0	7	0	0
Spring Run 3	31	14	10	24	47	5	35
Western Shore	42	8	14	22	51	7	11
Spring Island	23	16	8	24	57	26	39
Upper Spring Run	0	0	0	0	0	0	0
Totals	96	38	32	70	163	38	86

Figure 1. Braden West processing a Texas blind salamander that was collected and released at Johnson's Well, San Marcos, Texas.

Figure 2. New *Daphnia* culture tanks at the Uvalde National Fish Hatchery.

Figure 3. Richelle Jackson, Braden West, and Shawn Moore preparing to collect San Marcos salamanders for the mark-recapture study below the eastern spillway of Spring Lake Dam, San Marcos, Texas.

Figure 4. Ruben Tovar (University of Texas at Austin), Desiree Moore, Shawn Moore, and Braden West at the Southwestern Association of Naturalists conference in San Antonio, Texas.

September 2023 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desirée Moore, Dominique Alvear, and Braden West

With contributions from

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

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

On September 8, Braden West and Dr. Katie Bockrath collected 105 Peck's cave amphipods from Spring Island, New Braunfels, Texas. All amphipods were retained for refugia at the San Marcos Aquatic Resources Center (SMARC).

On September 20, Richelle Jackson, Desiree Moore, and West collected 206 San Marcos fountain darters from the middle San Marcos River, San Marcos, Texas (Figure 1). All darters were retained for refugia at the SMARC.

On September 21, Adam Daw, Jonathan Donahey and Nicholas Yvon collected 105 San Marcos fountain darters from below the Spring Lake Dam, San Marcos River. All darters were taken to the Uvalde National Fish Hatchery (UNFH).

Jackson and West checked the Diversion net in Spring Lake, San Marcos, Texas on Mondays and Thursdays (Figure 2). Three adult San Marcos salamanders and one juvenile San Marcos salamander were captured. All salamanders were released into Spring Lake.

Husbandry

<u>*Uvalde*</u>

Alvear, Daw, Donahey, and Meador built new storage rack systems for the refugia and quarantine buildings.

Daw and Donahey installed new sinks for the refugia.

Alvear and Meador set up seven hospital tanks and transferred 26 San Marcos salamanders from various refugia tanks all exhibiting severe spinal deformities and malnourishment.

Alvear and West went through all Comal Springs riffle beetle boxes to confirm species identification. All non-target species were discarded.

UNFH staff hosted Jackson, D. Moore, West, and Erin Lowenberg (Student Conservation

Association) on September 26 for a tour of recent improvements made at the UNFH.

<u>SMARC</u>

Jackson and S. Moore finished quarterly inventories of Peck's cave amphipod boxes at the SMARC.

West completed construction of a recirculating nursery system in the SMARC refugia space. Recirculating nursery systems allow staff to monitor and observe up to 40 clutches of captive-produced salamander eggs.

West completed two additional recirculating raceway systems in the SMARC refugia.

Jackson, S. Moore, and West mocked up a revised captive food production area in the SMARC refugia.

Animal Health

On September 11, Alvear, Daw, and Meador met with Dr. David Huffman (Texas State University) at the SMARC for a fountain darter parasite identification workshop. Thirty wild Comal Springs fountain darters were sacrificed for the workshop and examined for parasites. The fountain darters that were examined did not contain parasites but may have been too young for the purposes of parasite identification.

Task 2 Research

Dryopid Life History

Dr. Matt Pintar (BIO-WEST) continued choice experiments examining consumption rates of paired species of leaves by Comal Springs dryopid beetle larvae. Leaves from three tree species were included in the experiments.

San Marcos Salamander Mark Recapture

Several members of the SMARC staff, interns, and volunteers contributed to the collection, processing, and release of San Marcos salamanders in the San Marcos River and Spring Lake (Figure 3, 4). Salamanders were collected from Spring Lake near the Diversion pipe September 6 (Table 2). Salamanders were collected from Spring Lake near the Hotel site September 13 and 25. Salamanders were collected from the San Marcos River below the eastern spillway of the Spring Lake Dam September 14 and 27. All salamanders were released back to the area they were captured after they fully recovered. Across all sites, 10 salamander recaptures occurred in August (Table 2).

Reproductive Gene Expression in San Marcos Salamanders

Ruben Tover (University of Texas, Austin) continued RNA isolation of previously diceCT-scanned embryos of Texas blind and San Marcos salamanders. A small RNA test batch returned as a significant result, indicating Tovar can move forward with current samples.

Comal Springs Riffle Beetle Population Genetics

No updates to report.

Tagging Aquatic Invertebrates

D. Moore met with Dr. Shannon Brewer (Auburn University) to plan a trip to the SMARC in October to set up the long-term Comal Springs riffle beetle tagging study. They also discussed potential housing designs and logistics.

Genetic Assessment of Peck's Cave Amphipod

Dr. Bockrath and West collected eight Peck's cave amphipods from the Western Shore of Landa Lake, New Braunfels, Texas.

Additional Accomplishments

Dr. Bockrath conducted grants management duties for partnered research studies and discussed annual report timelines with research partners.

Dr. Bockrath also assessed the potential for 2023 research activities to be continued in 2024 based on current success, organism availability, and funding.

D. Moore began drafting 2023 research reports and 2024 research proposals.

D. Moore submitted a manuscript of the 2022 salamander tagging study to the journal Amphibian and Reptile Conservation.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Task 6 Meetings and Presentations

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

Jackson, D. Moore, and West gave a tour of the SMARC EARP buildings to 40 Texas Department of Transportation employees (Figure 5).

Dr. David Britton, Dr. Jennifer Howeth, Daw, Jackson, D. Moore, and West met with Kristy Smith and Scott Storment of the Edwards Aquifer Authority to discuss the status of current research projects and possible future research needs.

Summary of September Activities

- The EARP collected 105 Peck’s cave amphipods from Spring Island retained for the SMARC

- The EARP collected 206 San Marcos fountain darters from the middle San Marcos River retained for the SMARC
- The EARP collected 105 San Marcos fountain darters from below the San Marcos River retained for the UNFH
- The EARP checked the Diversion net in Spring Lake on Mondays and Thursdays

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for September 2023. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	206	105	0	311	0	0	59	21	145	360
Fountain darter: Comal	0	NT	--	--	0	168	36	36	192	328
Comal Springs riffle beetle	NT	NT	--	--	0	0	NA	0	36	16
Comal Springs dryopid beetle	NT	NT	--	--	0	0	NA	NA	2	8
Peck's cave amphipod	105	NT	0	105	0	0	33	NA	72	187
Edwards Aquifer diving beetle	NT	NT	--	--	--	--	0	0	0	0
Texas troglobitic water slater	NT	NT	--	--	--	--	0	0	0	0
Texas blind salamander	NT	NT	--	--	0	0	0	1	87	62
San Marcos salamander	NT	NT	4	4	0	0	0	0	179	167
Comal Springs salamander	NT	NT	--	--	0	0	1	0	57	90
Texas wild rice plants	NT	NT	--	--	0	10	0	1	187	185

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

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

Figure 1. Richelle Jackson in the San Marcos River holding a crayfish that was collected as bycatch during fountain darter collection.

Figure 2. Braden West in a boat on Spring Lake, San Marcos, Texas to check the Diversion net.

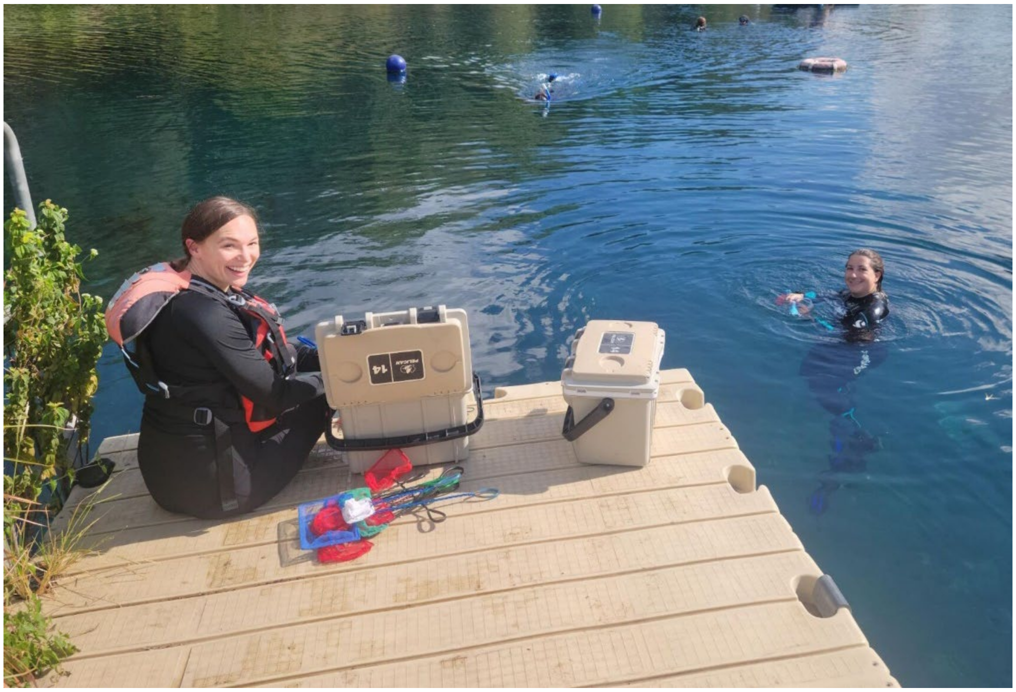


Figure 3. Sasha Doss (USFWS) and Dr. Katie Bockrath (snorkeling) collecting San Marcos salamanders for the mark-recapture study.

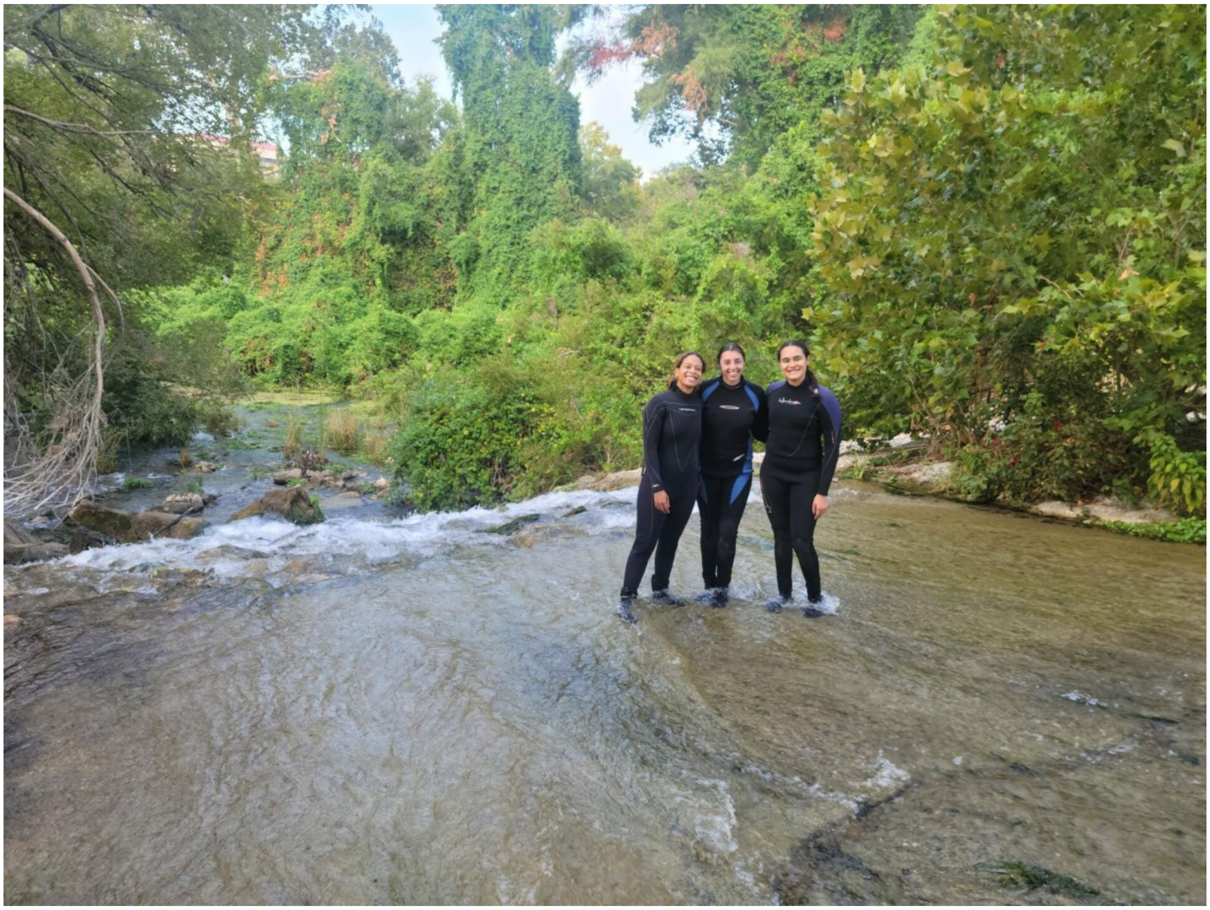


Figure 4. Richelle Jackson, Erin Lowenberg (Student Conservation Association), and Priscilla Inostroza (SMARC volunteer) at the eastern spillway of the San Marcos River, San Marcos, Texas preparing to snorkel for salamanders.

Figure 5. Braden West hosting several Texas Department of Transportation employees in the SMARC refugia room.

October 2023 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desirée Moore, Dominique Alvear, and Braden West

With contributions from

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

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

On October 5, Adam Daw, Heidi Meador, and Braden West collected three Texas wild rice plants from the San Marcos River, San Marcos, Texas for the Uvalde National Fish Hatchery (UNFH) refugia.

On October 31, BIO-WEST field staff finished their San Marcos River biomonitoring for fountain darters. A total of 133 fish were collected across two days. SMARC staff maintained 60 fish separately for Southwestern Fish Health Unit analysis. The remaining 73 fish were retained for quarantine and incorporation at the SMARC.

Edwards Aquifer Refugia Program (EARP) staff diligently maintained twice weekly sampling of the Diversion Springs net in Spring Lake, San Marcos, Texas. Staff collected 8 San Marcos salamanders from the net. All adult individuals captured were examined for p-Chips, and all individuals were released in a safe location in Spring Lake.

Husbandry

<u>*Uvalde*</u>

Dominique Alvear conducted several Peck's cave amphipod inventories and discovered one female with three juveniles in the brooding pouch. The juveniles were big enough to be removed from the mother and placed in a separate box to be monitored for growth and survival.

Alvear continued with San Marcos and Comal Springs fountain darter necropsies and tracking trends in mortalities. A necropsy workshop was held at the SMARC to train husbandry staff on basic techniques on how to assess fountain darters for various parasites and tissue abnormalities (Figure 1).

Jonathan Donahey began assembling a controller box after receiving training from Daw.

Donahey repaired chips in the fiberglass of a Texas wild rice tank (Figure 2).

Alvear and Meador designed and built a new amphipod culture system to test using various sponges as habitat and allow easier harvesting of the amphipods.

Meador conducted the semiannual inventory of all salamanders at the UNFH.
Daw demonstrated how to make drainage gutters for the new racks that will be installed in the refugia (Figure 3). Daw led a tour of the refugia program to Zoology students from Southwest Texas Junior College (Figure 4).

SMARC

Daw demonstrated the installation of various sensors into the tank system controller to SMARC husbandry staff.

Daw and West initiated improvements to increase system security in display aquaria at the SMARC. Their new design incorporated a clear PVC lid to prevent animals from escaping. West finished improvements to one display aquarium.

Richelle Jackson, Shawn Moore, and West worked to plan and build a captive food production area in the SMARC refugia space. The new captive food production area was built to include *Daphnia* production tanks, *Artemia* culture units, and a refreshed blackworm tank.

West finished construction of a recirculating rack in the SMARC refugia space.

Jackson and S. Moore conducted revolving inventories and incorporations of Peck's cave amphipod.

S. Moore trained other SMARC refugia staff in mortality archive protocol and the new Survey123 form to begin archiving preserved mortalities. S. Moore finished modifying the tissue archive survey and continued to clean the database as more mortalities were archived. This month, staff archived over 200 historical refugia mortalities and preserved tissue samples.

SMARC refugia staff began fall cleaning. Jackson organized the main refugia shelves for water quality and animal care supplies. Desiree Moore cleaned the breezeway and organized field gear. S. Moore cleaned the greenhouse and replaced a breaker switch with SMARC Facilities Operations Specialist Juan Martinez. S. Moore and West replaced a Texas wild rice system pump, and West replaced each tank's water supply hose.

Task 2 Research

Dryopid Life History

Dr. Katie Bockrath met with Dr. Matt Pintar (BIO-WEST) to discuss 2023 and future research on this project. Dr. Pintar continued paired choice experiments examining Comal Springs dryopid beetle microhabitat use (e.g., wood, leaves, rocks, flow, light) compared to *Stenelmis* sp.

San Marcos Salamander Mark Recapture

Several members of the SMARC staff, interns, and volunteers contributed to the collection, processing, and release of San Marcos salamanders in the San Marcos River and Spring Lake (Figure 5). Salamanders were collected from the San Marcos River below the eastern spillway of the Spring Lake Dam October 10 and 24 (Table 2). Salamanders were collected from Spring Lake near the Diversion pipe October 11. Salamanders were collected from Spring Lake near the Hotel site October 12 and 23. All salamanders were released back to the area they were captured after they fully recovered. Across all sites, 10 salamander recaptures occurred in October (Table 2).

Reproductive Gene Expression in San Marcos Salamanders

Dr. Bockrath met with Ruben Tovar (University of Texas, Austin) to discuss the ongoing RNA sequencing effort for the San Marcos salamander. They discussed targeting reproductive tissues of San Marcos salamanders at different reproductive and life stages.

Comal Springs Riffle Beetle Population Genetics

Dr. Bockrath met with Dr. Pintar to coordinate which Landa Lake and Spring Run locations would be used for genetic and refugia collections of Comal Springs riffle beetles.

Tagging Aquatic Invertebrates

Dr. Shannon Brewer (Auburn University) developed a prototype of a housing tube for tagged Comal Springs riffle beetles. This prototype was designed to allow passive scanning of p-Chips while also providing optimal conditions for the beetles.

Genetic Assessment of Peck's Cave Amphipod

Dr. Bockrath met with Dr. Chris Nice (Texas State University) to discuss the status of this project. Dr. Nice indicated he is ready to receive the samples collected by the EARP and begin DNA extractions. Dr. Kate Bell (Texas State University) worked with Dr. Nice to set up sequencing and analysis pipelines.

Additional Accomplishments

Dr. Bockrath and D. Moore continued drafting 2023 research reports and 2024 research proposals.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Dr. Bockrath worked to revise the 2024 workplan and budget and began the process to extend partnered research into 2024.

Task 6 Meetings and Presentations

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

Dr. Bockrath attended a meeting to discuss *Eurycea* research and collaborations among researchers to achieve common goals.

Dr. Bockrath and D. Moore presented over EARP work at the Texas Groundwater Invertebrate Forum October 20.

All EARP staff attended the annual Edwards Aquifer Habitat Conservation Plan appreciation event October 26. Thank you to the EAA for hosting.

Summary of October Activities

- The EARP collected three Texas wild rice plants from the San Marcos River retained for the UNFH
- The EARP received 133 San Marcos fountain darters from BIO-WEST, 60 of which were maintained for health analysis, and 73 of which were retained for the SMARC
- The EARP collected 8 San Marcos salamanders from the Diversion Springs net, all of which were released into Spring Lake

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for October 2023. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	133	NT	0	133	134	27	142	25	197	340
Fountain darter: Comal	NT	NT	--	--	0	79	50	20	54	381
Comal Springs riffle beetle	NT	NT	--	--	--	0	NA	NA	36	16
Comal Springs dryopid beetle	NT	NT	--	--	--	0	NA	NA	2	8
Peck's cave amphipod	NT	NT	--	--	47	65	NA	15	119	252
Edwards Aquifer diving beetle	NT	NT	--	--	--	--	--	--	0	0
Texas troglobitic water slater	NT	NT	--	--	--	--	--	--	0	0
Texas blind salamander	NT	NT	--	--	1	0	0	0	88	62
San Marcos salamander	NT	NT	8	8	0	0	3	4	174	177
Comal Springs salamander	NT	NT	--	--	6	0	0	2	63	88
Texas wild rice plants	NT	3	0	3	0	0		0	187	185

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

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

Figure 1. Dominique Alvear hosting a fountain darter necropsy workshop at the SMARC with Shawn Moore, Heidi Meador, Jonathan Donahey, and Braden West in attendance.

Figure 2. Heidi Meador cutting a drainage gutter for the new Hospital system in the Uvalde refugia.

Figure 3. Jonathan Donahey repairing chips in a Texas wild rice holding tank.

Figure 4. Adam Daw explaining the basis of the controller boxes to student tour at the UNFH.

Figure 5. Sarah Donelson (City of Austin), Braden West, Desiree Moore, Justin Crow, Matt Johnson (USFWS Ecological Services), Nate Bendik (City of Austin), Randy Gibson, and Dr. Katie Bockrath at Spring Lake after collecting San Marcos salamanders for the mark-recapture project.

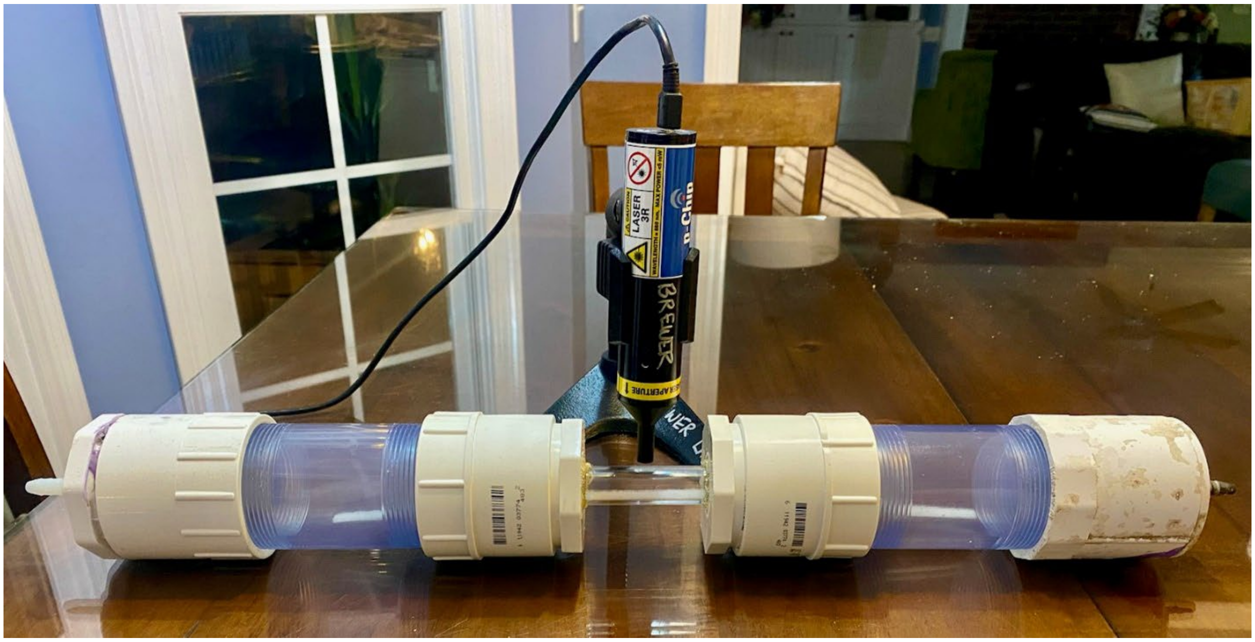


Figure 6. The prototype beetle housing with the p-Chip laser wand in a mounting stand to passively scan p-Chips.

November 2023 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desirée Moore, Dominique Alvear, and Braden West

With contributions from

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

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

Richelle Jackson, Shawn Moore, and Braden West maintained twice-weekly sampling of the Diversion net in Spring Lake, San Marcos, Texas. Two juvenile San Marcos salamanders were collected and released in accordance with EARP protocol. On November 2, a large (~50mm SVL) adult Texas blind salamander was collected from the Diversion net. Texas blind salamanders were not observed by USFWS in the Diversion net since 2019 until now. Animals of this size are likely very rare in their habitat and even more so to be caught in the drift net.

S. Moore and West worked with BIO-WEST to collect Comal River fountain darters in coordination with biomonitoring efforts on November 2 and 3. A total of 159 fountain darters were collected. All darters were retained for the refugia at the SMARC.

Jackson, S. Moore, and West set traps on November 13 for Texas blind salamander in Johnson's Well in the Purgatory Creek Natural Area, San Marcos, Texas. Traps were checked on November 15, 17, 20, 22, and 24. Traps were removed from Johnson's Well on November 27. A total of six salamanders were collected, and three were retained for the refugia at the SMARC.

Desirée Moore collected excess Comal Springs riffle beetles and Peck's cave amphipods from the Comal Springs, New Braunfels, Texas in coordination with the Comal Springs riffle beetle and Peck's cave amphipod genetics projects. Animals collected in excess of genetic targets were retained for the refugia at the SMARC. Three Peck's cave amphipods were retained from Spring Run 3, and three were retained from Spring Island. Seven Comal Springs riffle beetles were retained from Spring Run 3, seven were retained from Western Shore, and 22 were retained from Spring Island.

Husbandry

<u>*Uvalde*</u>

Heidi Meador incorporated three rice plants that were collected in October. The rice looked healthy in the refugia tanks.

Adam Daw finished plumbing the rest of the updated hospital tank rack system for the refugia with assistance from husbandry staff (Figure 1). Dominique Alvear made 20 hospital tanks for the rack and began running the system to monitor for leaks before animals were moved into the new system (Figure 2).

While continuing quarterly inventory of the Peck's cave amphipods, Alvear placed six amphipods in small holding containers and placed two *Daphnia* with each amphipod for six hours. The amphipods seemed to have eaten one *Daphnia* each. It was decided that further observations and trials are needed to help understand their dietary choices.

Jonathan Donahey finished constructing his first controller box after receiving training from Daw.

<u>*SMARC*</u>

Daw traveled to the SMARC to provide training to Jackson and S. Moore on repairing plumbing in tight spaces.

Daw and West continued work on the Texas wild rice physical filtration project. West completed installation of 120-watt UV bulbs, pipe supports, and a variable speed drive pump. Daw and West retrofitted the new filtration system into existing SMARC plumbing. West filled the tank with well water in preparation to conduct a leak test on the new system.

Jackson, Erin Lowenberg (SMARC Student Conservation Association intern), S. Moore, and West repotted 25% of the total refugia population of Texas wild rice on November 9.

Jackson and West continued making improvements to the captive food culture space in the SMARC refugia. Jackson finished cleaning and setting up the blackworm holding tank. West finalized edits to the protocol for blackworm culture.

Jackson and S. Moore used training obtained from Daw to repair leaks on the plumbing of one Texas wild rice tank. S. Moore repaired the recirculation system on QT-Rack 3 hospital system in the SMARC quarantine.

West finished construction on a new recirculating nursery system in the SMARC refugia. This new system helped alleviate deteriorating equipment and space restrictions in the SMARC refugia.

West finished construction on a third recirculating raceway system in the SMARC refugia.

West began designing a freestanding recirculating raceway design for use in areas where chiller units are not immediately available.

Daw and West designated space for the supersaturation diversion project in the SMARC refugia. West finished construction of the diversion mechanism. Daw provided training on the manipulation of the internal valve.

Animal Health

Fountain darters collected during the BIO-WEST biannual survey of the San Marcos and Comal Rivers in October and November were shipped to the Southwest Fish Health Unit (Dexter, New Mexico) for parasite/pathogen analysis. A total of 119 darters were shipped, 60 from the Comal River and 59 from the San Marcos River.

Task 2 Research

Dryopid Life History

Dr. Matt Pintar continued paired choice experiments examining Comal Springs dryopid beetle microhabitat use (e.g., wood, leaves, rocks, flow, light) compared to *Stenelmis* sp.

Dr. Pintar submitted a draft report for work on this project in 2023 and a draft proposal and budget for the work proposed for 2024.

San Marcos Salamander Mark Recapture

Several members of the SMARC staff, interns, and volunteers contributed to the collection, processing, and release of San Marcos salamanders in the San Marcos River and Spring Lake (Figure 3). Salamanders were collected from Spring Lake near the Diversion pipe November 8 (Table 2). Salamanders were collected from the San Marcos River below the eastern spillway of the Spring Lake Dam November 14. Salamanders were collected from Spring Lake near the Hotel site November 16. The second week of sampling at the eastern spillway and Hotel sites was cancelled due to staff availability. All salamanders were released back to the area they were captured after they fully recovered. Across all sites, six salamander recaptures occurred in October (Table 2).

Reproductive Gene Expression in San Marcos Salamanders

Tovar submitted a draft report for work on this project in 2023 and a draft proposal and budget for the work proposed for 2024.

Comal Springs Riffle Beetle Population Genetics

Desiree Moore joined Dr. Matt Pintar, Israel Prewitt, and Logan Leedham (BIO-WEST) to process lures and collect Comal Springs riffle beetles for genetic analysis. A total of five adults and 51 larvae were collected for this project.

Dr. Bockrath and Erin Lowenberg (Student Conservation Association intern) extracted and quantified DNA from all Comal Springs riffle beetles collected for this project.

Tagging Aquatic Invertebrates

Dr. Shannon Brewer (Auburn University) finalized the designs for the housing tubes for tagged and control Comal Springs riffle beetles. Production of several of these housing tubes began. The housing tube for tagged beetles encourages beetles to move along a narrow velcro path. This alignment facilitates automatic p-Chip scanning as the beetles pass under the laser scanner. The control housing tube allows for opening and visually inventorying the beetles.

Dr. Bockrath and Randy Gibson (SMARC biologist) set lures in springs at the Devil's River to collect *Heterelmis glabra* to serve as a surrogate species for Comal Springs riffle beetle. Using a surrogate will allow the examination of tagging effects on a species with similar morphology without the need to remove excess amounts of an endangered species from the wild.

Dr. Brewer submitted a draft report for work on this project in 2023 and a draft proposal and budget for the work proposed for 2024.

Genetic Assessment of Peck's Cave Amphipod

Desiree Moore joined Dr. Pintar, Prewitt, and Leedham to process lures and collect Peck's cave amphipods for genetic analysis. A total of 35 amphipods were collected for this project. Dr. Bockrath and Lowenberg transferred preserved Peck's cave amphipods to Dr. Chris Nice (Texas State University) for genetic analysis.

Additional Accomplishments

Desiree Moore gave an interview to John Boggess for an article and podcast in the EAHCP Steward Newsletter.

Dr. Bockrath and D. Moore met with Alvear and Daw to discuss a potential refugia research project investigating parasites in Comal Springs fountain darters.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Drafts of all 2023 EARP research reports and 2024 research proposals were submitted to the EAA.

Dr. Bockrath updated the 2024 Work Plan and Budget.

Task 6 Meetings and Presentations

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

All EARP staff met with Dr. Andy Gluesenkamp and the team at the San Antonio Zoo. The zoo team provided a tour and discussed future needs for the species both groups have in their care (Figure 4).

Summary of November Activities

- The EARP collected a Texas blind salamander from the Diversion net retained for the SMARC
- The EARP received 159 Comal River fountain darters from BIO-WEST, which were retained for the SMARC
- The EARP collected three Texas blind salamanders from Johnson's Well retained for the SMARC
- The EARP collected six Peck's cave amphipods and 36 Comal Springs riffle beetles from the Comal Springs retained for the SMARC
- Drafts of all 2023 research reports and 2024 research proposals were submitted to the EAA

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for November 2023. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	--	--	116	27	5	21	308	319
Fountain darter: Comal	159	NT	0	159	0	79	17	5	37	376
Comal Springs riffle beetle	36	NT	0	36	--	0	NA	0	36	16
Comal Springs dryopid beetle	NT	NT	--	--	--	0	NA	0	2	8
Peck's cave amphipod	6	NT	0	6	0	65	NA	30	119	222
Edwards Aquifer diving beetle	NT	NT	--	--	--	--	--	--	0	--
Texas troglobitic water slater	NT	NT	--	--	--	--	--	--	0	--
Texas blind salamander	3	NT	3	6	0	0	0	0	88	62
San Marcos salamander	NT	NT	2	2	0	0	3	9	171	168
Comal Springs salamander	NT	NT	--	--	0	0	1	4	62	84
Texas wild rice plants	NT	NT	0	--	0	3	9	0	178	188

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

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

Figure 1. Dominique Alvear, Jonathan Donahey, and Heidi Meador making airline manifold for the new hospital tank rack.

Figure 2. Dominique Alvear installing air and water lines for tanks on the new hospital tank rack.

Figure 3. David Thomasson, Erin Lowenberg, Desiree Moore, Somerley Swarm, Mark Fisher, Justin Crow, and Isaiah Trevino at Spring Lake, San Marcos, Texas for the San Marcos salamander mark-recapture study. Photo credit: Edwards Aquifer Habitat Conservation Plan.

Figure 4. Heidi Meador, Adam Daw, Dominique Alvear, Dr. Katie Bockrath, and Dr. Andy Gluesenkamp at the San Antonio Zoo during a tour.

December 2023 Monthly Activity Report:

Edwards Aquifer Refugia Program

Contract No. 16-822-HCP

Desirée Moore, Dominique Alvear, and Braden West

With contributions from

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

San Marcos Aquatic Resources Center

500 East McCarty Lane

San Marcos Texas, 78666

Phone: 512-353-0011

Task 1 Refugia Operations

Species Collection

On December 1, Adam Daw, Richelle Jackson, Heidi Meador, and Shawn Moore collected 20 Texas wild rice plants, 10 plants were taken back to each facility.

On December 21, Dominique Alvear, Daw, Meador, Jackson, and S. Moore, collected 89 adult Peck's cave amphipods and 10 juveniles from the Spring Island area of the Comal River (Figure 1). The juveniles and 39 adults were transported back to the Uvalde National Fish Hatchery (UNFH), 50 adults were taken to the San Marcos Aquatic Research Center (SMARC), and 6 adults were released.

Three San Marcos salamanders were collected from the Diversion net in Spring Lake, San Marcos, Texas. All three individuals were released.

Husbandry

<u>*Uvalde*</u>

Alvear, Jonathan Donahey, and Meador transferred Texas wild rice pots from tank 12 to tank 14 to reduce the buildup of algae and other non-targeted species (Figure 2).

Donahey cut eight more drainage gutters for new systems throughout the refugia. Donahey conducted the semi-annual inventory of all the fountain darter on site.

Donahey and Meador began replumbing tank 12 in the refugia, seeking guidance from Daw as needed.

Alvear tore down quarantine racks 5 and 8 to make room for the new sump tank and to begin replumbing systems. Alvear also kept up with semi-annual acid washing of the refugia systems to keep them running optimally. Literature review of the use of various dietary requirements for salamanders was also conducted as well as the use of disinfectants to effectively kill microsporidia and various parasites.

Daw started building a new rack in the quarantine building to hold incoming organisms.

Alvear and Daw worked on updating various SOPs.

SMARC

Braden West updated field collection SOPs for invertebrates.

West began writing SOPs for construction and maintenance of recirculating systems used in the SMARC refugia.

Jackson, S. Moore, and West collaborated with Alvear and Daw to ensure consistent updates to shared SOPs.

S. Moore trained Jackson and Erin Lowenberg (Student Conservation Association intern) on moving Texas wild rice from quarantine containers to refugia containers.

Jackson disassembled two aging rack systems in the SMARC refugia.

Jackson, S. Moore, and West conducted year-end inventories for all species held in refugia.

Jackson and S. Moore conducted inventories of captive-produced salamanders.

Task 2 Research

Dryopid Life History

Dr. Matt Pintar (BIO-WEST) collected one Comal Springs drypoid beetle over three collection events.

Dr. Pintar conducted initial laboratory experiments evaluating dryopid responses to presence of caged conspecifics and heterospecifics (*Stenelmis sexlineata*).

Dr. Pintar planned and obtained materials for reorganization of BIO-WEST's experimental space at the SMARC in preparation for new habitat preference trials and life history studies in 2024.

San Marcos Salamander Mark Recapture

Several members of the SMARC staff, interns, and volunteers contributed to the collection, processing, and release of San Marcos salamanders in the San Marcos River and Spring Lake (Figure 3). Salamanders were collected from Spring Lake near the Hotel site December 11 (Table 2). Salamanders were collected from the San Marcos River below the eastern spillway of the Spring Lake Dam December 12. Salamanders were collected from Spring Lake near the Diversion pipe December 13 (Figure 3). The second week of sampling at the Eastern Spillway and Hotel sites was cancelled due to staff availability. All salamanders were released back to the area they were captured after they fully recovered. Across all sites, five salamander recaptures occurred in December (Table 2).

Reproductive Gene Expression in San Marcos Salamanders

Desiree Moore and S. Moore helped Ruben Tover (University of Texas Austin), Brittany Dobbins (Texas State University), and Nisa Sindhi (Texas State University) to choose a wild stock gravid female Texas blind salamander and three F1 gravid female San Marcos salamanders for comparative gene expression analysis. The selected salamanders were humanely euthanized and dissected to remove reproductive tissues (Figure 4). Additionally, D. Moore and S. Moore identified males and females for dissection in the future.

Comal Springs Riffle Beetle Population Genetics

Dr. Katie Bockrath and Lowenberg completed Comal Springs riffle beetle DNA extractions. Enzyme shearing assays were tested on DNA extracted from F1 Comal Springs riffle beetles to ensure the reactions were efficient before being applied to the samples critical to this project.

Tagging Aquatic Invertebrates

Dr. Shannon Brewer and Brian De La Torre (Auburn University) continued production of control and experimental housing for the Comal Springs riffle beetle tagging trials.

Dr. Bockrath, Randy Gibson (SMARC biologist), and D. Moore planned a trip to the Devil's River to collect *Heterelmis glabra* from lures set in November to serve as a surrogate species for Comal Springs riffle beetle.

D. Moore coordinated with Dr. Brewer to schedule a trip for Dr. Brewer and De La Torre to travel to the SMARC to set up Comal Springs riffle beetle tagging trails in January (17-19).

Genetic Assessment of Peck's Cave Amphipod

Peck's cave amphipods collected in November were sorted and prepared for transfer to Dr. Chris Nice (Texas State University).

Additional Accomplishments

Dr. Bockrath awarded partnered research with 2024 funds to be available on January 1, 2024.

Task 4 Species Reintroduction

No work was completed this month for reintroduction.

Task 5 Reporting

All EARP staff contributed to the monthly report.

Alvear, Dr. Bockrath, Daw, D. Moore, and West worked on drafting the EARP 2023 annual report.

Dr. Bockrath and D. Moore resolved EAA comments and edits to the EARP 2023 research reports and 2024 research proposals.

Task 6 Meetings and Presentations

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

EARP staff attended the EAA End of Year Meeting. Daw presented salvage protocols and D. Moore presented the San Marcos salamander mark-recapture study at the meeting.

Summary of December Activities

- The EARP collected 20 Texas wild rice plants, 10 plants were retained at each facility

- The EARP collected 89 adult Peck's cave amphipods and 10 juveniles from the Spring Island area, 39 adults were retained for the UNFH, and 50 adults were retained for the SMARC
- All EARP 2023 research reports and 2024 research proposals were revised
- Partnered research project funds were awards for 2024

Tables and Figures

Table 1. New collections and total census of Edwards Aquifer organisms taken to facilities for refugia by species and facility for December 2023. "NT" indicates that species were not targeted for collection this month. "NA" indicates that inventory was not conducted this month.

Species	SMARC kept	UNFH kept	Released	Total collected	SMARC incorporated	UNFH incorporated	SMARC Mortalities	UNFH mortalities	SMARC census	UNFH census
Fountain darter: San Marcos	NT	NT	--	--	0	0	93.	19	89	300
Fountain darter: Comal	NT	NT	0	0	126	0	14	5	149	371
Comal Springs riffle beetle	NT	NT	0	0	32	0	36	NA	32	16
Comal Springs dryopid beetle	NT	NT	--	--	--	0	2	NA	0	8
Peck's cave amphipod	50	49	6	105	26	0	NA	20	145	202
Edwards Aquifer diving beetle	NT	NT	--	--	--	--	--	--	0	--
Texas troglobitic water slater	NT	NT	--	--	--	--	--	--	0	--
Texas blind salamander	NT	NT	--	0	0	0	0	0	88	62
San Marcos salamander	0	NT	3	3	0	0	8	4	163	164
Comal Springs salamander	NT	NT	--	--	0	0	4	1	58	83
Texas wild rice plants	10	10	0	20	0	0	0	0	178	188

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

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

Figure 1. Salamander egg found under a rock at Spring Island, Comal River, New Braunfels, Texas.

Figure 2. Jonathan Donahey holding two pots of Texas wild rice at the UNFH.

Figure 3. Emily Horan with a cooler of San Marcos salamanders collected by divers from the Diversion Springs area at Spring Lake.

Figure 4. Nisa Sindhi, Ruben Tovar, and Brittany Dobbins working to dissect a humanely euthanized San Marcos salamander.

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

4/6/2023

In Reply Refer To:
FWS/R2/FR-SFHU/1055
Memorandum

To: Adam Daw, San Marcos Aquatic Resource Center

From: Dave Hampton, Southwest Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 23-31)

On March 16, 2023, the Southwestern Fish Health Unit (SFHU) received 10 fountain darters (*Etheostoma fonticola*) collected from the San Marcos River in Hays County, TX. These fish were collected by staff at the San Marcos ARC on 3-14-2023 and shipped live to the SFHU laboratory for bi-annual gill parasite enumerations. The San Marcos ARC staff recorded collection of the fountain darters at latitude 29.889705 and longitude -97.934331° in Hays County, Texas. Water temperature at the collection site was recorded as 23°C.

Upon receipt of the fish, the SFHU staff examined the gills on the left side of the fish and enumerated the parasite load. Final numbers are reported on the following page.

If you have any questions about test methodology and results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 23-31.

cc: Huseyin Kucuktas, Southwestern Fish Health Unit (Acting)
David Britton,

FOD Parasite Data Sheet - Form P-03

Case History No. 23-30

Date examined: 03-16-2023

Date Collected: 3/14/2023

Collection site: Comal River, TX

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	91	181	228	250	131	152	68	141	119	60
Total Length (mm)	22	30	30	31	26	26	20	25	24	20

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

Mature (left gills only)	L	0	0	0	0	0	0	0	0	0	0
Immature (left gills only)	L	0	0, 6, 5, 2	0	0	0	0	0	0	0	0

Monogenea	L	0	0, 0, 3, 0	0, 2, 0, 0	0, 1, 0, 1	0	0, 0, 0, 1	0, 1, 0, 0	1, 0, 2, 0	0	0
Myxobolus sp.	L	0	0	0	0	0	0	0	0	0	0
Other	L	0	0	0	0	0, 0, 0, 8	0	0	0	0	0

Examiner signature Jason Woodland & [illegible]

Revised on 9/20/2017

FOD Parasite Data Sheet - Form P-03

Case History No. 23-31

Date examined: 03-16-2023 Date Collected: 3/14/2023

Collection site: San Marcos River, TX

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	504	361	320	408	371	513	412	347	160	111
Total Length (mm)	40	35	34	33	35	41	36	34	26	24

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

Mature (left gills only)	L	0	0	0	0	0	0	0	0	0	0
Immature (left gills only)	L	0, 1, 1, 0	0, 0, 0, 1	0	0	0	0	0	0	0	0

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

Examiner signature Jason Woodland & McB[illegible]

Revised on 9/20/2017

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

June 16, 2023

In Reply Refer To:
FWS/R2/FR-SFHU/1056
Memorandum

To: Braden West, San Marcos Aquatic Resource Center

From: Matthew Bagley, Southwestern Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the Comal River, Texas (Case Number 23-63).

On May 3, 2023, Southwestern Fish Health Unit (SFHU) staff received 58 fountain darters (*Etheostoma fonticola*) collected from Landa Lake (GNIS ID: 1372140). These fish were collected by drop net by staff from the San Marcos ARC and shipped live to the SFHU laboratory. The location was recorded at latitude 29.714284° and longitude -98.136028° Comal County, Texas, and river water temperature at the time of collection was 24°C.

Assays and examinations for the sampled fish included virology and parasitology. Viral screening of 58 fish included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected in the cell lines used for detecting targeted viral pathogens. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC for the Edwards Aquifer Refugia Program. Screening for *Centrocestus formosanus* was conducted by examination of all left side gills from 10 fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2020 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 2 of 10 fish examined and monogenean parasites were observed in 1 of 10 fish examined. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo. No viruses were detected by cell culture.

If you have any questions about test methodology and results, please do not hesitate to contact SFHU staff, and refer to the case history number 23-63 for all follow up correspondence.

cc:

David Britton, San Marcos Aquatic Resource Center
Adam Daw, San Marcos Aquatic Resource Center
Huseyin Kucuktas, Southwestern Fish Health Unit

FOD Parasite Data Sheet - Form P-03

Case History No. ~~23-60~~ 23-63 HK

Date examined: 5/10/23

Date Collected:

Collection site: Comal River

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	.085g	.068g	.080	.099	.065	0.126	.075	.076	.065	.037
Total Length (mm)	23 mm	21	22	24	21	35	22	21	21	19

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

		Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Mature (left gills only)	L	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —
Immature (left gills only)	L	0 —	0 —	0,0,0,1	0 —	0 —	1,0,0,0	0 —	0 —	0 —	0 —

		Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Monogenea	L	0 —	0 —	0 —	0 —	0 —	0 —	1,0,0,0	0 —	0 —	0 —
Myxobolus sp.	L	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —
Other	L										

Examiner signature [signature]

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

June 16, 2023

In Reply Refer To:
FWS/R2/FR-SFHU/1057
Memorandum

To: Braden West, San Marcos Aquatic Resource Center

From: Matthew Bagley, Southwestern Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 23-64).

On April 20, 2023, Southwestern Fish Health Unit (SFHU) staff received 59 fountain darters (*Etheostoma fonticola*) collected from San Marcos River (GNIS ID: 1375972). These fish were collected by drop net by staff from the San Marcos ARC and shipped live to the SFHU laboratory. The location was recorded at latitude 29.876415° and longitude -97.932155° Hays County, Texas, and river water temperature at the time of collection was 24°C.

Assays and examinations for the sampled fish included virology and parasitology. Viral screening of 59 fish included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected in the cell lines used for detecting targeted viral pathogens. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC for the Edwards Aquifer Refugia Program. Screening for *Centrocestus formosanus* was conducted by examination of all left side gills from 10 fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2020 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 3 of 10 fish examined and monogenean parasites were observed in 10 of 10 fish examined. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo. No viruses were detected by cell culture.

If you have any questions about test methodology and results, please do not hesitate to contact SFHU staff, and refer to the case history number 23-64 for all follow up correspondence.

cc:

David Britton, San Marcos Aquatic Resource Center
Adam Daw, San Marcos Aquatic Resource Center
Huseyin Kucuktas, Southwestern Fish Health Unit

FOD Parasite Data Sheet - Form P-03

Case History No. ~~23~~ 23-~~64~~ 64 KK

Date examined: 5/10/2023

Date Collected:

Collection site: ~~Comal River~~ San Marcos

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	.230	.249	~~.054~~ .209	.079	.153	.216	.153	.075	.058	.081
Total Length (mm)	30	31	DH ~~27~~ 30	22	28	30	27	23	22	24

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

		Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Mature (left gills only)	L	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —
Immature (left gills only)	L	0,0,0,0	1,0,0,0	0 —	0,0,0,0	1,0,0,0	1,0,0,0	0 —	0 —	0 —	0 —

		Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Monogenea	L	2,0,0,0	0,3,0,1	1,1,0,1	1,1,3,1	0,1,0,0	1,2,1,0	0,1,0,0	0,2,1,1	0,1,0,0	1,1,2,3
Myxobolus sp.	L	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —
Other	L										0 —

Examiner signature DH, MB

1.240

San Diego Zoo Wildlife Alliance

Molecular Diagnostics Report
Amphibian Disease Laboratory
Beckman Center for Conservation Research

Set ID: 231851 07/21/23

USFWS San Marcos Aquatic Resources Center

Bd, Bsal [qPCR] multiplex Verified on: 07/21/23

Sample ID	Species	Sample	Collection Date	Test	Result
23S001	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S001	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S002	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S002	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	confirmation required
23S003	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S003	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	positive
23S004	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S004	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S005	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S005	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S006	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S006	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	positive
23S007	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S007	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	confirmation required
23S008	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S008	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S009	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S009	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S010	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S010	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S011	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S011	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected

23S012	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S012	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S013	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S013	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	confirmation required
23S014	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S014	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	confirmation required
23S015	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S015	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S016	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S016	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	confirmation required
23S017	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S017	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S018	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S018	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S019	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S019	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	positive
23S020	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S020	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S021	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S021	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S022	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S022	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	confirmation required
23S023	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S023	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S024	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S024	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	confirmation required
23S025	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S025	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S026	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S026	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	confirmation required
23S027	San Marcos Salamander	skin swab	03/30/23	Bsal [qPCR]	not detected
23S027	San Marcos Salamander	skin swab	03/30/23	Bd [qPCR]	not detected
23S028	San Marcos Salamander	skin swab	06/08/23	Bsal [qPCR]	not detected
23S028	San Marcos Salamander	skin swab	06/08/23	Bd [qPCR]	not detected
23S029	San Marcos Salamander	skin swab	06/08/23	Bsal [qPCR]	not detected
23S029	San Marcos Salamander	skin swab	06/08/23	Bd [qPCR]	confirmation required

23S030	San Marcos Salamander	skin swab	06/08/23	Bsal [qPCR]	not detected
23S030	San Marcos Salamander	skin swab	06/08/23	Bd [qPCR]	positive
23S031	San Marcos Salamander	skin swab	06/08/23	Bsal [qPCR]	not detected
23S031	San Marcos Salamander	skin swab	06/08/23	Bd [qPCR]	positive
23S032	San Marcos Salamander	skin swab	06/08/23	Bsal [qPCR]	not detected
23S032	San Marcos Salamander	skin swab	06/08/23	Bd [qPCR]	positive
23S033	San Marcos Salamander	skin swab	06/08/23	Bsal [qPCR]	not detected
23S033	San Marcos Salamander	skin swab	06/08/23	Bd [qPCR]	positive
23S034	San Marcos Salamander	skin swab	06/08/23	Bsal [qPCR]	not detected
23S034	San Marcos Salamander	skin swab	06/08/23	Bd [qPCR]	positive
23S035	San Marcos Salamander	skin swab	06/08/23	Bsal [qPCR]	not detected
23S035	San Marcos Salamander	skin swab	06/08/23	Bd [qPCR]	not detected
23S036	San Marcos Salamander	skin swab	06/08/23	Bsal [qPCR]	not detected
23S036	San Marcos Salamander	skin swab	06/08/23	Bd [qPCR]	positive
23S037	San Marcos Salamander	skin swab	06/08/23	Bsal [qPCR]	not detected
23S037	San Marcos Salamander	skin swab	06/08/23	Bd [qPCR]	not detected
23S038	San Marcos Salamander	skin swab	06/08/23	Bsal [qPCR]	not detected
23S038	San Marcos Salamander	skin swab	06/08/23	Bd [qPCR]	not detected
23S039	San Marcos Salamander	skin swab	06/08/23	Bsal [qPCR]	not detected
23S039	San Marcos Salamander	skin swab	06/08/23	Bd [qPCR]	not detected
23S040	San Marcos Salamander	skin swab	06/08/23	Bsal [qPCR]	not detected
23S040	San Marcos Salamander	skin swab	06/08/23	Bd [qPCR]	confirmation required
23S041	San Marcos Salamander	skin swab	07/07/23	Bsal [qPCR]	not detected
23S041	San Marcos Salamander	skin swab	07/07/23	Bd [qPCR]	not detected
23S042	San Marcos Salamander	skin swab	07/07/23	Bsal [qPCR]	not detected
23S042	San Marcos Salamander	skin swab	07/07/23	Bd [qPCR]	positive
23S043	San Marcos Salamander	skin swab	07/07/23	Bsal [qPCR]	not detected
23S043	San Marcos Salamander	skin swab	07/07/23	Bd [qPCR]	confirmation required
23S044	San Marcos Salamander	skin swab	07/07/23	Bsal [qPCR]	not detected
23S044	San Marcos Salamander	skin swab	07/07/23	Bd [qPCR]	positive

Confirmatory: Bd [qPCR] Verified on: 07/21/23

Sample ID	Species	Sample	Collection Date	Test	Result
23S002	San Marcos Salamander	skin swab	03/30/23	Confirmatory - Bd	indeterminate

Confirmatory: Bd [qPCR] Verified on: 07/21/23

Sample ID	Species	Sample	Collection Date	Test	Result
23S007	San Marcos Salamander	skin swab	03/30/23	Confirmatory - Bd	indeterminate

Confirmatory: Bd [qPCR] Verified on: 07/21/23

Sample ID	Species	Sample	Collection Date	Test	Result
23S013	San Marcos Salamander	skin swab	03/30/23	Confirmatory - Bd	not detected

Confirmatory: Bd [qPCR] Verified on: 07/21/23

Sample ID	Species	Sample	Collection Date	Test	Result
23S014	San Marcos Salamander	skin swab	03/30/23	Confirmatory - Bd	indeterminate

Confirmatory: Bd [qPCR] Verified on: 07/21/23

Sample ID	Species	Sample	Collection Date	Test	Result
23S016	San Marcos Salamander	skin swab	03/30/23	Confirmatory - Bd	indeterminate

Confirmatory: Bd [qPCR] Verified on: 07/21/23

Sample ID	Species	Sample	Collection Date	Test	Result
23S022	San Marcos Salamander	skin swab	03/30/23	Confirmatory - Bd	indeterminate

Confirmatory: Bd [qPCR] Verified on: 07/21/23

Sample ID	Species	Sample	Collection Date	Test	Result
23S024	San Marcos Salamander	skin swab	03/30/23	Confirmatory - Bd	indeterminate

Confirmatory: Bd [qPCR] Verified on: 07/21/23

Sample ID	Species	Sample	Collection Date	Test	Result
23S026	San Marcos Salamander	skin swab	03/30/23	Confirmatory - Bd	indeterminate

Confirmatory: Bd [qPCR] Verified on: 07/21/23

Sample ID	Species	Sample	Collection Date	Test	Result
23S029	San Marcos Salamander	skin swab	06/08/23	Confirmatory - Bd	positive

Confirmatory: Bd [qPCR] Verified on: 07/21/23

Sample ID	Species	Sample	Collection Date	Test	Result
23S040	San Marcos Salamander	skin swab	06/08/23	Confirmatory - Bd	not detected

Confirmatory: Bd [qPCR] Verified on: 07/21/23

Sample ID	Species	Sample	Collection Date	Test	Result
23S043	San Marcos Salamander	skin swab	07/07/23	Confirmatory - Bd	not detected

A "confirmation required" result means a follow up up test with more technical replicates is in process. A final report will follow.
Lab contact: phone 760-291-5470 or x5471 or x5472, email AmphibianLab@sdzwa.org

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

In Reply Refer To:
FWS/R2/FR-SFHU/1063 August 29, 2023

Memorandum
To: Braden West, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the Comal River, Texas (Case Number 23-84).

On August 15, 2023, Southwestern Fish Health Unit (SFHU) staff received 20 fountain darters (*Etheostoma fonticola*) collected from the Comal River (GNIS ID: 1372140), Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for health testing as part of an ongoing parasite enumeration study. San Marcos ARC staff recorded collection of fountain darters at the Landa Lake site at latitude 29.7142° and longitude -98.1358° (corrected longitude) in Comal County. The river water temperature was 24°C. Originally collected from the river on May 5, 2023, these fish have since been maintained in a 250-gallon flow-through tank, tank# QT-5, as part of the Edwards Aquifer Refugia Program. Following incorporation into refugia these fish were treated with 170µL/L formalin on 7/25/2023.

Aside from four fish that were received dead on arrival, all live fish were euthanized in buffered MS-222. Gill necropsies and microscopic observations for parasites were completed by SFHU staff on 10 of the fish submitted. Screening for *Centrocestus formosanus* was conducted by examination of the left side set of gills for each fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2020 edition) and standard SFHU protocols.

Results:
Immature *Centrocestus formosanus* were observed in 9 of 10 fish examined. Monogenetic trematodes were also observed on the gills from 9 of 10 fish examined. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is also included in a separate file with my email of this report.

If you have any questions about test methodology or results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 23-84 for all follow-up correspondence.

cc: Huseyin Kucuktas, Southwestern Fish Health Unit/ Southwestern Native ARRC
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 23-84

Date examined: 8/15/23   Date Collected: 5-3-23

Collection site: Comal River, TX (Landa Lake)

		Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)		259	159	148	88	133	161	140	150	103	54
Total Length (mm)		30	28	30	25	29	29	26	28	24	23

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

Mature (left gills only)	L	0	0	0	0	0	0	0	0	0	0
Immature (left gills only)	L	1, 0, 1, 0	0, 1, 0, 0	2, 0, 0, 0	0, 1, 0, 0	0, 0, 5, 1	0, 1, 0, 0,	0	0, 0, 0, 2	0, 1, 0, 0	0, 2, 1, 2

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

Examiner signature DAVID HAMPTON

Digitally signed by DAVID HAMPTON
Date: 2023.08.24 16:11:05 -06'00'

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

In Reply Refer To:
FWS/R2/FR-SFHU/1064 August 29, 2023

Memorandum
To: Braden West, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 23-85).

On August 15, 2023, Southwestern Fish Health Unit (SFHU) staff received 20 fountain darters (*Etheostoma fonticola*) collected from the San Marcos River (GNIS ID: 1375972), Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for health testing as part of an ongoing parasite enumeration study. San Marcos ARC staff recorded collection of fountain darters at approximately 100m below a dam spillway for Spring Lake at latitude 29.8897° and longitude -97.9342° in Hays County. The river water temperature was 24°C. Originally collected from the river on July 6, 2023, these fish have since been maintained in a 250-gallon flow-through tank, tank QT-3, as part the Edwards Aquifer Refugia Program. No fish treatments were indicated prior to submission.

Aside from three fish that were received dead on arrival, all live fish were euthanized in buffered MS-222. Gill necropsies and microscopic observations for parasites were completed by SFHU staff on 10 of the fish submitted. Screening for *Centrocestus formosanus* was conducted by examination of the left side set of gills for each fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2020 edition) and standard SFHU protocols.

Results:
One immature *C. formosanus* was observed in 1 of 10 fish examined. Moderate to high numbers of monogenetic trematodes were also observed on gills from all 10 fish examined. Large numbers of myxozoan parasites, possibly a *Myxobolus* sp., were found on one fish. Furthermore, large numbers of *Ichthyobodo* sp. parasites were also observed on another fish. The parasite data sheet that contains the specific number, unless indicated as “Too Numerous To Count” (TNTC), and type of parasites isolated from each fish is also included in a separate file with my email of this report.

If you have any questions about test methodology or results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 23-85 for all follow-up correspondence.

cc: Huseyin Kucuktas, Southwestern Fish Health Unit/ Southwestern Native ARRC
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 23-85

Date examined: 8/15/23 Date Collected: 7-6-23

Collection site: San Marcos River, TX

		Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)		243	124	137	301	270	158	155	137	177	321
Total Length (mm)		32	29	29	38	33	32	27	28	30	36

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

Mature (left gills only)	L	0	0	0	0	0	0	0	0	0	0
Immature (left gills only)	L	0	0	0	1, 0, 0, 0	0	0	0	0	0	0

Monogenea	L	0, 4, 1, 2,	6, 4, 4, 1	7, 13, 17, 8	7, 18, 23, 8	0, 1, 10, 3	2, 6, 3, 3	5, 7, 5, 8	6, 7, 5, 3	1, 2, 1, 0	2, 2, 3, 3
Myxobolus sp.	L	0	0	0	0	0	0	0	0,*TNTC,*TNTC,0	0	0
Other	L	0	0	0	0	0	0	Ichthyobodo everywhere/*TNTC	0	0	0

Examiner signature DAVID HAMPTON Digitally signed by DAVID HAMPTON Date: 2023.08.24 16:08:11 -06'00'

Revised on 9/20/2017

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

In Reply Refer To:
FWS/R2/FR-SFHU/1068 December 11, 2023

Memorandum

To: Adam Daw, San Marcos Aquatic Resource Center

From: Matthew Bagley, Southwestern Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the Comal River, Texas (Case Number 24-02).

On November 01, 2023, Southwestern Fish Health Unit (SFHU) staff received 36 fountain darters (*Etheostoma fonticola*) collected from the Comal River (GNIS ID: 1372140). These fish were collected using drop net by staff from the San Marcos ARC and shipped live to the SFHU laboratory. The location was recorded at latitude 29.7112° and longitude -98.1276° Comal County, Texas, and river water temperature at the time of collection was 24°C.

Assays and examinations for the sampled fish included virology and parasitology. Viral screening of 36 fish included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected in the standard cell lines used. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC for the Edwards Aquifer Refugia Program. Screening for *Centrocestus formosanus* was conducted by examination of all left side gills from 10 fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2020 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 6 of 10 fish examined and monogenean parasites were observed in 3 of 10 fish examined. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo. No viruses were detected by cell culture.

If you have any questions about test methodology and results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 24-02 for all follow up correspondence.

cc: Huseyin Kucuktas, Southwestern Fish Health Unit
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 24-02

Date examined: 11/02/23

Date Collected: 11/1/23

Collection site: Comal River, TX

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	179	218	93	239	222	248	197	175	125	64
Total Length (mm)	28	30	22.5	31	30	31	29	28	25	20

Centrocestus formosanus **cysts**

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

Mature (left gills only)	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Immature (left gills only)	L	0,0,0,0	0,1,0,0	0,0,0,0	0,0,0,0	0,0,2,0	0,0,0,1	0,1,0,0	1,0,0,0	0,2,1,0	0,0,0,0

Monogenea	L	0,0,0,0	0,0,0,0	0,0,0,0	1,0,1,0	0,0,1,0	0,0,0,0	0,0,0,0	1,0,0,0	0,0,0,0	0,0,0,0
Myxobolus sp.	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Other	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0

Examiner signature DAVID HAMPTON Digitally signed by DAVID HAMPTON Date: 2023.12.14 10:56:26 -07'00'

Revised on 9/20/2017

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

In Reply Refer To:
FWS/R2/FR-SFHU/1049 November 11, 2023

Memorandum
To: Adam Daw, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 23-02).

On October 26, 2022, Southwestern Fish Health Unit (SFHU) staff received 49 fountain darters (*Etheostoma fonticola*) collected from the San Marcos River (GNIS ID: 1375972), Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for fish health testing. San Marcos ARC staff recorded collection of fountain darters at latitude 29.8764° and longitude -97.9320° in Hayes County, Texas, and the river water temperature was 23°C.

Assays and examinations for the sampled fish included virology and parasitology. Viral screening of 49 fish included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected using standard cell lines. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC. External examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of the left side set of gills for 10 fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2020 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 4 of 10 fish examined. In addition to *C. formosanus*, one fish was observed with myxosporidean parasites associated with the gills. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo. No viruses were detected by cell culture.

If you have any questions about test methodology or results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 23-02 for all follow up correspondence.

cc: Huseyin Kucuktas, Southwestern Fish Health Unit/ Southwestern Native ARRC
David Britton, San Marcos Aquatic Resources Center

FOD Parasite Data Sheet - Form P-03

Case History No. 23-02

Date examined: 10-26-2022

Date Collected: 10-19-22

Collection site: San Marcos River

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	268	305	371	317	306	194	259	221	252	157
Total Length (mm)	23	26	27	26	25	23	25	22	25	20

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

		Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Mature (left gills only)	L	2, 1, 1, 0	0	0	0	0	0	0	0	0	0
Immature (left gills only)	L	0	0	2, 8, 6, 7	2, 3, 4, 2	0	0	0	0	0	0, 1, 6, 1

		Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Monogenea	L	0	0	0	0	0	0	0	0	0	0
Myxobolus sp.	L	0, 2, 16, 3	0	0	0	0	0	0	0	0	0
Other	L	0	0	0	0	0	0	0	0	0	0

Examiner signature [signatures]

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

In Reply Refer To:
FWS/R2/FR-SFHU/1069 December 11, 2023

Memorandum
To: Adam Daw, San Marcos Aquatic Resource Center

From: Matthew Bagley, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 24-03).

On November 02, 2023, Southwestern Fish Health Unit (SFHU) staff received 59 fountain darters (*Etheostoma fonticola*) collected from the San Marcos River (GNIS ID: 1375972), Texas. These fish were collected by dip net by staff at the San Marcos ARC and shipped live to the SFHU laboratory for fish health testing. San Marcos ARC staff recorded collection of fountain darters at latitude 29.8767° and longitude -97.9320° in Hayes County, Texas, and the river water temperature was 24°C.

Assays and examinations for the sampled fish included virology and parasitology. One fish of 59 fish received was dead on arrival and was therefore not included for testing. Viral screening of 58 fish included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected using standard cell lines. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC. External examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of the left side set of gills for 10 fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2020 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 1 of 10 fish examined. In addition to *C. formosanus*, seven fish was observed with monogenean parasites associated with the gills. *Ichthyobodo sp.* was also seen in two of the fish that were too numerous to count. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo. Largemouth bass virus (LMBV) was detected by cell culture and confirmed by PCR. This finding is important as LMBV has not been detected in fountain darters collected from the San Marcos watershed, however it has been detected in other species of fish from other watersheds in Texas (Southard, GM et al. "Largemouth bass virus in Texas: distribution and management issues." Journal of Aquatic Animal Health 21.1 (2009): 36-42.) San Marcos ARC staff indicated that husbandry issues where fountain darters are held may exist and are being monitored closely. Additionally, staff indicated that logistic issues during the shipment of samples are also being reviewed. A second set of fountain darters

collected from San Marcos River kept in quarantine at San Marcos ARC will be tested for LMBV in late December.

If you have any questions about test methodology or results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 24-03 for all follow up correspondence.

cc: Huseyin Kucuktas, Southwestern Fish Health Unit/ Southwestern Native ARRC
David Britton, San Marcos Aquatic Resources Center

FOD Parasite Data Sheet - Form P-03

Case History No. 24-03

Date examined: 11/02/23

Date Collected: 10/31/23

Collection site: Lower San Marcos River

		Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)		451	320	168	202	299	271	79	147	291	101
Total Length (mm)		37	34	29	32	36	32	25	27	32	27

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

Mature (left gills only)	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Immature (left gills only)	L	0,0,0,0	0,2,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0

Monogenea	L	1,0,1,0	0,0,0,0	2,3,1,1	1,1,4,2	7,7,4,1	1,0,0,0	1,9,1,7	0,0,0,0	0,0,0,0	0,5,8,3
Myxobolus sp.	L	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Other	L	0,0,0,0	0,0,0,0	0,0,*TNTC,60	0,0,0,0	0,0,0,0	0,0,0,0	*TNTC	0,0,0,0	0,0,0,0	0,0,0,0

Examiner signature MATTHEW BAGLEY Digitally signed by MATTHEW BAGLEY Date: 2023.12.14 10:52:50 -07'00'

*TNTC= Too Numerous To Count; Ichthyobodo

Revised on 9/20/2017

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

In Reply Refer To:
FWS/R2/FR-SFHU/1050 November 30, 2022

Memorandum

To: Adam Daw, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the Comal River, Texas (Case Number 23-03).

On October 26, 2022, Southwestern Fish Health Unit (SFHU) staff received 60 fountain darters (*Etheostoma fonticola*) collected from the Comal River (GNIS ID: 1372140). These fish were collected using dip net by staff from the San Marcos ARC and shipped live to the SFHU laboratory. The location was recorded at latitude 29.7142° and longitude -98.1358° Comal County, Texas, and river water temperature at the time of collection was 23°C.

Assays and examinations for the sampled fish included virology and parasitology. One fish of the 60 fish received was dead on arrival and was therefore not included for testing. Viral screening of 59 fish included those listed as USFWS national targeted pathogens as well as any other viruses that may be detected in the standard cell lines used. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC for the Edwards Aquifer Refugia Program. Screening for *Centrocestus formosanus* was conducted by examination of all left side gills from 10 fish. Testing was performed per the American Fisheries Society-Fish Health Section Bluebook (2020 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 9 of 10 fish examined and monogenean parasites were observed in 4 of 10 fish examined. The parasite data sheet that contains the specific number and type of parasites isolated from each fish is attached to the end of this memo. No viruses were detected by cell culture.

If you have any questions about test methodology and results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 23-03 for all follow up correspondence.

cc: Huseyin Kucuktas, Southwestern Fish Health Unit
David Britton, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 23-03

Date examined: 10-26-2022

Date Collected: 10-25-22

Collection site: Comal River

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	275	380	295	186	186	202	254	190	161	218
Total Length (mm)	32	35	33	30	29	30	32	28	27	30

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

Mature (left gills only)	L	1, 1, 0, 2	0	0, 1, 4, 0	0, 1, 2, 0	0	2, 0, 0, 0	6, 2, 2, 1	0	0, 0, 1, 0	0
Immature (left gills only)	L	0	0, 3, 2, 3	0, 0, 0, 1	0	0	0	0	1, 2, 0, 1	1, 2, 0, 1	0, 2, 0, 1

Monogenea	L	0, 3, 1, 1	0	0	0	0	0	1, 0, 1, 0	1, 0, 0, 0	0, 1, 0, 0	0
Myxobolus sp.	L	0	0	0	0	0	0	0	0	0	0
Other	L	0	0	0	0	0	0	0	0	0	0

Examiner signature

Revised on 9/20/2017

David Britton
Center Director
San Marcos Aquatic Resource Center
U.S. Fish & Wildlife Service
500 East McCarty Lane
San Marcos, Texas 78666

Chad Furl
Chief Science Office
Edwards Aquifer Authority HCP
San Antonio, Texas 78215

February 3, 2023

Dr. Furl:

On Sunday, January 22, the San Marcos Aquatic Resources Center experienced a power failure that impacted both wells simultaneously and led to the loss of many animals held in refugia. This occurred when the station was closed and after our weekend walk-through staff had already completed their checks for the day. Staff were not on duty when this event occurred.

We have two wells that provide water to the station: one serving as the primary water source and the second as a reserve in case the other fails. Both wells have generators designed to supply power in case of a power outage. In this case, however, the power failure was a brown out caused by a bird coming in contact with conductors. This unusual situation was sufficient to stop the well pumps and damage the communication system between the wells, but not trigger the generators. Unprecedented, the entire system, including the built-in redundancy, failed, leaving the station without a source of water. Restoration took more than two hours.

Water pressure is monitored by Security One Inc., who notified us of the drop in pressure. Our facilities operations specialist came to the station and manually started a generator to restore function to one of the well pumps. The system remained powered by our generator until the utility company eventually repaired the electrical system. We later discovered that the restored water pressure led to a supersaturation of gasses in the well line. We remotely monitor for supersaturation, but the event was sudden and unexpected. By the time we could respond, we found animals already succumbing to gas bubble disease.

In total, we lost many wild-stock animals in refugia, including 180 Comal Springs Fountain Darters, 56 San Marcos Salamanders, 52 Comal Springs Salamanders, 82 Texas Blind Salamanders, and 2 Peck's Cave Amphipods.

We also lost many of our captive-bred stock (held for research), including 46 Texas Blind Salamanders, 12 San Marcos Salamanders, 3 Comal Springs Salamanders, 1 San Marcos Fountain Dater, and 12 Comal Springs Fountain Darters.

In order to bring our standing stock and refugia numbers back to target values, we will need to devote extra effort in collections in 2023. Our staff has reached out to the Meadows Center at Spring Lake to coordinate salamander collections using the Diversion Springs net in the lake and two hand-collection events in Spring Lake below the dam. We will increase our Comal and San Macros Fountain Darter collections outside of the BIO-WEST monitoring surveys in order to increase refugia numbers. Exact dates are unknown at this point, but we are expecting to conduct Fountain Darter collections at least twice a month until refugia target numbers are reached.

We have made operational changes to prevent future impacts with future events. We conducted a debriefing meeting with EARP staff and created a new communications plan. Program leads and our refugia manager will be added to our Security One call list to ensure relevant staff are aware of what is happening and can allow for faster response and improved communication.

We are currently having our generators and their communications systems analyzed, upgraded and repaired. We have created new Standard Operating Procedures for electrical outages, taking into consideration this event. We will test all the control/alarm/automation systems, plumbing (blead/bypass valves), and generators on set interval to make sure they are operating properly. Our refugia staff will modify input flow in the salamander tanks from single source to a spray bar set up to improve the removal of supersaturated gas.

This event was unprecedented and unfortunate. Our staff strive to provide the best possible care for all animals on station. We are committed to maintaining and improving facilities and operations associated with the agreement with the Edwards Aquifer Authority. We are confident that we can recover from this loss and are sure that we will be better prepared for unexpected events in the future.

Respectfully,

David Britton