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

ANNUAL REPORT 2022

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EXECUTIVE SUMMARY

BACKGROUND

On January 1, 2017, a contract (Contract # 16-822-HCP) between the Edwards Aquifer Authority (EAA) and the U.S. Fish and Wildlife Service (USFWS) was initiated for the operation and maintenance of a series of refugia for ten species endemic to the Edwards Aquifer. These refugia were required 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 sixth annual report of the contract covering the calendar year of 2022. The sixth year of the contract focused on maintaining the existing standing stocks and conducting research while facing challenges of an ongoing global pandemic of Covid-19, significant staff changes, and drought.

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 2022. 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 2022; all other covered species were collected in 2022.

Figure 1. Adam Daw, Braden West, and Jennifer Whitt collecting Peck's cave amphipods at Spring Island, New Braunfels, Texas.

Table 1. Counts of individuals collected in 2022 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 collected 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	0/219								
FEB		0/11				10/0			11/0
MAR		0/2	0/163						
APR					125/0				4/5
MAY			12/0	77/0		8/0			0/3
JUN	52/0	4/0	16/0						
JUL					73/0				
AUG			95/0	475/378	87/152	2/0	2/0	0/124	10/20
SEP				0/119				127/0	
OCT			110/0	165/0	52/0				
NOV			0/37	0/78		4/0			
DEC									

RESEARCH

We conducted eight research projects in 2022, several with external partners. These research projects focused on species covered by the Edwards Aquifer Habitat Conservation Plan, including Comal Springs riffle beetles (CSRB), three salamander species (Texas blind salamanders, San Marcos salamanders, and Comal Springs salamanders), and fountain darters.

All research was conducted to improve successful completion of their life cycles, promote reliable reproduction, and establish methods for future genetic assessments.

USFWS staff wrote two captive propagation handbooks that describe all previous refugia and partnered research on captive propagation of San Marcos salamanders and CSRB. The San Marcos salamander captive propagation handbook is in Appendix B. The CSRB captive propagation report is in Appendix C.

Texas State University (TXST) and USFWS staff completed the study examining the effects of bacteria exposure on larval survival and pupation of the CSRB. Results showed the microbiomes of larvae from the Uvalde National Fish Hatchery (UNFH) were more similar to wild larvae than those from the San Marcos Aquatic Resources Center (SMARC), and microbiome composition of larvae was different than the adult CSRB microbiome. The microbiome of CSRB larvae was impacted by monocultural bacterial exposure. A report for this research is included in Appendix D.

BIO-WEST and SMARC staff continued research on increasing pupation success of CSRB in captivity. Building on 2021 research, which determined flow-through tubes were preferred over an alternative housing design (box with larger air-water interface). Wild-caught adult CSRB were collected and held in refugia to produce F1 larvae for 2022 research focused on the effects of larval density and wild biofilm on pupation rates. Density did not have a significant effect on pupation success and at least 40 larvae can be held in a single tube. Biofilm did not significantly impact pupation success. The EARP will continue to offer CSRB captive inoculated feeding materials. A report for this research is included in Appendix E.

SMARC staff created a fountain darter tissue archive using preserved individuals stored in various conditions on station. This project also assessed the viability of preserved tissues for future genetic analyses. Results show variable total body lengths by collection location with captive bred individuals being the largest group. Preserved samples were approximately 50/50 ratio of males to females. Samples stored in non-climate-controlled conditions resulted in very poor DNA quality, regardless of preservation method. These samples are not suitable for future research. This project resulted in protocols for preserving and storing future samples and extracting DNA from samples preserved with either formalin or ethanol. The DNA extraction protocols will be critical for gathering genetic data from museum samples, if necessary. A report of this research is included in Appendix F.

There were two research projects involving salamanders. The first project addressed the problem of tag retention in aquatic salamanders, which is known to decline over time due to movement, loss, and tag degradation. P-Chips were examined as an alternative tagging option for Texas blind and Comal Springs salamanders. This study determined p-Chips provided high survival, tag retention, and tag readability for both species. The second project addressed the problem of Chytrid (Bd) fungal infections pose as health and biosecurity risk for amphibians on station and in areas where reintroductions may occur, if necessary. Treating aquatic salamanders post wild collection may mitigate this threat. The efficacy of itraconazole in treating Bd in San Marcos salamanders was tested at two doses. The treatments had no effect on the Bd status, and higher doses of itraconazole might be needed to treat Bd in aquatic salamanders. A report for the p-Chip tagging study is included in Appendix G and a report for the Bd treatment study is included in Appendix H.

A two-year genetic assessment of the CSRB in Landa Lake began in 2022. Spring openings where lures will be set were identified, but lure deployment was delaying until 2023 due to drought conditions. CSRB mortalities from other efforts were preserved for DNA extraction. An interim report for this research is included in Appendix I.

BUDGET

The Aquifer Refugia Program did not exceed the allocated budget defined in the 2022 Refugia Work Plan previously approved by the EAA Board of Directors. The Refugia Program spent approximately $1,192,620 in 2022. Research activities accounted for $459,666, and approximately $679,392 was spent on collections, husbandry, and propagation. Approximately $53,562 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*	Petitioned

* 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.

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 facilities: the SMARC and UNFH (Table 3). Although both facilities are administratively under the direction of a single Center Director, Dr. David Britton. Dr. Jennifer Howeth was hired as the new Deputy Center Director in 2022. Dr. Britton is responsible for the Edwards Aquifer Refugia Program in San Marcos and is assisted by 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 2022. 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 2022. The Edwards Aquifer Refugia Program underwent staff changes in 2022. Jennifer Whitt and Tommy Funk left the program to join other FWS and Park Service offices. Braden West and Desiree Moore transitioned from term positions to permanent positions. The program welcomed two new employees, Shawn Moore at SMARC and Dominique Alvear at UNFH.

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 Fish Biologist
Braden West	Refugia Biologist
Shawn Moore	Refugia Biotechnician
Vacant	Refugia Biotechnician
Uvalde National Fish Hatchery	
Scott Walker	Uvalde National Fish Hatchery Project Leader
Adam Daw	Refugia Husbandry and Collections Team Lead
Dominique Alvear	Refugia Biologist
Benjamin Thomas	Refugia Biotechnician
Vacant	Refugia Biotechnician

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

Desiree Moore, Research Biologist at the SMARC, worked with Dr. Bockrath to design and implement research projects across stations. Moore prepared 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.

Jennifer Whitt, Ben Thomas, Tommy Funk, and Braden West, 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.

Figure 2. EARP Staff and Interns. Top row, right to left: Desiree Moore, Adam Daw, Braden West, Malory Theurer, Tommy Funk, Jennifer Whitt, and Ben Thomas. Bottom row, right to left: Eleanor Krellenstein, Dr. Katie Bockrath, Shawn Moore.

BUILDING CONSTRUCTION

Minor modifications to the EARP building occurred in 2022. A wall was constructed to separate the office space into an isolated room. The office space was then transformed into the Genetics Lab. Minor system modifications occurred on an as needed basis to accommodate research and refugia housing needs. The quarantine systems at the SMARC began to be modified to increase usable space and allow individual isolation in incoming wild salamanders. Three tank racks used to house refugia invertebrates were built, one at the SMARC and two at the UNFH. We started the transition of water quality monitoring systems, from Hydrolab sondes to Walchem controllers/monitors during 2022. One of the new Walchem, Iwaki America Inc. controllers/monitors was installed at the UNFH and SMARC. The new systems are more cost effective per unit and add additional features, including the ability to control equipment in addition to monitoring water quality parameters.

Figure 3. Left: Modifications to the EARP building begin. Juan Martinez (SMARC) instructing Braden West and Tommy Funk on how to properly create a doorway in a wall. Right: Braden West reinforcing the doorway after modifying a hallway.

COVERED SPECIES ANALYSIS

Collections of the Covered Species continued this year to achieve standing stock targets as outlined in the Contract and the 2022 EA Refugia Work Plan (Table 3 and Table 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 Spring system.

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 4. Texas blind salamander

Table 3. Number of organisms incorporated in the SMARC Refugia Standing Stock in 2022, 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*		246	402	71%
Fountain darter – Comal Springs *Etheostoma fonticola*		300	313	73%
Comal Springs riffle beetle *Heterelmis comalensis*		48	36	51%
Comal Springs dryopid beetle *Stygoparnus comalensis*		2	2	100%
Peck's cave amphipod *Stygobromus pecki*		120	139	58%
Edwards Aquifer diving beetle *Haideoporus texanus*		0	0	--
Texas troglobitic water slater *Lirceolus smithii*		0	0	--
Texas blind salamander *Eurycea rathbuni*		10	172	92%
San Marcos salamander *Eurycea nana*		0	96	60%
Comal Springs salamander *Eurycea* sp.		0	110	96%
Texas wild rice *Zizania texana*		33	205	92%

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 = (1 -(refugia mortality / (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 2022, 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*		149	454	81%
Fountain darter – Comal Springs *Etheostoma fonticola*		166	177	95%
Comal Springs riffle beetle *Heterelmis comalensis*		58	48	53%
Comal Springs dryopid beetle *Stygoparnus comalensis*		12	10	83%
Peck's cave amphipod *Stygobromus pecki*		197	232	66%
Edwards Aquifer diving beetle *Haideoporus texanus*		0	0	--
Texas troglobitic water slater *Lirceolus smithii*		0	0	--
Texas blind salamander *Eurycea rathbuni*		0	65	94%
San Marcos salamander *Eurycea nana*		0	167	84%
Comal Springs salamander *Eurycea* sp.		35	93	93%
Texas wild rice *Zizania texana*		53	205	96%

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 = (1 -(refugia mortality / (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	4	5
Texas wild rice	5	5	5	5	5
Texas blind salamander	4	5	4	4	1
San Marcos salamander	5	4	4	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	1
Peck's cave amphipod	4	4	4	3	2
Edwards Aquifer diving beetle	1	0	0	0	1

FOUNTAIN DARTER (*ETHEOSTOMA FONTICOLA*), ENDANGERED

Our Standing Stock goal for fountain darters is 1,000 fish per river (San Marcos and Comal) divided between the two facilities. Standing stock goals for San Marcos fountain darters were slightly below target numbers in 2022. High mortality rates for both incoming Comal Springs fountain darters and those in refugia inhibited reaching target goals for Comal Springs fountain darters. In 2019, the managing biologist, in concert with Refugia biologists and supervisors at the SMARC, made the decision to cease collection of fountain darters from the Comal River until further studies were completed to investigate potential causes of these increased mortalities. We received approval from the EAA to suspend target goals for the Comal Springs fountain darters in the interim. In the summer, due to a drought, the Comal River spring flow conditions reach critical 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 6.

Table 6. Fountain darter refugia population figures

		Beginning of Year Census	Incorporated 2022[1]	End of Year Census	Target Goal 2022 Work Plan	Percent Survival [2]
San Marcos River	SMARC	415	246	402	500	71%
	UNFH	483	149	454	500	81%
Comal River	SMARC	125	300	313	*	73%
	UNFH	35	166	177	*	95%

* 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 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. Fish removed from the refugia as part each of the facilities yearly animal health inspection are not included in the moralities and Percent Survival.

[2] Survival rate = (1 -(refugia mortality / (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.

COLLECTIONS

In 2022, the collection of fountain daters was increased due to the low spring flows of both the Comal and San Marcos Rivers. In addition to the animals donated to the refugia by BIO_WEST Inc. during their bi-annual surveys in May/April and October, we collected San Marcos fountain darters in August. Comal Springs fountain darters were collected by refugia staff in August, September, and November from around Spring Island and Landa Lake.

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. These fountain darters were not included in the counts for the refugia. In April-May and October 2022, a subset 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 and viral analysis.

Figure 5. Braden West and Dr. Katie Bockrath collect Comal Springs fountain darters at Landa Lake using a siene net.

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. A subset (~60) of newly collected fountain darters were separated (not given a formalin dip) and sent to the USFWS SFHU for routine parasitology and health screening before the larger group of collected fish were incorporated into the refugia.

HUSBANDRY

All culture systems were monitored multiple times daily for proper water flow, acceptable temperature, and mortalities. Fish mortalities were immediately removed from the systems. If warranted, deaths were necropsied for external parasites, and preserved in vials containing 95% denatured ethanol. If external parasites were noted during the necropsy or there was an increase in mortality in a tank, then either a 1-hour static bath of either 1% sea 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, but not limited to, 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 Monday, Wednesday, and Friday, varying between live amphipods, live black worms, live *Artemia*, live *Daphnia* sp., and frozen mysid shrimp.

Figure 6. Two displaying male Fountain Darters at the San Marcos Aquatic Resources Center

SURVIVAL RATES

In 2019, 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 on-going pattern for Comal Springs fountain darters since collections were restarted in 2017 after Comal Springs fountain darters were found to test positive for Large Mouth Bass Virus (LMBV). Given the history of low intake survival rates, we suspended collections of Comal Springs fountain darters for the refugia stock in the fall of 2019. In the summer of this year, Comal River fountain darters were collected again in larger numbers as a result of low spring flow. Once in quarantine there have been mixed survival rates, but in general higher than previously with some collections having >85% survival during the quarantine period.

The reason for the higher survival rates is unknown. The Comal Springs fountain darters sampled for LMBV in October tested negative which is the first time since 2016. The LMBV negative Comal Springs fountain darters, collected in 2016, had a high survivorship and did not exhibit symptoms or mortalities of Comal Springs fountain darters collected from 2017 to 2021.

The 2022 survival rates for fountain darters in refugia at the SMARC was 71% for the San Marcos River population and 73% for the Comal River population. At the UNFH, the survival rate was 81% for the San Marcos population and 95% for the Comal River population.

MAINTENANCE OF SYSTEMS

Refugia systems were deep cleaned annually with 20 to 30% vinegar (SMARC) or muriatic acid (UNFH) to remove calcium carbonate deposits that have formed within the tank, plumbing, chiller, and pump casing that can affect functionality. When a system was empty, they were bleached with 20ppm free chlorine for 24 hours followed by neutralization with Sodium Thiosulfate (UNFH) or the tank surface sprayed with 0.5% 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 2022, 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 collections by EARP staff for standing and refugia stocks occurred one time in June from around Spring Island. In January, Randy Gibson (USFWS) donated individuals from lures he placed in Spring Run 1, 2, and 3 as part of a non-refugia project.Riffle beetles were collected with cotton lures following EAHCP standard operating procedures (Hall 2016) or from in-situ wood. No specific spring orifice was sampled two times in a row. Standing stock numbers were reduced to 75 individuals per station until propagation methods are refined and 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 2022	End of Year Census	*Target Goal 2022 Work Plan	Percent Survival
SMARC	23	48	36	--	43%
UNFH	32	58	48	--	53%

* for 2022 there was no net end of the year goal, as we planned on collecting CSRB mainly to support research, until survival is increased in captivity

COLLECTIONS

On December 13, staff assisted Randy Gibson (USFWS) with the retrieval of cotton lures from Spring Run 3 of the Comal River, which were placed by Gibson as part of an non-refugia sampling effort. From these lures 219 CSRB where captured, 152 of which were retained for the

UNFH refugia. On June 15, staff collected 61 CSRB from wood debris around Spring Island (Comal River), 52 of these were taken to the SMARC for the refugia.

Figure 7. Will Coleman, Randy Gibson, Amelia Hunter, and Braden West at collecting Comal Springs riffle beetle at Spring Island, New Braunfels, Texas.

QUARANTINE

Incoming CSRB were quarantined in the quarantine room 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, then CSRB joined the Refugia population in its own separate container labeled by collection date at the end of the 30-day quarantine period to observe survival rates over time. Due to limited space during the construction of new invertebrate systems at the SMARC, the beetles from different collections were sometimes aggregated in the same box.

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, we did not follow a traditional feeding schedule. Alternatively, leaves and cotton cloth containing biofilm were used in each system, providing food. Inventories were conducted every other month and new leaf and cotton material was added as needed. Conditioned wood was incorporated into refugia containers.

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, biofilm cloth, and mesh for structure and habitat. 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. Air space and emergent structure was provided in box containers housing larvae.

SURVIVAL RATES

Because CSRB have an average life span of approximately a 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 sixth 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 2022 survival rates for CSRB in refugia at the SMARC was 51% and 53% at the UNFH.

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. We are observing 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 not set 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 2022	End of Year Census	In Quarantine End of Year	Target Goal 2022 Work Plan	Percent Survival
SMARC	0	2	2	0	*	100%
UNFH	0	12	10	0	*	83%

**No set target as catch rates and hatchery survival are uncertain given the rarity of the species*

COLLECTIONS

In 2022, sampling events occurred for CSDB at Spring Island by checking in-situ submerged wood. We collected eleven CSDB in February and two March, which were retained for the UNFH refugia population. In June, four CSDB were collected and taken to the SMARC refugia.

QUARANTINE

Incoming CSDB were quarantined in the Invertebrate Refugia area at the UNFH. CSDB were acclimated to quarantine water conditions at a rate of 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, then 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 the 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, we did not follow a traditional feeding schedule. Alternatively, leaves, wooden dowels, and cotton cloth containing biofilm were placed in containers that provided a constant food source. Conditioned wood pieces were added. 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.

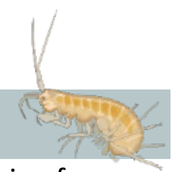
SURVIVAL RATES

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 2022 survival rates for CSDB in refugia at the SMARC was 100% and 83% at the UNFH.

CAPTIVE PROPAGATION

No larvae or eggs were observed in 2022 during inventories.

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



Peck's cave amphipods (PCA) were collected from Comal Springs by hand during four collection events. The refugia also received PCA caught in drift nets during biomonitoring activities. 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 2022	End of Year Census	Target Goal 2022 Work Plan	Percent Survival
SMARC	121	120	139	250	58%
UNFH	153	197	232	250	66%

COLLECTIONS

There were five collection events conducted in 2022 for Peck's cave amphipods (PCA) by refugia staff. These took place around Spring Island of the Comal River, New Braunfels, TX. A total of 311 PCA were captured and 293 transferred to the SMARC and UNFH for the refugia. In addition to the refugia collections, SMARC biologist Randy Gibson donated 12 PCA in May and 110 PCA to the SMARC refugia program in October 2022. These were collected from the Comal River headwaters via net during spring and fall Comal River biomonitoring event.

Figure 8. Left: Eleanor Krellenstein collecting Peck's cave amphipod. Right: Braden West collecting Peck's cave amphipods with a dip net at Spring Island in New Braunfels, Texas.

QUARANTINE

Incoming PCA were quarantined in separate systems than existing refugia stock in the SMARC Refugia Invertebrate area or the quarantine room at the 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, then 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 one to two times a 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 top 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 2022 survival rates for PCA in refugia at the SMARC was 58% and 66% 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. Biologist were confident, given observed growth rates, that juveniles that survived could be located, identified, and moved to an F1 container.

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

No Edwards Aquifer diving beetles were collected during 2022. 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

Will Coleman, a doctoral student at Texas State University, discovered a non-lethal way to distinguish *L. smithii* from other species based on the characteristics of the pleotelson. In 2019, using Coleman's method, we determined the refugia population consisted primarily of *Lirceolus hardeni* (no common name). Further, Mr. Coleman's 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, 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 2022. In the future, if *L. smithii* are collected from Texas Sate Artesian Well, the refugia will employ documented husbandry procedures that were very 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 we projected it would take up to 10 years to reach our standing stock goal. In 2019, we observed 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, TX. 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 same-age class. Some individuals of this age class were transferred to the UNFH to discourage inbreeding in the refugia. Numbers incorporated, end of the year census, and survival rates can be found in Table 10.

Table 10 Texas blind salamander refugia population figures

	Beginning of Year Census	Incorporated 2022	End of Year Census	In Quarantine End of Year	Target Goal 2022 Work Plan	Percent Survival
SMARC	192	10	172	4	250	92%
UNFH	70	0	65	0	60	94%

COLLECTIONS

Texas blind salamanders are collected from caves, wells, fissures, and driftnets on high flow springs. Traps were deployed quarterly in Primer's Fissure, Johnson's Well, Rattlesnake Cave, and Rattlesnake Well. Traps were 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 were retained for refugia. Any gravid females were retained due to their rarity. During 2022 there no drift net collections of TBS due to low spring flows.

In 2022, Primer's Fissure and Johnson's Well were both sampled in February and May, but on Johnson's Well was only sampled in August and November due to low water in Primer's Fissure in those months. Rattlesnake Cave and Rattlesnake Well were sampled in January. No TBS were observed in either Rattlesnake Cave or Well. All sites were trapped for two weeks during each collection event and biologists collected tail clips of salamanders released from these sites for future genetic analysis.

QUARANTINE

Texas blind salamanders were transported directly to the quarantine space at the SMARC after collection. The quarantine area is a separate, biologically secure area away from the refugia systems, preventing the spread of disease and aquatic nuisance species. Salamanders were acclimated to quarantine water conditions over the course of several hours after arrival. All newly collected larva 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 visible implant elastomer (VIE) tags so that collection origin was known. Corbin (2020) completed a genetic analysis of wild caught Texas blind salamanders and shows low genetic diversity and no genetic differentiation between sampling locations. Thus, all collected 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 gas 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, 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 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 VIE tags (for individual identification) under sedation and then were combined with other newly tagged individuals of equivalent sizes. Salamanders continued their grow-out in these groups. Once salamanders were large enough for individual triplet tags, they were then moved out of their groups, retaining their individual data. The triplet tags allow for quick identification of individuals to access sex, collection location, and year of collection.

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 92% at the SMARC and 94% at the UNFH in 2022. Survival rates during quarantine period are not included in annual survival rates.

HEALTH MONITORING

Biologists monitored salamanders for changes in appearance and behavior including anorexia, 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 or formalin 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 to 30% vinegar (SMARC) or muriatic acid (UNFH) to remove calcium carbonate deposits that have 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 location is known, and they were housed in group systems to encourage production of offspring for future research. Females were checked periodically for presence of visible eggs. Offspring produced can be identified by maternal origin but not paternal. 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 27 clutches of eggs and no clutch was produced at the UNFH in 2022. Clutch data are reported in Table 11.

Table 11. Texas blind salamander clutches produced during 2022

Site	Date	*Parent Generation*	*Offspring Generation*	*# Deposited*	*# Hatched*	*(%) Survival*
SMARC	1/07/2022	WS	F1	NA	NA	NA
SMARC	1/17/2022	WS	F1	3	NA	NA
SMARC	2/07/2022	WS	F1	15	NA	NA
SMARC	2/12/2022	WS	F1	13	NA	NA
SMARC	2/20/2022	WS	F1	25	0	0%
SMARC	3/11/2022	WS	F1	47	NA	NA
SMARC	3/28/2022	WS	F1	16	NA	NA
SMARC	3/30/2022	WS	F1	19	NA	NA
SMARC	4/18/2022	WS	F1	48	4	8%
SMARC	4/28/2022	WS	F1	NA	NA	NA
SMARC	4/29/2022	WS	F1	NA	NA	NA
SMARC	4/30/2022	WS	F1	6	NA	NA
SMARC	5/16/2022	WS	F1	31	NA	NA
SMARC	5/24/2022	WS	F1	NA	NA	NA
SMARC	5/26/2022	WS	F1	NA	NA	NA
SMARC	5/30/2022	WS	F1	NA	NA	NA
SMARC	6/6/2022	WS	F1	NA	NA	NA
SMARC	6/13/2022	WS	F1	NA	NA	NA
SMARC	6/20/2022	WS	F1	NA	NA	NA
SMARC	6/27/2022	WS	F1	NA	NA	NA
SMARC	7/03/2022	WS	F1	NA	NA	NA
SMARC	7/18/2022	WS	F1	19	5	26%
SMARC	7/21/2022	WS	F1	12	4	33%
SMARC	8/02/2022	WS	F1	29	4	14%
SMARC	10/12/2022	WS	F1	14	2	14%

SMARC	12/12/2022	WS	F1	33	*	*
SMARC	12/30/2022	WS	F1	11	*	*

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, we collect San Marcos salamanders twice each year in amounts sufficient to cover the expected loss given average mortality. The number of collections was increased in 2022, due to the low flow rates of the San Marcos River in the Summer. 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 2022	End of Year Census	In Quarantine End of Year	Target Goal 2022 Work Plan	Percent Survival
SMARC	161	0	96	87	250	60%
UNFH	199	0	167	48	250	84%

COLLECTIONS

In 2022, there were two collections in September, both below Spring Lake Dam. In November there were 2 collection events. One in Spring Lake with SCUBA divers collecting around Diversion Springs and snorkelers collecting around the Meadow Center. The other collection was below Spring Lake Dam.

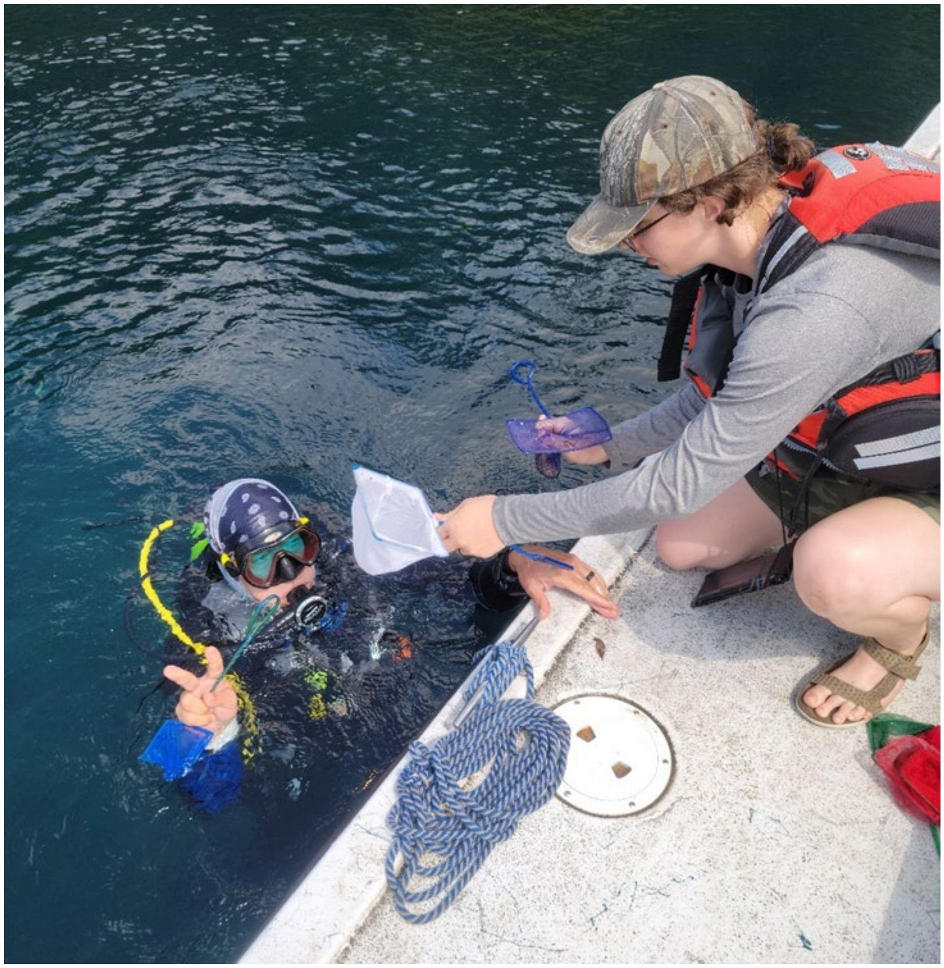


Figure 9. Student Conservation Association Intern Celeste Palmquist trading nets with a USFWS diver, Channing St. Abuin, collecting San Marcos salamanders at Spring Lake.

QUARANTINE

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 below the Spring Lake dam and the Diversion Spring net 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 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). 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. Upon reaching a minimum of 30 to 40 mm in TL, juveniles were given VIE tags (for sex and year-collected identification) under sedation and combined with other newly tagged individuals of equivalent sizes. 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 60% at the SMARC and 84% at the UNFH. Survival rates during their quarantine period are not included in the annual survival rates. The increased mortality of egg-bound females continued at both refugia facilities.

HEALTH MONITORING

Biologists monitored salamanders for changes in appearance and behavior including anorexia, 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 or formalin 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 to 30% vinegar (SMARC) or muriatic acid (UNFH) 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

In 2022, wild-stock salamanders produced 25 clutches at the SMARC and three clutches at the UNFH. Clutch info is presented in Table 13.

Table 13. Clutches of San Marcos salamanders

Site	Date	Parent Generation	Offspring Generation	Eggs Deposited	# Hatched	(%) Survival
SMARC	1/02/2022	WS	F1	NA	NA	NA
SMARC	1/13/2022	WS	F1	5	NA	NA
SMARC	1/20/2022	WS	F1	22	NA	NA
SMARC	2/03/2022	WS	F1	NA	NA	NA
SMARC	2/16/2022	WS	F1	30	4	13%
SMARC	2/21/2022	WS	F1	24	17	71%
SMARC	3/07/2022	WS	F1	27	14	52%
SMARC	3/12/2022	WS	F1	15	0	0%
SMARC	3/25/2022	WS	F1	70	50	71%
UNFH	3/30/2022	WS	F1	20	17	85%
SMARC	4/11/2022	WS	F1	5	NA	NA
SMARC	4/23/2022	WS	F1	10	NA	NA
SMARC	5/02/2022	WS	F1	10	NA	NA
SMARC	5/12/2022	WS	F1	NA	NA	NA
SMARC	6/01/2022	WS	F1	NA	21	NA
SMARC	6/18/2022	WS	F1	NA	NA	NA
SMARC	6/22/2022	WS	F1	NA	2	NA
SMARC	6/27/2022	WS	F1	NA	NA	NA
SMARC	7/06/2022	WS	F1	4	NA	NA
UNFH	8/01/2022	WS	F1	34	20	59%
SMARC	9/07/2022	WS	F1	NA	NA	NA
SMARC	9/17/2022	WS	F1	NA	NA	NA
SMARC	9/20/2022	WS	F1	11	7	64%
SMARC	9/28/2022	WS	F1	3	NA	NA
SMARC	10/13/2022	WS	F1	8	6	75%
SMARC	10/25/2022	WS	F1	17	4	24%
SMARC	12/5/2022	WS	F1	21	NA	NA
UNFH	12/13/2022	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 utilized 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 2022	End of Year Census	In Quarantine End of Year	Target Goal 2022 Work Plan	Percent Survival
SMARC	114	0	110	2	135	94%
UNFH	65	35	93	0	105	93%

COLLECTIONS

USFWS staff snorkeled to collect adult Comal Springs salamanders using dip nets around the Spring Island area of Landa Lake. In August 2022, staff collected 2 individuals, which were taken to the SMARC refugia. Few Comal Spring salamanders were observed during the collection event, possibly due to low spring flow.

QUARANTINE

In 2022, after collection all Comal Springs salamanders were transported directly to the quarantine facility at the UNFH. 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, but not limited to, dissolved oxygen, pH, and total gas pressure, were checked weekly.

Habitat enrichment items, including natural and artificial rocks, plastic plants, and meshes 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. Upon reaching a minimum of 30 to 40 mm in TL, salamanders are given VIE tags (for sex and year-collected identification) under sedation and combined with other newly tagged individuals of equivalent sizes. 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 2022, with 94% at the SMARC and 93% at the UNFH. There were few cases of mortality due to tank escapement compared to previous years, indicating that the modified system design for the salamanders at the SMARC was beneficial.

HEALTH MONITORING

Biologists monitored salamanders for changes in appearance or behavior including anorexia, 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 or formalin 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 to 30% vinegar (SMARC) or muriatic acid (UNFH) 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 2022, 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. Five clutches of eggs were produced at the SMARC and three clutches at the UNFH (Table 15).

Table 15. Propagation of Comal Springs salamanders

Site	***Date***	***Parent Generation***	***Offspring Generation***	***# Deposited***	***# Hatched***	***(%) Survival***
SMARC	1/23/2022	WS	F1	20	NA	NA
SMARC	2/17/2022	WS	F1	6	NA	NA
SMARC	2/28/2022	WS	F1	7	NA	NA
SMARC	5/2/2022	WS	F1	2	NA	NA
SMARC	7/3/2022	F1	F2	5	0	0%
UNFH	8/17/2022	WS	F1	37	8	22%
UNFH	8/22/2022	WS	F1	33	5	15%
UNFH	11/10/2022	WS	F1	28	NA	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.

TEXAS WILD RICE (*ZIZANIA TEXANA*), ENDANGERED

The standing-stock goal for Texas wild rice (TWR) is 430 plants divided between the two facilities. Native habitat for Texas wild rice is divided into alphabetical sections of the San Marcos River, determined by Texas Parks and Wildlife. Texas Parks and Wildlife categorizes TWR in alphabetical (A–K) river segments of the San Marcos River (0). 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 full report of this study can be found in Appendix F. 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.

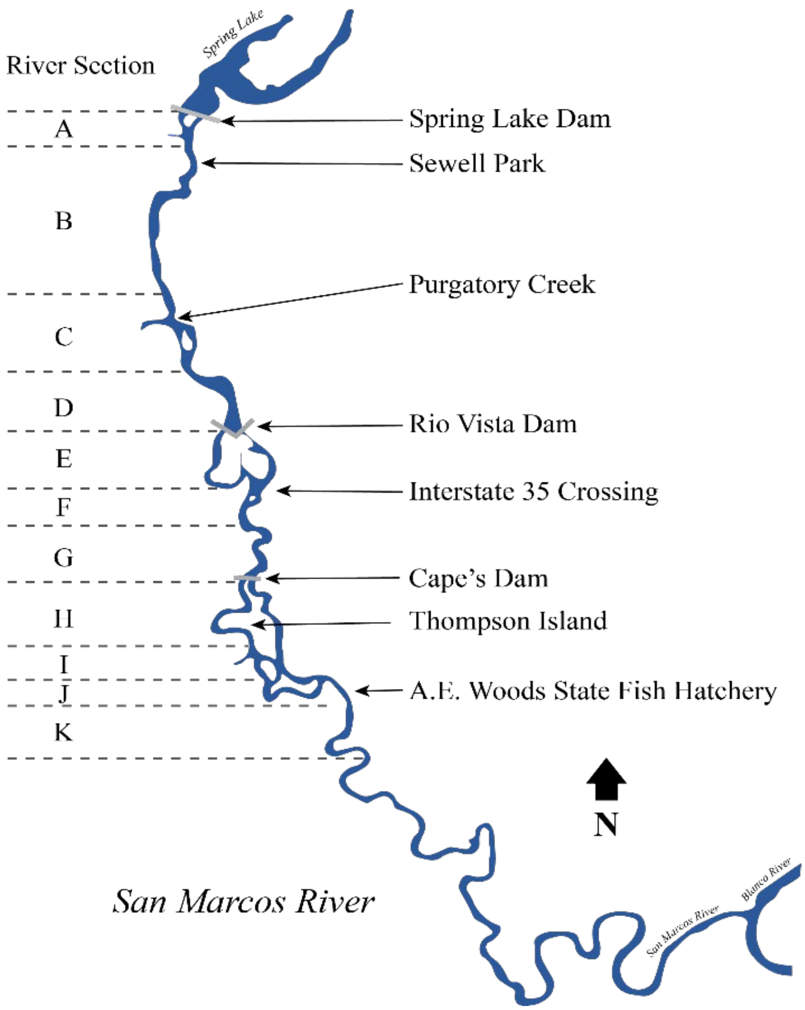


Figure 10. Lettered sections of the San Marcos River designating Texas wild rice habitat established by Texas Parks and Wildlife Department.

Table 16. Texas wild rice refugia population figures

	Beginning of Year Census	Incorporated 2021	End of Year Census	In Quarantine End of Year	Target Goal 2020 Work Plan	Percent Survival
SMARC	191	33	205	0	215	92%
UNFH	169	53	205	0	215	96%

COLLECTIONS

Tiller collections in the San Marcos River occurred in February, April, May, and August 2022. 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 (enabled with Wide Area Augmentation System (WAAS), providing 3-meter position accuracy). 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.

Figure 11. Student Conservation Association Interns, Elleanor Krellenstein and Kevin Rubio, collecting Texas wild rice data in the San Marcos River.

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 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.

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 96%. 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 pumps to circulate water through units and produce flow throughout the tanks. At the UNFH, recirculation manifolds were maintained to facilitate flow throughout the tanks, driven by 1/5 to 3/4 HP submersible pumps.

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 Refugia Program did not engage in propagation of TWR by sexual reproduction through seed production in 2022. 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 consolidating information on programmatic efforts for the San Marcos salamander and CSRB, increasing survival and pupation rates of CSRB, developing tissue archive and DNA extraction SOPs, using p-Chip tags in salamander species, treating Bd in the San Marcos salamander, and a genetic assessment of CSRB. Much of this research was built on knowledge gained in previous studies. Below are summaries for each project approved within the 2022 Work Plan (Appendix A).

Figure 12. Jennifer Whitt, Dr. Katie Bockrath, and Ben Thomas sexing and measuring pairs of salamanders (one male, one female) for the third trial of the San Marcos salamander captive habitat project.

SAN MARCOS SALAMANDER (*EURYCEA NANA*) REPRODUCTION: REFUGIA HABITAT AND CAPTIVE PROPOGATION

Our objectives were to 1) complete the study investigating the effects of darkened tanks, textured tank bottoms, and a combination of the two on reproduction events of San Marcos salamanders to determine if we can use these conditions to promote propagation on demand, and 2) to create a document summarizing all programmatic efforts to understand reproduction of San Marcos salamanders. The final trial examining tank modifications was completed, but no oviposition occurred throughout the study. Therefore, no conclusions could be made about tank modifications affecting propagation. The summary document discussing San Marcos salamander reproduction is in Appendix B.

CAPTIVE HUSBANDRY AND PROPAGATION OF THE COMAL SPRINGS RIFFLE BEETLE

The purpose of this project was to create a document summarizing all programmatic efforts to understand the husbandry and propagation of CSRB. The summary document is in Appendix C.

ASSESSING THE EFFECT OF *STAPHYLOCOCCUS* EXPOSURE ON COMAL SPRINGS RIFFLE BEETLE (*HETERELMIS COMALENSIS*) CAPITVE SURVIVAL AND PROPOGATION

This project was a collaboration between the U.S. Fish and Wildlife Service and Dr. Camila Carlos-Shanley's lab at Texas State University. The objective of this project was to determine if exposure to *Staphylococcus sciuri* affects the survival and pupation of wild CSRB larvae compared to a no bacteria added control and a *Bacillis* sp. control. Larvae survival was lowest in the no bacteria group, followed by the *Staphylococcus* group. Survival was highest in the *Bacillus* group. Certain strains of *Bacillis sp*. have been identified as probiotics with anti-microbial properties that reduce the abundance of pathogenic bacterium in humans. This, along with the increased survivorship in the *Bacillis* treatment group, suggests that *Bacillis* may be a beneficial bacterium in the CSRB larval microbiome. Because larval survival was low for the *Staphylococcus* group, increased biosecurity standards, such as wearing gloves and increased sanitation, should be expanded to routine procedures in the Refugia. Some larvae escaped their containers during the first trial, which was attributed to the frequency of inventory events. No pupation occurred in Trial 1, but four larvae pupated across treatments in Trial 2 after they were transferred to the SMARC and placed on a flow-through system. The results indicated increasing biosecurity measures, performing fewer inventory events, and moving CSRB to flow-through systems could provide higher survival and pupation for CSRB and fewer escaped larvae.

Larvae microbiomes were compared to previously collected wild adults, SMARC larvae, and UNFH larvae. The microbiome of the larvae in this study (wild) were most similar the UNFH larvae, indicating the water at the SMARC might be different from Comal Springs water. Additionally, larvae microbiomes are different from those of adults. It is possible that larvae and adults have different diets or occupy different areas of natural and captive habitat. The observed differences in larval and adult microbiomes may have varying impacts on innate immunity, survival, and overall health and behavior. Additional research would be required to investigate further. The final report can be found in Appendix D.

Figure 13. Individual flowthrough containers used to conduct bacterial exposure trials.

INCREASING COMAL SPRINGS RIFFLE BEETLE (*HETERELMIS COMALENSIS*) F1 ADULT PRODUCTION

BIO-WEST, Incorporated and the U.S. Fish and Wildlife Service collaborated on this project. The overarching goal of this study was to increase pupation of CSRB larvae in the refugia population. The objectives of this study were to 1) determine if higher densities of larvae in flow-through tubes can maintain or improve pupation/eclosion rates, and 2) compare the effects of providing wild-cultivated and captive-cultivated biofilm (on leaves, wood, and cloth) on pupation rates.

The results indicated there was no difference in pupation/eclosion rates when larvae were held at densities of 20, 30, and 40. Therefore, EARP standard operating procedures should be changed to permit up to 40 larvae in each flow-through tube. This change allows more larvae to be held given the same amount of space and water usage. Larvae provided with captive-cultivated biofilm had a higher pupation rate than larvae provided with wild-cultivated biofilm, indicating the methods used to obtain wild biofilm were not more beneficial than the methods used to cultivate biofilm at the SMARC. The final report is located in Appendix E.

Figure 14. Israel Prewitt (BIO-WEST) working on the Comal Springs riffle beetle density and biofilm trials to assessing impacts on increasing F1 captive production.

HISTORICAL FOUNTAIN DARTER (*ETHEOSTOMA FONTICOLA*) TISSUE ARCHIVE

The goals of this study were to 1) create a sample archive that enables more efficient mortality storage and tracking, provides a record of fountain darter health and variation, and to generate Standard Operating Procedures for future sample preservation and archive for effective usage in future efforts, and 2) assess the viability of preserved tissues preserved and stored under various conditions for genetic analyses and to develop DNA extraction protocols of these samples. The sample archive was created, and a Standard Operating Procedures document was developed to guide future sample management. Approximately 1,050 fountain darter samples were entered into the archive database. Total body length varied by collection location and, for the most part, sex ratio remained approximately 50/50 across collection years. Existing DNA extraction protocols were modified and refined to extract viable DNA from formalin preserved tissues stored in non-climate-controlled conditions. These modifications were recorded, and Standard Operating Procedures were generated for future use in non-ideally preserved specimens, such as museum collections. Fountain darters stored in 95% ethanol and in climate-controlled conditions provided the best DNA extraction and PCR amplification results, and this storage method will be implemented for all future preserved fountain darters. Preserved samples stored in non-climate-controlled conditions were not viable. These samples may be suitable for non-genetic assessments but should not be considered for inclusion in genetic assessments. The final report is located in Appendix F.

EVALUATING THE EFFECTS OF P-CHIP TAGS ON SMALL-BODIED SALAMANDERS (*EURYCEA* SPP.)

The objectives of this study were to examine the 1) survival, 2) tag retention, and 3) the effects on growth associated with p-Chip microtransponder tags in Texas blind and Comal Springs salamanders at the San Marcos Aquatic Resources Center. Survival was 97-100%, with one mortality in the Comal Springs salamander control group, and one in the Texas blind salamander tagged group. Tag retention was 100% in Texas blind salamanders and 98% in Comal Springs salamanders. Growth was not affected by tagging. Novice readers had approximately an 80% reading success rate. The time it took novice readers to scan a subset of the tagged salamanders was highly variable and ranged from 40 minutes to 2 hours. The final report is located in Appendix G.

Figure 15. A Texas blind salamander with a p-Chip tag. The p-Chip is circled in black.

TREATMENT TRIALS FOR BATRACHOCHYTRIUM DENDROBATIDIS INFECTIONS IN AQUATIC SALAMANDERS

This study was designed to test the efficacy of itraconazole and dosing strategies to treat Batrachochytrium dendrobatidis (Bd) infections in San Marcos salamanders. A pilot study was conducted to determine if San Marcos salamanders could tolerate three candidate treatment doses. No negative effects were recorded during the pilot study. The full study was completed using the two highest itraconazole doses, here after 'low' and 'high' dose. Salamanders were tested for Bd before and after treatment to determine any changes in Bd status as a result of Itraconazole treatment. Itraconazole treatment had no effect on Bd status, regardless of dose, compared to control salamanders. Salamanders either had no change in Bd status (47%), tested positive before and negative after treatment (24%), or tested negative before and positive after treatment (29%). One mortality occurred in each of the low and high dose groups. No mortalities occurred in the control group. This suggests the Itraconazole doses used for this study are too low to have a measurable impact on the presence/detection of Bd in aquatic salamanders. Higher doses of Itraconazole should be tested to further assess its efficacy for Bd treatments. The final report is located in Appendix H.

Figure 16. Student Conservation Association Inter Shawn Moore and Dr. Bockrath measure, sex, and swab San Marcos salamanders before placing them in experimental tank for Bd treatment testing.

GENETIC ASSESSMENT OF THE COMAL SPRINGS RIFFLE BEETLE IN LANDA LAKE

This is a two-year project where the overall objective is to assess the genetic diversity of the CSRB across Landa Lake and estimate effective population size to inform Refugia collection and CSRB conservation needs. Spring openings across Landa Lake were identified for candidate locations to set poly-cotton lures in 2023. Mortalities of larval and adult CSRB reserved from other efforts were preserved in 95% ethanol for downstream DNA extraction. The interim report is located in Appendix I.

BUDGET

Task	U.S. Fish and Wildlife Service 2022	Budget Spent	Total Task Budget Spent
1	**Refugia Operations**		**$682,679.37**
	SMARC Refugia & Quarantine Bldg.		
	Construction	-	
	Equipment	$13,784.02	
	Utilities	$5,972.59	
	UNFH Renovation Refugia & Quarantine Bldg.		
	Construction	-	
	Equipment	$10,792.41	
	Utilities	$24,020.60	
	SMARC Species Husbandry and Collection	$113,321.44	
	UNFH Species Husbandry and Collection	$201,227.77	
	Diver Salaries	$0	
	Water Quality Monitoring System	$709.30	
	Fish Health Unit	$9,336.15	
	SMARC Reimbursables	$94,575.34	
	UNFH Reimbursables	$85,833.63	
	Subtotal	*$556,879.0*	
	Admin Cost	*$123,106.12*	
2	**Research**		**$459,477.88**
	BIO-WEST: CSRB propagation	$8,689.68	
	BIO-WEST: CSRB pupation (2021 Rollover)	$49,183.07	
	BIO-WEST: CSRB Handbook	$0	
	TXST: CSRB pupation	$13,590.15	
	USFWS Research Projects	$305,312.17	
	Subtotal	*$376.775.07*	
	Admin Cost	*$82,890.52*	
3	**Species Propagation and Husbandry**	-	**-**
4	**Species Reintroduction**	-	**-**
5	**Reporting**		**$45,787.76**
	SMARC Staff	$30,111.18	
	UNFH Staff	$7,419.77	
	Subtotal	*$37,530.95*	
	Admin Cost	*$8256.81*	
6	**Meetings and Presentations**		**$7,774.35**
	SMARC Staff	$4,232.53	
	UNFH Staff	$2,139.88	
	Subtotal	*$6,372.41*	
	Admin Cost	*$1,401.94*	
		TOTAL	**$ 1,195,719.36**

ACRONYMS AND ABBREVIATIONS

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

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PROFESSIONAL PRESENTATIONS FROM STAFF AND COLLABORATORS

- **Bockrath, K.D.** – Genetic Assessment of Comal Springs riffle beetles: A 2022 proposal. Edwards Aquifer Habitat Conservation Plan Research Work Group Committee Meeting.
- **Dobbins, B**., Floyd EE, Rehan MH, Wiebe DE, Tovar RU, García DM, Hillis DM. Texas Conservation Symposium. "Pax6 Expression Among Sighted and Blind Salamanders of the Genus *Eurycea.*" (Jan. 13)
- **Dobbins, B.**, Floyd EE, Rehan MH, Wiebe DE, Tovar RU, García DM, Hillis DM. Texas Academy of Science. "Pax6 expression among sighted and blind salamanders of the genus *Eurycea*." (Poster Presentation, Systematics and Evolutionary Biology Section).
- **Dobbins, B.**, Tovar RU, Devitt TJ, García DM, Hillis DM. Texas Herpetological Society. "Feeling Their Way In the Dark: Differences in Neuromast Numbers between Surface and Subsurface Salamanders (*Eurycea*)". (Oct. 22)

- **Dobbins, B.**, Rogers MN, Tovar RU, Devitt TJ, García DM, Hillis DM. American Physiological Society. "Expansion of the Lateral Line System Among Blind Salamanders of the Genus *Eurycea*." (Poster Presentation, Comparative and Evolutionary Physiology Section).
- **Moore, D. M.**, K. Bockrath, and E. Kosnicki. 2021. Comal Springs riffle beetle propagation. Research Work Group for the Edwards Aquifer Habitat Conservation Plan, San Marcos, TX.
- **Moore, D. M.**, S. K. Brewer, and K. Bockrath. 2022. Evaluation of p-Chip tags on small-bodied federally listed aquatic species. R2 Fish and Aquatic Conservation Science Symposium, Region 2, USFWS
- **Moore, D. M.**, S. K. Brewer, and K. Bockrath. 2022. Evaluation of p-Chip tags on small-bodied federally listed aquatic species. Fish Technology Center First Quarterly Meeting, USFWS
- **Moore, D.M.** 2022. P-Chip tagging in small-bodied aquatic organisms. Workshop taught at the San Marcos Aquatic Resources Center, San Marcos, Texas. (June 23, 2022)
- Oddo B, Jones R, Dobbins B, **Tovar RU**, Devitt TJ, Hillis DM, Garcia DM. Texas Herpetological Society. "Pax6 localization in the eyes and neuromasts of sighted and blind salamanders of the genus *Euyrcea*" October 22, 2022. (Poster Presentation).
- **Ruben U. Tovar**. Texas A&M San Antonio, Dr. Charles Watson, Herpetology class. "Crossing to the dark side, evolution and development of blind salamanders from central Texas."
- Hartman N, **Tovar RU**, Devitt TJ, Garcia, DM, Hillis DM. Texas Conservation Symposium. Convergent eye reduction among Central Texas *Eurycea.* Poster Presentation. (Nick won best undergraduate Poster Presentation).
- **Ruben U. Tovar**, Thomas J. Devitt, Dana M. Garcia, David M. Hillis. Texas Herpetological Society. Deep homology in early eye development across divergent, adaptive morphologies of *Eurycea* salamander species". (Oct. 22)
- **Ruben U. Tovar**. CBSL Habitat Enhancement Committee and Partners Meeting. "Exploring the diversity and evolution of the central Texas brook salamanders (Genus: *Eurycea*)."
- **Ruben U. Tovar**, Dana M. Garcia, Thomas J. Devitt, David M. Hillis. Joint SDB-PASEDB Meeting, Vancouver, Canada: "Deep homology in early eye development across divergent, adaptive morphologies of *Eurycea* salamander species". (July 18, 2022. Program Abstract #271)
- **Ruben U. Tovar.** Wild Neighbors Speaker Series: "Diversity and Evolution of Texas Salamanders" Hosted by Jaya Torres (Sep. 30). (Wild Neighbors Speaker Series)
- **West, B.** - Available Survey123 forms for hatchery use. Presentation to the USFWS Data Management Team for Standardization of Survey123 forms.