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

ANNUAL REPORT 2021

CONTRACT NO. 16-822-HCP

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Figure 1. Founding Edwards Aquifer Refugia Program staff: Linda Moon, Amelia Hunter, Kelsey Anderson, and Dr. Lindsay Glass Campbell.

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 fifth annual report of the contract covering the calendar year of 2021. The fifth 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 and significant staff changes.

The major objectives of the USFWS Refugia Program are to 1) develop and provide fully functioning refugia for the Covered Species; 2) conduct research to expand knowledge of the Covered Species with a focus on Refugia needs; 3) develop and refine animal rearing methods and captive propagation techniques for the Covered Species; 4) reintroduce species, in the event of a loss of species populations in their native environment, and monitor recovery; and 5) attend meetings and provide oral presentations to EAHCP Science Committee, Implementing Committee, and EAA Board of Directors as requested by the EAHCP Program Manager.

COLLECTIONS

Collection events occurred in every month of 2021. 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 2021; all other covered species were collected in 2021.

Table 1. Counts of individuals collected in 2021 by species and month

	CSRB	CSDB	PCA	CSFD	SMFD	TXBS	CSS	SMS	TWR
JAN	0/16								
FEB						11/0			0/5
MAR	34/0	0/1	13/0				0/45	126/0	
APR	701*/0				148/147				0/22
MAY	0/9			258/0		7/0			0/15
JUN	0/42						0/15		
JUL	23/0		3/0					56/0	
AUG						6/0			
SEP			68/0				0/63		
OCT				60/0	182/62	1/0		2/0	0/19
NOV			0/78			8/0	0/16		
DEC	11/0								

Notes: 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. *Not all individuals collected were adults or incorporated into the refugia. A majority of these individuals were larvae that were used in research or released.

RESEARCH

We conducted six research projects in 2021, several with external partners. These research projects focused on species covered by the Edwards Aquifer Habitat Conservation Plan, including two invertebrate species (Comal Springs riffle beetles and Comal Springs dryopid beetles), San Marcos salamanders, and Texas wild rice. All research was conducted to improve successful completion of their life cycles, promote reliable reproduction, and measure how well the refugia reflects the wild population.

USFWS staff investigated how modifications to the refugia habitat may promote reproductive activity in San Marcos salamanders. Two different light conditions in combination with two different substrate conditions were tested. A total of three trials are to be completed, spanning the San Marcos salamander breeding season. The third trial is currently underway. An Interim report for this research is included in Appendix B.

BIO-WEST, Incorporated (BIO-WEST) continued their work to increase survival rates of Comal Springs dryopid beetles in captivity. This work was a continuation of a 2020 effort to design and construct captive holding chambers. The 2021 effort focused on dryopid beetle habitat choice and the addition of sycamore saplings. A final report is located in Appendix C.

Texas State University (TXST) and USFWS staff studied the effects of *Staphylococcus* sp. exposure on larval survival and pupation of the Comal Springs riffle beetle. Three treatments were tested: an infectious staph exposure treatment, a benign *Bacillus* sp. exposure treatment, and a no exposure treatment control. The staph exposure treatment and no exposure treatment had higher mortality, while the *Bacillus* exposure treatment had lower mortality across two trials. Once transferred to the SMARC, surviving larvae on a flow-through system pupated irrespective of their exposure treatment. A subset of larvae from each bacterial exposure treatment was sacrificed for genetic analysis. Samples were sent to Microbial Genome Sequencing Center in Pittsburg, Pennsylvania for sequencing but were lost in transit. Remaining samples from the first trial will be sent off for genomic analysis in 2022. An interim report for this research is included in Appendix D.

BIO-WEST and SMARC continued research on increasing pupation success of Comal Springs riffle beetles in captivity. Building on 2020 research, which concluded that an air-water interface is important for riffle beetle pupation, an alternative housing design was tested to determine if a larger air interface would improve pupations rates. Wild caught adult Comal Springs riffle beetles were collected and held in refugia to produce F1 larvae for 2022 research focused on the effects of density and biofilm on pupation rates. A report for this research is included in Appendix E.

Texas State University continued examining the life history of Comal Springs riffle beetles to assess factors which affect pupation rates. The research addresses two main questions. First, do pupae need access to air-water interface areas to successfully pupate in captivity? Second, does frequent handling of larvae, and more specifically pupae, lead to lower adult eclosion rates? The final report for this research is included in Appendix F.

A genetic assessment of Wild and Refugia populations of Texas wild rice was carried out in partnership with the US Fish and Wildlife Service Southwestern Native Aquatic Resources and Recovery Center (SNARRC). Individuals across the full Texas wild rice range as well as all individuals held in refugia at the San Marcos Aquatic Resources Center and the Uvalde National Fish Hatchery were included in the study. All sampled individuals were genetically assessed using the same methods as two previous genetic assessments of Texas wild rice. The goal of this study was to assess contemporary population structure and genetic diversity of wild Texas wild rice, assess how well the refugia reflects the wild population, and how well each refugia mirror each other. A report for this research is included in Appendix G.

BUDGET

The Aquifer Refugia Program did not exceed the allocated budget defined in the 2021 Refugia Work Plan previously approved by the EAA Board of Directors. The Refugia Program spent approximately $924,150 in 2021. Research activities accounted for $333,700, and approximately $528,450 was spent on collections, husbandry, and propagation. Approximately $62,000 was spent on reporting, meetings, and presentations. Most unspent funds in Task 1 will move to a Task 1 Reserve Fund 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: SMARC and UNFH (Table 3). Although both facilities are administratively under the direction of a single Center Director, each facility is directed by its own project leader. Dr. Ken Ostrand was the Center Director until he accepted another position within the USFWS. Dr. David Britton, previously the Deputy Center Director at the SMARC, became Acting Center Director until he officially became the Center Director in October 2021. Chris Hathcock assumed the role of Acting Deputy Center Director. Dr. Britton is responsible for the Edwards Aquifer Refugia Program in San Marcos and is assisted by Acting Deputy Center Director Chris Hathcock. After the departure of the Project Leader at the Uvalde National Fish Hatchery, Dr. Scott Walker was hired to fill this role. Adam Daw, based at the UNFH, led the Refugia Husbandry and Collections team for both facilities in 2021. Dr. Katie Bockrath, the Refugia Research team lead and point of contact for the Edwards Aquifer Refugia Program, coordinated all research activities, project plans, reporting and budgets beginning in June 2021.

Table 3 USFWS Refugia Program Staff

San Marcos Aquatic Resources Center	
Dr. David Britton	Center Director
Chris Hathcock	Acting Deputy Center Director
Dr. Katie Bockrath	Refugia Research Team Lead
Desiree Moore	Research Fish Biologist
Braden West	Refugia Biotechnician
Thomas Funk	Refugia Biotechnician

Uvalde National Fish Hatchery	
Scott Walker	Uvalde National Fish Hatchery Project Leader
Adam Daw	Refugia Husbandry and Collections Team Lead
Jennifer Whitt	Refugia Biotechnician
Benjamin Thomas	Refugia Biotechnician

Day to day operations were managed by two Lead Biologists (permanent positions funded through the Contract with the EAA) 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 and Dr. David Britton, prepared the annual report, annual work plans, and monthly reports, developed research activities and reports, developed and managed the Refugia Program budget, and oversaw outside research agreements.

Adam Daw, Refugia Husbandry and Collections Lead at UNFH, coordinated the husbandry and collections across stations. Mr. 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 Fish Biologist at the SMARC, worked with Dr. Bockrath to design and implement research projects across stations. Ms. 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 constructed, maintained, and monitored holding systems for refugia species. The technicians performed daily husbandry duties, promoted biosecurity, and assisted with research activities. Additionally, they managed logs and databases, authored, and edited Standard Operating Procedures (SOPs), and contributed to monthly reports.

BUILDING CONSTRUCTION

Final building construction and remodels were completed in 2021. Minor system modifications occur on an as needed basis to accommodate research and refugia housing needs. The system at the UNFH that houses the Peck's cave amphipods and Comal Springs riffle beetles were modified by moving the system's chillers outside the room to reduce heat buildup in the room and provide more space for housing refugia organisms.

COVERED SPECIES ANALYSIS

Collections of the Covered Species continued this year to achieve standing stock targets as outlined in the Contract and the 2021 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 Comal Springs riffle beetles 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 in 2021 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 has the highest knowledge score of all covered species and is in complete refugia.

Figure 2. Texas blind salamander

Table 3. Number of organisms incorporated in the SMARC Refugia Standing Stock in 2021, 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*		202	415	67%
Fountain darter – Comal Springs *Etheostoma fonticola*		0	124	85%
Comal Springs riffle beetle *Heterelmis comalensis*		230	23	6%
Comal Springs dryopid beetle *Stygoparnus comalensis*		0	0	--
Peck's cave amphipod *Stygobromus pecki*		134	121	30%
Edwards Aquifer diving beetle *Haideoporus texanus*		0	0	--
Texas troglobitic water slater *Lirceolus smithii*		0	0	--
Texas blind salamander *Eurycea rathbuni*		6	192	94%
San Marcos salamander *Eurycea nana*		76	161	56%
Comal Springs salamander *Eurycea* sp.		0	114	94%
Texas wild rice *Zizania texana*		13	191	87%

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 = (100 - refugia mortality) / (start of year inventory + # incorporated). 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 2021, 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*		205	483	74%
Fountain darter – Comal Springs *Etheostoma fonticola*		22	35	65%
Comal Springs riffle beetle *Heterelmis comalensis*		101	32	27%
Comal Springs dryopid beetle *Stygoparnus comalensis*		1	0	0%
Peck's cave amphipod *Stygobromus pecki*		0	153	49%
Edwards Aquifer diving beetle *Haideoporus texanus*		0	0	--
Texas troglobitic water slater *Lirceolus smithii*		0	0	--
Texas blind salamander *Eurycea rathbuni*		44	70	92%
San Marcos salamander *Eurycea nana*		0	199	82%
Comal Springs salamander *Eurycea* sp.		21	65	93%
Texas wild rice *Zizania texana*		42	169	78%

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 = (100 - refugia mortality) / (start of year inventory + # incorporated). 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	3	3	0
Comal Springs salamander	5	4	3	3	0
Comal Springs riffle beetle	5	4	3	2	0
Comal Springs dryopid beetle	3	2	1	1	0
Texas troblibitic water slater	1	1	0	1	0
Peck's cave amphipod	4	4	4	2	0
Edwards Aquifer diving beetle	1	0	0	0	0

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 were met for San Marcos fountain darters in 2021. 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 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. If drought or flow conditions reach critical levels, we will collect Comal Springs 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 2021[1]	End of Year Census	Target Goal 2021 Work Plan	Percent Survival
San Marcos River	SMARC	601	202	415	500	67%
	UNFH	480	206	483	500	79%
Comal River	SMARC	172	0	125	*	85%
	UNFH	27	22	35	*	65%

* We postponed collecting Comal Springs fountain darters 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.

COLLECTIONS

SMARC staff collected fountain darters from the San Marcos River and the Comal River in August 2021 for routine testing for *Centrocestus* sp., a trematode parasite. These fountain darters were not included in the counts for the refugia. In April-May and October 2021, BIO-

WEST Incorporated donated fountain darters from the San Marcos River and Comal River, collected during their biomonitoring activities. A subset of fish from both collections were sent directly to the USFWS Southwestern Fish Health Unit (SFHU) for parasite and viral analysis. A group of Comal Springs fountain darters from the first collection in May were brought into the UNFH for observation, after which they were incorporated into the UNFH refugia population after quarantining.

Figure 3. Tommy Funk collecting fountain darters in Spring Lake, San Marcos, Texas.

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 in 2021. 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 Southwest Fish Health Unit, in Dexter, New Mexico, for routine parasitology and health screening before the larger group of collected fish were incorporated into the refugia.

SURVIVAL RATES

In 2019, at both 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. The collected fountain darters from the Comal River in 2021 went for parasitic testing. The group that was transferred to the UNFH for observation had high initial mortality.

LMBV negative Comal Springs fountain darters, collected in 2016, have high survivorship and did not exhibit symptoms or mortalities of Comal Springs fountain darters collected from 2017 to present. The LMBV negative fountain darters have been in refugia for over three years and were brought in as adults. Mortalities in this group may be due to natural senescence. After the initial high mortality in the group of Comal Springs fountain darters brought in for observation in May 2021, the surviving individuals have had low mortality after their quarantine period.

The 2021 survival rates for fountain darters in refugia at SMARC was 67% for the San Marcos River population and 85% for the Comal River population. At UNFH, the survival rate was 79% for the San Marcos population and 65% for the Comal River population.

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*, and frozen mysid shrimp. A culture and use of live *Moina* sp. was evaluated as a food item for fountain darters at the UNFH with good success.

MAINTENANCE OF SYSTEMS

Refugia systems were deep cleaned annually with 20 to 30% vinegar (at SMARC) or muriatic acid (at 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 (at UNFH) or the tank surface sprayed with 0.5% Virkon (at 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 2021, 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

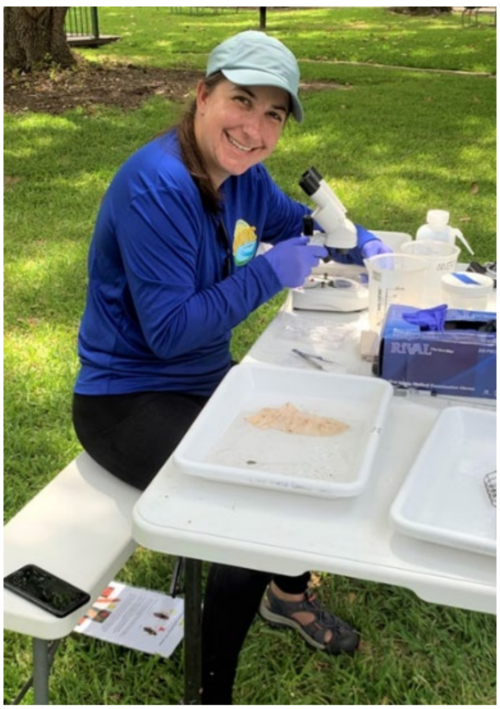


Figure 4. Dr. Katie Bockrath identifying Comal Springs riffle beetles in Landa Park, New Braunfels, Texas.

Comal Spring riffle beetle collections for standing and refugia stocks occurred seven times in 2021 (January, March, April, May, June, July, and December) from the following locations: Spring Run 1, Spring Run 3, Western Shore, and areas surrounding Spring Island. 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 2021	End of Year Census	*Target Goal 2021 Work Plan	Percent Survival
SMARC	0	230	23	--	6%
UNFH	14	101	32	--	27%

* for 2021 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 January 23, 2021, staff collected cotton lures with Comal Springs riffle beetles (CSRB) at Spring Run 3. A total of 16 adult CSRB were collected, with 15 transported to the UNFH for the refugia population. On March 25, 2021 refugia staff collected 34 adult CSRB from around Spring Island and were kept for the SMARC refugia population . On April 1 and 13, 2021, SMARC staff collected 627 juvenile and 74 adult CSRB on cotton lures originally placed by Dr. Ely Kosnicki at Spring Run 3. Of those collected, 89 juveniles were retained for the Staph exposure research project and 50 adults for the SMARC refugia. In April, refugia staff placed cotton lures in Spring Run 3. These lures were checked and reset on May 20, 2021, and 2 juvenile and 9 adult CSRB were collected. The 2 juveniles were

Figure 5. Jennifer Whitt, Braden West, and Desiree Moore identifying and enumerating beetle species collected from Landa Lake, New Braunfels, Texas.

transferred to the SMARC for research purposes the 9 adults were retained for the UNFH refugia. On June 6, 2021 cotton lures were set on the Western shore of Landa Lake. On June, 24, 2021 the lures in Spring Run 3 were retrieved, and 15 juviniles and 42 adult CSRB were captured. One juvenile and 35 adults were transferred to the UNFH for the refugia.

On June, 24, 2021, cotton lures were set in Spring Run 3. On July, 27, 2021, CSRB were collected from wood around Spring Island and the lures on the Western shore of Spring Lake were retreived. From the wood around Spring Island, 60 juveniles and 23 adult CSRB were collected and from the lures, 33 juveniles and 2 adults were collected. All the juveniles from both locations were kept for research and 23 adults were retained for the SMARC refugia. On September 30, 2021, lures were retrieved from Spring Run 3, 18 juvenile CSRB were captured and released. On the same day, 71 adult CSRB were collected from wood around Spring Island with all individuals kept for propogation of F1 larvae to be used for research at the SMARC. On October, 28, 2021, cotton lures were set in Spring Run 3. On December 14, 2021, the lures in Spring Run 3 were retrieved, three juvenile and six adult CSRB were collected. The six adults were transported to the UNFH for the refugia.

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.

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. At the SMARC, a high mortality event occurred in February 2021; likely caused by equipment and power failure during a heavy freeze event that lasted several days.

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.

Figure 6. Close-up of Comal Springs riffle beetles.

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. Because our research in 2019 showed that larvae burrowed through wood; we incorporated wood into the larvae containers.

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. These larvae were provided to our research partners for their research. At the UNFH, seven F1 larvae pupated, and those adults produced F2 larvae.

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 2021	End of Year Census	In Quarantine End of Year	Target Goal 2021 Work Plan	Percent Survival
SMARC	1	0	0	0	*	0%
UNFH	0	1	0	0	*	0%

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

COLLECTIONS

In 2021, sampling events occurred for CSDB at Spring Run 3 and Spring Island by checking in-situ submerged wood. We collected one CSDB in March 2021 from Spring Island, which was retained for the UNFH refugia population.

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.

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 riffle beetles was assessed.

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

Figure 7. Adam Daw examining in-situ submerged wood for Comal Springs dryopid beetles.

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.

CAPTIVE PROPAGATION

There were insufficient numbers of individual CSDB collected in 2021 to attempt captive propagation in the refugia. Research efforts did produce some captive propagated individuals (See Appendix B).

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

Peck's cave amphipods (PCA) were collected from Comal Springs by hand during three collection events in 2021. 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 2020	End of Year Census	Target Goal 2020 Work Plan	Percent Survival
SMARC	265	134	121	250	30%
UNFH	322	0	153	250	49%

COLLECTIONS

Three collection events were conducted in 2021 for Peck's cave amphipods (PCA). SMARC staff collected PCA in March 2021 around Spring Island in the Comal River, New Braunfels, TX. A total of 13 PCA were captured and transferred to the SMARC for the refugia. In May 2021, SMARC biologist Randy Gibson donated 41 adult PCA to the SMARC refugia program. These were collected during biomonitoring work conducted in Comal Springs, New Braunfels, TX. In September 2021, PCA were collected from around Spring Island, 71 were collected with 64 transferred to the SMARC for the refugia. In October 2021, SMARC biologist Randy Gibson donated 20 PCA to the SMARC refugia program. These were collected during biomonitoring work conducted in Comal Springs, New Braunfels, TX. Refugia staff collected PCA in November 2021 from Spring Island in the Comal River, with 78 collected and 73 transferred to the UNFH for the refugia.

Figure 8. Braden West transferring Peck's cave amphipods collected from Landa Lake, New Braunfels, Texas to Jennifer Whitt for examination and enumeration.

QUARANTINE

Incoming PCA were quarantined in separate systems than existing refugia stock in the SMARC Refugia Invertebrate area or the quarantine room at 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.

SURVIVAL RATES

While PCA have consistently had higher survival rates on average than other Refugia invertebrate species, we still strive for improvement each year. PCA are known to cannibalize smaller individuals, which lower survival rates. Biologist also estimated an optimum density (0.5 to 0.6 per liter) for PCA in containers based on survival records and observations of cannibalism at higher densities. Because PCAs are small and cannibalistic, mortality is difficult to assess by simply counting dead individuals. 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.

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).

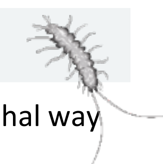
Plastic totes were used as culture containers to house PCA, with PVC piping that delivered water in a manner to mimic upwellings. The systems did not have a traditional cleaning or siphoning schedule, but alternatively, were cleaned during inventory. At this time, staff checked water lines, hoses, and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

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 2021. 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 2021. 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 UNFH to discourage inbreeding in the refugia.

Figure 9. Desiree Moore and Tommy Funk during a Diversion Spring net check.

Due to the large number of Texas blind salamanders held at the SMARC from the 2019 collection, 44 were transferred to the UNFH to make room at the SMARC for new individuals. Numbers incorporated, end of the year census, and survival rates can be found in 10.

Table 10 Texas blind salamander refugia population figures

	Beginning of Year Census	Incorporated 2021	End of Year Census	In Quarantine End of Year	Target Goal 2021 Work Plan	Percent Survival
SMARC	269	6	192	7	250	93%
UNFH	29	44	70	0	60	92%

COLLECTIONS

Texas blind salamanders were 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 2021 there was no sampling at Rattlesnake Cave and Well due to COVID-related closures and safety precautions.

Figure 10. Tommy Funk taking the cover off Johnson's well (San Marcos, Texas) to sample for salamanders.

Primer's Fissure and Johnson's Well were both sampled in February, May, August, and November 2021, as these sites can be accessed while easily maintaining social distancing procedures. These 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.

The USFWS has a large drift net on Diversion Spring in Spring Lake to collect salamanders and invertebrates coming from the spring. During periods when we were not trapping for Texas blind salamanders elsewhere, we placed a collection cup on the net and checked it two to three times a week. All live Texas blind salamanders caught from Diversion Spring net were returned to station under the assumption that any salamander leaving a spring orifice and entering the lake environment will ultimately succumb to predation. In 2021, the net was placed on the spring from July 1-29, 2021, with no Texas blind salamanders being collected.

Texas State University personnel set nets on Sessom Creek and Artesian Well for their own uses during 2021. They donated one Texas blind salamander to the refugia in November. Our collection site at Sessom Creek was not sampled in 2021.

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.

SURVIVAL RATES

The survival of all Texas blind salamanders was 93% at SMARC and 92% at UNFH in 2021. Survival rates during quarantine period are not included in annual survival rates.

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.

Figure 11. Conner McMichael, Jennifer Whitt, Adam Daw, and Ben Thomas practicing visible implant elastomer (VIE) tagging for salamander tagging events. Mask mandates were not in place at the time of this photo.

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 quickly 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, or frozen mysid shrimp. Juveniles were fed *Artemia* spp. nauplii or chopped blackworms as they increased in size.

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 (at the SMARC) or muriatic acid (at 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.

Figure 12. Texas blind salamander embryo

In total, Texas blind salamanders at the SMARC produced 16 clutches of eggs and one clutch was produced at the UNFH in 2021. Clutch data is reported in (Table 11). As we are over-capacity with juvenile F1 salamanders, ten of the clutches produced at the SMARC in 2021 were donated to Ruben Tovar and Dr. Tom Devitt at University of Texas for ongoing ocular development research. At the end of 2021, SMARC held 107 F1 individuals, 35 F1 individuals hatched during 2021 and there are no hatched F1 individuals at the UNFH.

Table 11. Individual clutches produced by Texas blind salamanders during 2021

Site	Date	*Parent Generation*	*Offspring Generation*	*# Deposited*	*# Hatched*	*(%) Survival*	*Comments*
SMARC	1/5/21	WS	F1	38	+	+	Taken by Ruben Tovar
SMARC	1/13/21	WS	F1	34	+	+	Taken by Ruben Tovar
SMARC	1/20/21	WS	F1	38	+	+	Taken by Ruben Tovar
SMARC	1/26/21	WS	F1	34	+	+	Taken by Ruben Tovar
SMARC	2/4/21	F1	F2	29	+	+	Taken by Ruben Tovar
SMARC	2/4/21	WS	F1	34	+	+	Taken by Ruben Tovar
SMARC	2/10/21	F1	F2	34	+	+	Taken by Ruben Tovar
SMARC	2/10/21	WS	F1	65	+	+	Taken by Ruben Tovar
SMARC	3/1/21	WS	F1	30	+	+	Taken by Ruben Tovar
SMARC	3/8/21	WS	F1	10	+	+	Taken by Ruben Tovar
SMARC	4/8/21	WS	F1	19	+	+	Taken by Ruben Tovar
SMARC	7/15/21	WS	F1	9	+	+	Taken by Ruben Tovar
SMARC	10/27/21	WS	F1	12	12	100	Sacrificed for Ruben Tovar
SMARC	11/3/21	WS	F1	14	11	78	Sacrificed for Ruben Tovar
SMARC	11/13/21	WS	F1	32	12	37	Kept at SMARC
SMARC	11/14/21	WS	F1	27	9	33	Kept at SMARC
SMARC	12/23/21	WS	F1	NA	*	*	Kept at SMARC
UNFH	12/28/21	WS	F1	26	*	*	Kept at UNFH

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
+Clutches donated to University of Texas

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. Only one San Marcos salamander collection was conducted in 2021, with the second collection being cancelled due to concerns with *Batrachochytrium dendrobatidis* prevalence in the wild population. Staff at the SMARC incorporated 28 adults in January that were collected in 2020. The SMARC and UNFH staff continued research on San Marcos salamander reproduction with the aim to produce offspring on demand (see Appendix B). Numbers incorporated, end of the year census, and survival rates can be found in Table 12.

Figure 13. Tommy Funk and Braden West collecting San Marcos salamanders in the San Marcos River, San Marcos, Texas.

Table 12. San Marcos salamander refugia population figures

	Beginning of Year Census	Incorporated 2021	End of Year Census	In Quarantine End of Year	Target Goal 2021 Work Plan	Percent Survival
SMARC	226	76	161	5	250	56%
UNFH	247	0	199	0	250	82%

COLLECTIONS

San Marcos salamanders were collected in March 2021. One hundred twenty-six adults were captured below Spring Lake Dam with 51 retained for the refugia. In July 2021, 56 individuals were captured from a drift net placed at Diversion Springs in Spring Lake with 3 retained for the refugia. In October 2021, two individuals were captured by Randy Gibson at Diversion Springs with a drift net and donated to the SMARC refugia. No sampling was conducted by USFWS SCUBA divers in Spring Lake during 2021.

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.

Figure 14. Ben Thomas swabbing salamanders for Bd and Bsal testing.

SURVIVAL RATES

The survival rate of San Marcos salamanders in the refugia population was 56% at the SMARC and 82% 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. At SMARC, there was a marked difference in survival rates between San Marcos salamanders collected after fall 2017 compared to those collected before fall 2017 (the "heritage group"). Most of these older salamanders were already at the facility before the new Refugia Program started in 2017. Salamanders collected after fall 2017 have not been mixed with the heritage salamanders in tanks or shared water systems. As of December 2021, only a single individual from this heritage group remained at the SMARC. Individuals from other younger populations are beginning to show similar issues as the heritage group; therefore, reproductive-related death may increase in probability with increasing animal age and/or time in captivity.

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.

Figure 15. Tommy Funk and Desiree Moore using visible implant elastomer (VIE) to tag salamanders.

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 (at SMARC) or muriatic acid (at 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

At the SMARC in 2021, wild-stock salamanders produced eleven clutches. At the end of 2021, SMARC had 3 San Marcos salamander offspring, almost all of which were small juveniles. UNFH held 19 F1 salamanders. As we have excess juvenile F1 salamanders, seven of the clutches produced at the SMARC in 2021 were donated to Ruben Tovar and Dr. Tom Devitt at University of Texas for ongoing ocular development research.

Table 13. Clutches of San Marcos salamanders

Site	Date	Parent Generation	Offspring Generation	Eggs Deposited	# Hatched	(%) Survival	Comments
SMARC	1/5/21	WS	F1	44	+	NA	Taken by Ruben Tovar
SMARC	1/8/21	WS	F1	14	+	NA	Taken by Ruben Tovar
SMARC	1/12/21	WS	F1	45	+	NA	Taken by Ruben Tovar
SMARC	1/26/21	WS	F1	43	+	NA	Taken by Ruben Tovar
SMARC	3/16/21	WS	F1	15	+	NA	Taken by Ruben Tovar
SMARC	5/5/21	WS	F1	23	+	NA	Taken by Ruben Tovar
SMARC	7/7/21	WS	F1	20	+	NA	Taken by Ruben Tovar
SMARC	8/13/21	WS	F1	17	+	NA	Taken by Ruben Tovar
SMARC	9/1/21	WS	F1	11	10	91	Taken by Ruben Tovar

SMARC	10/1/21	WS	F1	12	12	100	Taken by Ruben Tovar
SMARC	10/5/21	WS	F1	4	0	0	Kept at SMARC
SMARC	11/13/21	WS	F1	33	3	9	Kept at SMARC
SMARC	11/29/21	WS	F1	7	*	NA	Kept at SMARC
SMARC	12/3/21	WS	F1	24	*	NA	Kept at SMARC
SMARC	12/13/21	WS	F1	32	*	NA	Kept at SMARC
SMARC	12/15/21	WS	F1	44	*	NA	Kept at SMARC
SMARC	12/20/21	WS	F1	12	*	NA	Kept at SMARC
SMARC	12/28/21	WS	F1	NA	*	NA	Kept at SMARC

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
+Clutches donated to University of Texas.

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 2021	End of Year Census	In Quarantine End of Year	Target Goal 2021 Work Plan	Percent Survival
SMARC	122	0	114	0	135	94%
UNFH	49	21	65	35	105	93%

COLLECTIONS

USFWS staff snorkeled to collect adult Comal Springs salamanders using dip nets around the Spring Island area of Landa Lake. In March 2021, staff collected 45 individuals, followed by, 27 individuals in June, 63 individuals in September and 16 individuals in November. Once a salamander was captured, staff inspected it for abnormalities, injuries, or lesions. Any abnormal individuals were noted, enumerated, and returned to where they were found. Small individuals (<30 mm) were returned if collected by hand.

QUARANTINE

In 2021, 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.

SURVIVAL RATES

Survival rates of Comal Springs salamanders were high in 2021, 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.

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.

Figure 16. Adam Daw, Dr. Katie Bockrath, Jennifer Whitt, Ben Thomas, Tommy Funk, and Braden West standing next to the show tanks at the Uvalde National Fish Hatchery. Mask mandates were not in place at the time this photo was taken.

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 (at SMARC) or muriatic acid (at 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 2021, 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. Two clutches of eggs were produced at the SMARC.

Table 15. Propagation of Comal Springs salamanders

Site	***Date***	***Parent Generation***	***Offspring Generation***	***# Deposited***	***# Hatched***	***(%) Survival***	***Comments***
SMARC	1/12/21	WS	F1	8	+	NA	Taken by Ruben Tovar
SMARC	1/26/21	WS	F1	3	+	NA	Taken by Ruben Tovar

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
+Clutches donated to University of Texas.

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 (Figure 13). 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 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

Figure 17. Jennifer Whitt, Braden West, and Tommy Funk collecting Texas wild rice tillers in the San Marcos River, San Marcos, Texas.

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 17.

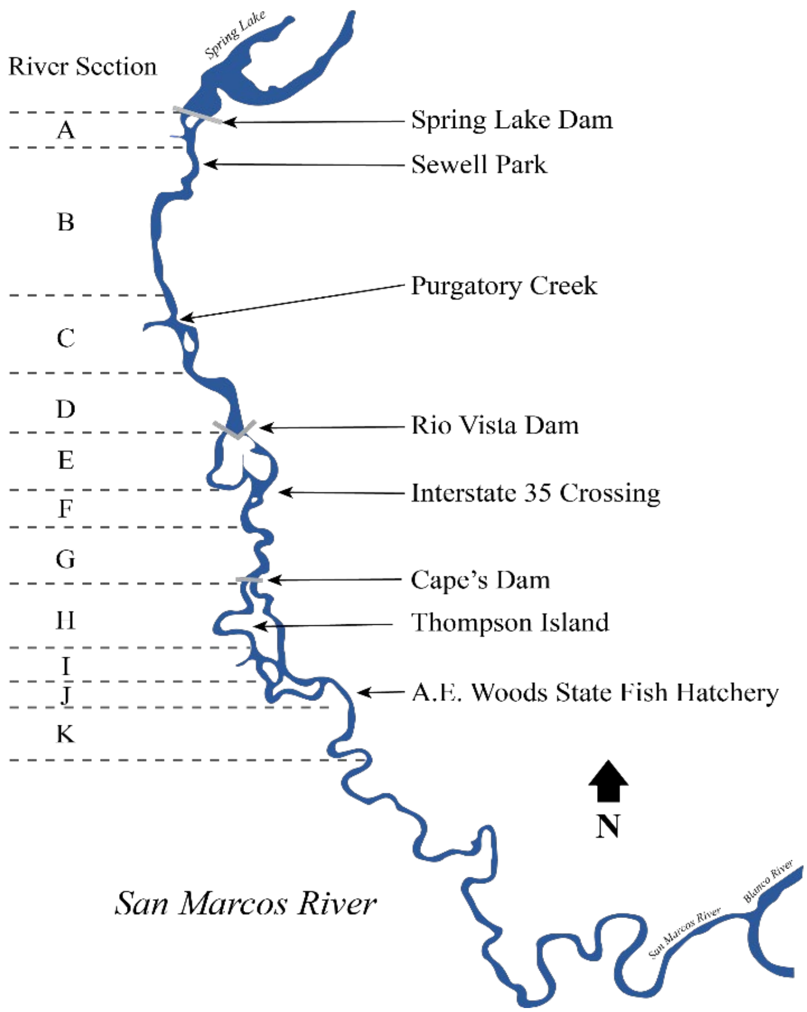


Figure 18. Lettered sections of the San Marcos River designating Texas wild rice habitat established by Texas Parks and Wildlife Department.

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	206	13	191	0	215	87%
UNFH	174	42	169	22	215	78%

COLLECTIONS

Tiller collections in the San Marcos River occurred in February, March, May, October, and December 2021. 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 19. Adam Daw recording data as Tommy Funk and Braden West collect Texas wild rice tillers.

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.

SURVIVAL RATES

Overall survival rate of TWR plants at the SMARC was 87%, with older plants more likely to succumb to mortality. The overall survival rate of TWR plants at the UNFH was 78%. 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.

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.

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 SMARC had individual heater-chiller units on tanks with 2 HP pumps to circulate water through units and produce flow throughout the tanks.

At 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. We also used suckermouth catfish (*Hypostomus plecostomus* and *Pterygoplichthys* spp.) in our refugia raceways to help control algae. 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 SMARC during the summer months to control algal growth in tanks. Shade

Figure 20. Ben Thomas holding Texas wild rice tillers collected from the San Marcos River

structures were installed over the TWR tanks at the UNFH in the winter of 2021.

Figure 20. Shade structures installed over the Texas wild rice tanks at the Uvalde National Fish Hatchery.

CAPTIVE PROPAGATION

The Refugia Program did not engage in propagation of TWR by sexual reproduction through seed production in 2021. However, the Plant Ecology and Restoration Program at the SMARC engaged in TWR plant propagation and continues to study and refine techniques.

Figure 21. Texas wild rice in the San Marcos River.

RESEARCH

Research activities for the Refugia program (USFWS and sub-contractors) focused on increasing survival and pupation rates of invertebrate species, implementing reliable propagation of the San Marcos salamander, and an up-to-date genetic study of Texas wild rice populations. Much of this research was built on knowledge gained in previous studies. Below are summaries for each project approved within the 2021 Work Plan (Appendix A).

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

Our objective in 2021 was to investigate 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 when needed. We asked Dr. Ruth Marcec-Greaves (DVM, Ph.D., and Director of the National Amphibian Conservation Center, Detroit Zoological Society) to evaluate our salamander husbandry practices and provide advice. She hypothesized that manipulating habitat within salamander tanks might stimulate reproduction. Therefore, this project was designed to examine two habitat characteristics hypothesized to relate to salamander reproduction, darkened tanks (covered) and a textured tank floor (textured liner). The research system consisted of 44 5.5-gallon aquaria with perforated polyvinyl chloride (PVC) lids. There were 11 aquaria in each treatment group (i.e., dark/textured, dark/smooth, light/textured, and light/smooth). Each aquarium contained rocks

Figure 22. Adam Daw, Dr. Katie Bockrath, Desiree Moore, and Jennifer Whitt sexing San Marcos salamanders and recording data for the habitat modification research project.

and artificial plants as salamander habitat. One adult male and female were assigned to each aquarium to monitor reproduction for 90 days. The trials were scheduled to span the entirety of the San Marcos salamander breeding season. The first trial ran from May 5 – August 3, 2021, the second ran from August 18 – November 16, 2021, the third will run from December 8, 2021 – February 8, 2022. No oviposition occurred in the first or second trials. The interim report is in Appendix B.

LIFE-HISTORY ASPECTS OF *STYGOPARNUS COMALENSIS* BARR AND SPANGLER, 1992 (COLEOPTERA: DRYOPIDAE): COMAL SPRINGS DRYOPID BEETLE RESEARCH 2021– DR. ELY KOSNICKI, BIO-WEST, INCORPORATED

The focus of this study was to build upon research of *Stygoparnus comalensis* conducted in 2017-2019. Due to the longevity of this species and growth rate, it was important to build on the existing data collected and reported by BIO-WEST in 2019. The main objectives were to: 1) continue monitoring adults, eggs, and larvae, 2) develop new aquaria for rearing all life stages, and 3) investigate natural habitats for reliable collections sites of this species.

A total of 288 eggs were produced among 16 female subjects. The longest surviving female lived for ca. 452 days and produced 66 eggs during that time. There was a strong relationship between the number of eggs produced and the length of time females were bred, producing an egg every 7-8 days. Extrapolating out to 630 days of captive breeding indicated the female reproductive potential of approximately 86 eggs. A total of 52 larvae were produced, representing an 18% hatching rate.

A total of 10 pupae were produced. The shortest duration from oviposition to pupation was 323 days while the longest duration was recorded over 513 days (387.7 ± 62.5 days; n = 10). Four adults (two of each sex) were produced, but only two of these were observed as pupae before eclosion and were noted to pupate for 14 and 19 days, respectively. The four adults were observed to take 422.5 ± 6.0 days to reach adulthood from oviposition (n = 4). Unfortunately, the adults produced from this study did not reproduce.

The number of instar stages were estimated by identifying inflection points in the second derivative of two different body measurements: body length and the Eigenvalue of the first principal component of multiple body length measurements. There was a considerable amount of variability in the larvae measurements. Principal components analysis of body lengths measured showed that axis 1 (PC1) explained 96.8% of the variation. There were insufficient data to make accurate instar estimations with no significant inflection points identified. When comparing implied inflection points between each measurement type, graphs based on the second derivative of PC1 suggested only four instars, while the graph of second derivative of body length suggested 5 instars. Body length of final instars were consistent with published data.

The new aquaria were constructed to house all life stages. These were referred to as BlackBoxes and were fashioned from 2.5-gal tanks. Each contained conditioned leaf and woody material as well as a sapling from the genus *Platanus*. The idea behind the BlackBox design was that females could oviposit anywhere in the aquarium and larvae would have a means of surviving. Only two eggs were produced at the time of this report, but the experiment is ongoing.

Four surveys to find reliable collecting locations for dryopid beetles were generally unsuccessful. Inspection of natural habitat from a known reliable collecting location revealed two late-stage larvae burrowed in a small scrape of submerged and degraded wood, suggesting dryopid eggs clearly developed even though completely submerged. This is not the first species of dryopid to have a submerged larval habitat, and it is likely that other species reside in such habitats but are difficult to study and therefore have gone unnoticed. The full report 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 aureus* affects the survival and pupation of Comal Springs riffle beetle (CSRB) larvae. Wild larvae were collected from cloth lures and woody debris in Comal Springs using disinfected equipment gloves to prevent contamination. Larvae were exposed to one of three treatment groups: *Staphylococcus* exposure, *Bacillus* exposure, or No Bacteria exposure. The *Bacillus* sp. group was included to examine the effects of increased bacterial load by adding harmless bacteria (*Bacillus subtilis* SID-166) instead of S. aureus 278. The No Bacteria group served as a negative control. Fifteen larvae were included in each treatment and two trials were conducted. A total of 90 larvae were used in this study.

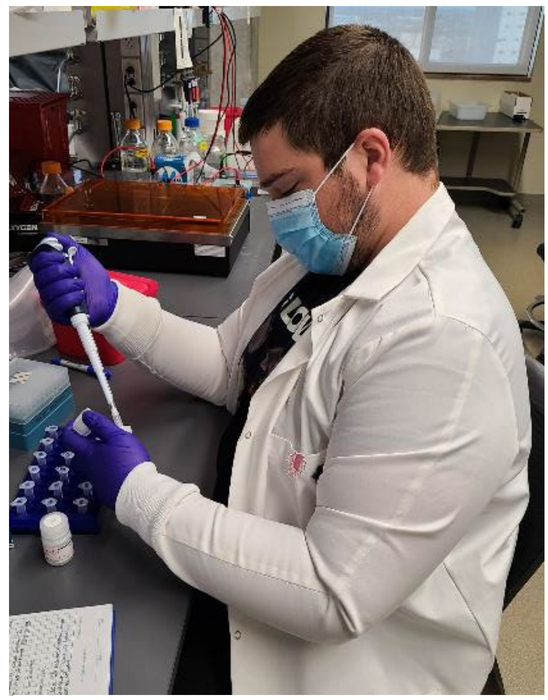


Figure 23. Sam Tye (Texas State University) preparing Comal Springs riffle beetle larvae DNA for sequencing. Photo credit: Arielle Johnson (Texas State University).

The larvae were brought to the Freeman Aquatic Building at Texas State University (FAB) and placed individually in 16-mL containers supplied with Edwards Aquifer water from the artesian well on campus. Each container held sycamore leaves (first trial) or cloth pieces (second trial) conditioned at the FAB in Edwards Aquifer water as food items for the larvae. Dr. Carlos-Shanley's lab prepared agarose using cultured *S. Aureus* 278 and *B. subtilis* 166 from isolated strains found in SMARC Comal Springs riffle beetles in the staph and *Bacillus* groups, respectively. Agarose with no bacteria added was prepared for the control group. Survival and pupation were monitored for 45 days for each trial.

Larvae survival was lowest in the No Bacteria group, followed by the *Staphylococcus* group. Survival was highest in the *Bacillus* group. 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 Comal Springs riffle beetles to flow-through systems could provide higher survival and pupation for Comal Springs riffle beetles and fewer escaped larvae. The interim report can be found in Appendix D.

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

Historically, Comal Springs riffle beetle larvae exhibit low pupation rates in captivity. BIO-WEST, Incorporated and the U.S. Fish and Wildlife Service are collaborating on this ongoing project. The overarching goal of this study is to increase pupation of CSRB larvae in the refugia population. This project is made up of three phases. The first phase evaluated a tube design modified as a small rectangular flow-through box. The second phase is to determine if higher densities of larvae in flow-through tubes can maintain or improve pupation/eclosion rates. The third phase of this research compares the effects of providing wild-cultivated and captive-cultivated biofilm (on leaves, wood, and cloth) on pupation rates.

Figure 24. Dr. Ely Kosnicki (BIO-WEST) adding aquifer water to an experimental box design for Comal Springs riffle beetle larvae.

The first phase was completed in 2021. Three flow-through boxes housing 20 larvae each were used to examine pupation rates. Flow issues occurred in each of the three boxes. Outflow clogging resulted in low flow six times and overflow once in the first box. The second box had six low flow events with three resulting in overflow. One low flow event with overflow was recorded for the third box. Two pupation events (one male and one female) were recorded in the third box. No other pupation events occurred. Due to the box design needing improvements, the original tubes were selected to be used for phases two and three of this project. This project is ongoing. At the end of 2021, BIO-WEST, Incorporated and the U.S. Fish and Wildlife Service were preparing to begin phase two of this project. The interium report for phase one is located in Appendix E.

FACTORS AFFECTING PUPATION RATES IN THE COMAL SPRINGS RIFFLE BEETLE – DR. WESTON NOWLIN, TEXAS STATE UNIVERSITY

The overall goal of this research was to examine how captive holding conditions and methods affect pupation rates and the successful eclosion of adult CSRB. The research addresses two main questions: 1) Does the configuration and type of incubation chamber affect larval survival and pupation rates of CSRB in captivity, and 2) does the frequency of handling larvae and pupae of the CSRB increase mortality?

The first objective, investigating air interfaces in incubation chambers, compared two treatments: larvae reared in a more "traditional" flow through chamber versus larvae reared in a growth chamber specifically designed to have a top area that facilitated the presence of air-filled gaps and spaces. Three late-instar larvae were placed in each chamber checked once every two weeks for a 20-week period. The number of surviving larvae, pupae, and adults were recorded at every check and pupae and adults were removed. Any missing, dead, or removed individuals were replaced with new larvae. The chambers with air-water interfaces had significantly higher (>10%) larval mortality rates than those held in the "old" chamber type. The air-water interface chambers had significantly greater number of pupae produced (15 pupae) across the experiment than the "old" chamber type (six pupae). However, the number of adults produced did not vary by treatment, potential due to small sample sizes.

The second objective, investigating the effects of handling frequency, examined two treatments: 1) checking larvae once every two weeks (bi-weekly), and 2) checking larvae once every month. Three late-instar larvae were placed in each chamber for an 18-week period. Each treatment was replicated five times. The number of surviving larvae, pupae, and adults were recorded at every check and pupae and adults were removed. Any missing, dead, or removed individuals were replaced with new larvae. The number of pupae produced did not significantly differ between treatments, but the number of adults did. Adults were produced in the monthly handling treatment while no adults were successfully produced in the bi-weekly handling treatment. This result indicates that handling on a less-frequent basis has a measurable effect on the number of adults produced in captivity. The full report is located in Appendix F.

USING MOLECULAR TECHNIQUES TO ASSESS GENETIC DIVERSITY AND POPULATION STRUCTURE IN TEXAS WILD RICE (*ZIZANIA TEXANA*)

Our goals were to determine if the refugia populations of Texas wild rice at the SMARC and UNFH capture the diversity of the wild population and compare the contemporary genetic analysis to the two previous genetic assessments. Tissue collections were performed by SMARC staff, and the genetic analyses were completed by staff at the U.S. Fish and Wildlife Service Southwestern Native Aquatic Resources and Recovery Center (SNARRC). Tissue samples were ≥10cm leaf clips and were frozen until they could be shipped to the SNARRC. All captive Texas wild rice

Figure 25. Tommy Funk navigating to the selected rice clip location in the San Marcos River, San Marcos, Texas.

plants at the SMARC and UNFH were sampled. The wild population was sampled by randomly selecting stands of Texas wild rice in each river section based on the proportion of rice in that section. Tissue samples were ≥10cm leaf clips and were frozen until they could be shipped to the SNARRC. Genomic DNA was extracted from the tissue samples and ten microsatellite markers were amplified, but three of those markers were discarded because they were not suitable for analysis. The genetic results of this study were compared to the results of two previous studies conducted in 2007 and 2012.

The results revealed that the wild population has not lost genetic diversity since the 2012 study, but any spatially separated subpopulations that existed before this study have now been mixed into one panmictic population. The results also showed that the refugia populations better represent the wild population than they did at the time of the first 2007 assessment, but additional work is needed to have adequate representation from all river sections in the refugia populations. To determine the minimum number of plants to keep in the refugia populations, genotypes from the wild population were randomly subsampled four times (representing 25%, 40%, 50%, and 75% of genotypes) and those subsamples were used in one-sided t-tests versus all wild genotypes to identify significant reductions in allelic richness. Those calculations determined that at least 200 unique individual plants must be kept in the refugia population to preserve existing wild genetic diversity. Additionally, the refugia populations at the SMARC and

Figure 26. Ashley Seagroves (SMARC) holding a Texas wild rice tissue clip she collected by diving in the San Marcos River, San Marcos, Texas.

UNFH are meant to be redundant failsafe populations, but several plants are not represented in both populations. The plants that are not redundant could be propagated and used to create redundancy between the two refugia populations. The full report is in Appendix G.

Task	U.S. Fish and Wildlife Service 2021		Budget Spent	Total Task Budget Spent
1	**Refugia Operations**			**$528,456.22**
	SMARC Refugia & Quarantine Bldg.			
	Construction		-	
	Equipment		$6,685.87	
	Utilities		$4,183.49	
	UNFH Renovation Refugia & Quarantine Bldg.			
	Construction		-	
	Equipment		$25,993.64	
	Utilities		$23,676.96	
	SMARC Species Husbandry and Collection		$124,348.36	
	UNFH Species Husbandry and Collection		$183,890.99	
	Diver Salaries		$0	
	Water Quality Monitoring System		$5,506.85	
	Fish Health Unit		$8,000.00	
	SMARC Reimbursables		$24,083.32	
	UNFH Reimbursables		$39,652.70	
		Subtotal	*$446,022.18*	
		Admin Cost	*$82,434.04*	
2	**Research**			**$333,700.07**
	BIO-WEST: CSRB pupation		$31,735.42	
	BIO-WEST: CSRB pupation (2019)		$4,782.25	
	BIO-WEST: Life History (2020)		$26,701.83	
	BIO-WEST: Dryopid life history		$61,812.87	
	TXST: CSRB pupation (2020)		$6,090.04	
	TXST: CSRB pupation		$24,080.38	
	USFWS Research Projects		$148,084.72	
		Subtotal	*$279,593.17*	
		Admin Cost	*$54,106.90*	
3	**Species Propagation and Husbandry**		-	-
4	**Species Reintroduction**		-	-
5	**Reporting**			**$51,373.75**
	SMARC Staff		$21,798.59	
	UNFH Staff		$21,408.81	
		Subtotal	*$43,207.40*	
		Admin Cost	*$8,166.35*	
6	**Meetings and Presentations**			**$10,621.83**
	SMARC Staff		$4,741.57	
	UNFH Staff		$4,186.97	
		Subtotal	*$8,928.54*	
		Admin Cost	*$1,693.29*	
			TOTAL	**$924,151.86**

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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APPENDICES

A. 2021 EA Refugia Work Plan
B. Continuation of San Marcos salamander (*Eurycea nana*) reproduction: refugia habitat and captive propagation
C. Life-history aspects of *Stygoparnus comalensis* Barr and Spangler, 1992 (Coleoptera: Dryopidae): Comal Springs dryopid beetle research 2021
D. Assessing the effect of *Staphylococcus* exposure on Comal Springs riffle beetle (*Heterelmis comalensis*) captive survival and propagation
E. Comal Springs riffle beetle research 2021-2022: increasing Comal Springs riffle beetle (*Heterelmis comalensis*) F1 adult production at the refugia level
F. Factors affecting pupation in the endangered Comal Springs riffle beetle
G. Using molecular techniques to assess genetic diversity and population structure in Texas wild rice (*Zizania texana*)
H. Monthly reports
I. Fish health unit reports