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

ANNUAL REPORT 2020

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David Britton, Lindsay Campbell, and Kelsey Anderson

U.S. Fish and Wildlife Service

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

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

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

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Figure 1. The four founding women who built our Aquifer Refugia Program at the San Marcos Aquatic Resources Center from the ground up: 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 fourth annual report of the contract covering the calendar year of 2020. The fourth 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.

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 2020 except May. Collection numbers by month and species are shown in Table 1. Comal Springs fountain darters, Edwards Aquifer diving beetles, San Marcos gambusia (presumed extinct), and Texas troglobitic water slaters were not collected in 2020; all other covered species were collected in 2020.

Table 1. Counts of individuals collected in 2020 by species and month

	CSRB	CSDB	PCA	SMFD	TXBS	CSS	SMS	TWR
JAN	98/100		4/120		6/0	12/0		
FEB	275/0		0/113		10/0			0/14
MAR					1/0		39/0	
APR				140/0				
MAY								
JUN			72/115			34/0		
JUL	17/0		171/0					
AUG				530/0	13/0			
SEP			125/50			14/0		
OCT		2/0	28/0	262/373				10/0
NOV			0/45		23/0	0/8		
DEC	133/41	1/0	14/0				33/0	13/0

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, 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.

RESEARCH

We conducted eight research projects in 2020, several with external partners. These research projects focused on three invertebrate species (Peck's cave amphipods, Comal Springs riffle beetles, and Comal Springs dryopid beetles) and the salamander species (San Marcos, Comal, and Texas blind salamanders) covered by the Edwards Aquifer Habitat Conservation Plan. All research was conducted to improve survival and health of our captive species and improve successful completion of their life cycles.

USFWS staff investigated increasing survival of captive Peck's cave amphipods. We found that medium density filtration media worked well as an artificial habitat to increase survival of this species. A final report is attached in Appendix B.

BIO-WEST, Incorporated (BIO-WEST) continued their work on increasing survival rates of Comal Springs dryopid beetles in captivity. This work began late in the year and will continue into 2021. To date, BIO-WEST has begun designing and constructing captive holding chambers for this species. A final report is anticipated next year.

USFWS staff studied San Marcos salamander reproduction. We attempted artificial application of luteinizing hormone releasing hormone to predictably induce reproduction in this species. We found no increase in egg deposition but will continue to explore ways to successfully predict reproductive output for captive members of this species. An interim report can be found in Appendix C.

USFWS staff continued research to improve Comal Springs riffle beetle nutrition. We found the captive riffle beetles preferred our standard diet of conditioned leaves over all other artificial diets tested. We also discovered that our artificial diets increased the necessity for maintenance of the holding systems to control healthy water quality. A report for this research is included in Appendix G.

Texas State University (TXST) studied the microbiome of Comal Springs riffle beetles for comparison with wild and captive biofilms. Dr. Carlos-Shanely isolated bacteria and successfully identified 142 isolates using partial 16S rRNA gene sequencing. In total, TXST identified 30 genera belonging to four phyla. Of these, 23 were found only in wild beetles, 41 only in captive beetles, and eight were found in both groups. The diversity of culturable bacteria was higher in the wild water samples than those from the refugium environment, but the wild beetles displayed lower diversity in their microbiome than their captive counterparts at the genus level. This work will continue into 2021. TXST is currently working on phylogenetic and functional analyses of these genomes. A brief report is included in Appendix E.

BIO-WEST continued research on increasing pupation success of Comal Springs riffle beetles in captivity. They concluded that an air water interface is important for riffle beetle pupation. They also determined that conditioned leaves alone were associated with better pupation, compared to conditioned cotton, leaves and wood. BIO-WEST estimated that a colony of 10 female Comal Springs riffle beetles surviving 60 days with unlimited access to mates would produce ca. 185 larvae. Of these 22 larvae would be expected to become adults, assuming a commonly observed 12% survival rate. A perpetual captive colony could be expected if these produce a 50:50 sex ratio. A report for this research is included in Appendix D.

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? At the end of 2020, Dr. Nowlin was finalizing his report with an anticipated completion date of February 28, 2021.

USFWS staff evaluated three different long-term tagging methods in aquatic salamander species. We tested visible implant elastomers and visible implant alphanumeric tags but decided not to consider passive integrated transponder tags because these tags were too large for most of our salamanders. We found that visible implant elastomer tags were the most efficacious because they were easily read, even by novel observers, and were generally retained (not shed) by the salamanders. A report for this research is included in Appendix F.

BUDGET

The Aquifer Refugia Program did not exceed the allocated budget defined in the 2020 Refugia Work Plan previously approved by the EAA Board of Directors. The Refugia Program spent approximately $908K in 2020. Research activities accounted for $366K, and approximately $542K was spent on staff, collections, husbandry and propagation, 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 2020 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 purpose of our Edwards Aquifer Refugia Program is to house and to protect adequate populations of the Covered Species in order to preserve the capacity 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. Funds cannot be moved between tasks but can be rolled forward or backwards through the years; however, total expenditures for the length of the contract cannot exceed the contract value.

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*	Endangered*
Comal Springs dryopid beetle	*Stygoparnus comalensis*	Endangered
Peck's Cave amphipod	*Stygobromus pecki*	Endangered
Texas wild-rice	*Zizania texana*	Endangered
Texas blind salamander	*Eurycea rathbuni*	Endangered
San Marcos salamander	*Eurycea nana*	Threatened
Edwards Aquifer diving beetle	*Haideoporus texanus*	Petitioned
Comal Springs salamander	*Eurycea pterophila*	None†
Texas troglobitic water slater	*Lirceolus smithii*	Petitioned

* The San Marcos gambusia was last collected in the wild in 1983 and may already be extinct. It is not included as part of the refugia at this time unless re-discovered.

†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, except the San Marcos gambusia, which is presumed extinct. 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 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, Dr. Ken Ostrand, each facility was directed by its own project leader. Dr. David Britton, the Deputy Center Director at SMARC was responsible for the Edwards Aquifer Refugia Program in San Marcos. Dr. Patricia Duncan, the Project Leader at UNFH, was responsible for the Edwards Aquifer Refugia Program in Uvalde. Dr. Duncan parted with the USFWS in November 2020. Until a replacement is made, Dr. Britton has assumed her role over the Refugia program staff at UNFH. Dr. Lindsay Campbell, the Managing Biologist for the Aquifer Refugia Program in San Marcos, coordinated the program as the Lead and Point of Contact for EAA Refugia operations, in addition to the duties of supervisor listed below.

Table 3 USFWS Refugia Program Staff

San Marcos Aquatic Resources Center	
Lindsay Campbell, Ph.D.	Managing Biologist for the Refugia Program San Marcos Program Supervisor
Kelsey Anderson	Biotechnician
Amelia Everett Hunter	Biotechnician – until May 2020
Linda Moon	Biotechnician – until October 2020
Joseph Barnett	Biotechnician – June 2020 to August 2020
Braden West	Biotechnician – from September 2020
Thomas Funk	Biotechnician – from September 2020

Uvalde National Fish Hatchery	
Mark Yost	Uvalde Program Supervisor – until June 2020
Adam Daw	Refugia Team Lead – from November 2020
Makayla Blake	Biotechnician – until May 2020
Benjamin Whiting	Biotechnician – until May 2020
Rachel Wirick	Biotechnician – until May 2020
Valerie Fischer	Biotechnician – June 2020 to August 2020
Juan Banda	Biotechnician – July 2020 to August 2020
Jennifer Whitt	Biotechnician – from August 2020
Benjamin Thomas	Biotechnician – from September 2020

Day to day operations were managed by two Supervisory Biologists (term positions funded through the Contract with the EAA), providing supervision, mentorship, and training to biological technicians at their respective facilities (see Table 3 for staffing chart). The supervisors managed and coordinated species husbandry, propagation, 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. They 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, they prepared all written materials required for reporting. They communicated regularly with the EAA, USFWS personnel, researchers, and other partners.

Dr. Lindsay Campbell coordinated efforts in San Marcos with those in Uvalde. Dr. Britton, with input of supporting staff, prepared the annual report. Dr. Campbell, with input of supporting staff, prepared yearly work plans, monthly reports, developed research activities and reports, developed and managed the Refugia Program budget, coordinated collection activities, and oversaw outside research agreements.

Mark Yost, Supervisory Biologist at UNFH, supervised the dedicated staff at UNFH and coordinated their efforts with Dr. Campbell in San Marcos. In addition to supervisory duties listed above, he provided written materials covering activities at UNFH to be incorporated into monthly reports and the annual report and offered input into the yearly work plan.

Biological Technicians (term positions funded through the Contract with the EAA), under the management of the lead Supervisory Biologist at each facility, assisted with collections, daily upkeep, maintenance, propagation, and research efforts for the refugia species at SMARC and UNFH. This included maintaining experimental and culture production systems, keeping records, entering data, and participating in research activities. The technicians also generated basic summary statistics, graphic analyses of data, and documented program accomplishments through the composition of standard operating procedures (SOPs), reports, and manuscripts. Kelsey Anderson, Amelia Hunter, and Linda

Moon, each contributed to separate research projects during 2020, including data analysis, writing, and presentation. All three, with the addition of Makayla Blake, a biological technician at UNFH, helped to develop the 2020 research proposals along with Dr. Campbell.

During the summer of 2020, a temporary biological technician, Joseph Barnett, was hired to help with refugia work at SMARC. This position was limited to a maximum of 60 days. He supported Refugia program efforts (husbandry and collections), allowing other SMARC staff to focus more time on research.

BUILDING CONSTRUCTION

All major construction at the Uvalde National Fish Hatchery (UNFH) and the San Marcos Aquatic Resources Center (SMARC) had been completed before calendar year 2020. However, some corrections were necessary to achieve sufficient cooling at the UNFH facilities. The heater/chiller units used to control temperature in aquatic holding tanks produced excessive waste heat in the quarantine area. The air conditioning system was unable to overcome this extra heat. Dr. Patricia Duncan, in consultation with USFWS Engineering, decided to install walls to separate the mezzanine (with the heater/chiller units) from the main quarantine space. Costs for this project were incurred by the USFWS. The project was awarded to Firstop, LLC, with project manager Oscar Maltos and project superintendent Raymond Bueno.

A pre-construction conference call was held on May 11, 2020. Ray Fletcher was assigned the role as the USFWS contracting officer. Mark Orton was assigned as Mr. Fletcher's technical representative. Dr. Patricia Duncan took the role as the field inspector for the USFWS. Work began April 1, 2020. The project included installing walls and doors around the mezzanine in the quarantine building where the heater/chiller units had been installed. These walls were installed to prevent waste heat generated from the heater/chiller units from entering the cooled quarantine space. Louvers in the exterior walls would supply fresh air from the outside and a new exhaust fan in the ceiling would remove waste heat from the building. The project was scheduled to be completed by May 18, 2020, but an extension was granted until June 12, 2020, following delays. The work was completed in June and the systems were functioning nominally throughout peak summer temperatures.

Figure 2. The mezzanine level of the quarantine space at the Uvalde National Fish Hatchery, where the heater/chiller units were installed. The top panel shows the room was open to the work area below. The bottom panel shows the room enclosed.

COVERED SPECIES ANALYSIS

Collections of the Covered Species continued this year to achieve standing stock targets as outlined in the Contract and the 2020 Work Plan (Table 4 and Table 5). For many species, the acclimation to captive systems can be achieved relatively quickly; this is particularly true for Texas wild rice, San Marcos fountain darters, and San Marcos salamanders.

After consultation with the EAA staff, our other partners, and experts in the field, we decided to reduce the number of invertebrate collection events and numbers of 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.

Figure 3. Texas blind salamander

Table 4 Number of organisms incorporated in the SMARC Refugia Standing Stock in 2020, 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*		417	601	58%
Fountain darter – Comal *Etheostoma fonticola*		1	172	80%
Comal Springs riffle beetle *Heterelmis comalensis*		345	0	0%
Comal Springs dryopid beetle *Stygoparnus comalensis*		4	1	6%
Peck's cave amphipod *Stygobromus pecki*		281	265	51%
Edwards Aquifer diving beetle *Haideoporus texanus*		0	0	--
Texas troglobitic water slater *Lirceolus smithii*		*	*	*
Texas blind salamander *Eurycea rathbuni*		18	269	95%
San Marcos salamander *Eurycea nana*		19	246	68%
Comal Springs salamander *Eurycea* sp.		59	49	33%
Texas wild rice plants *Zizania texana*		21	174	72%

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 does not include any organisms 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.

*Those previously held in refugia were of a different *Lirceolus* species, so these were disbanded, see Texas troglobitic water slater section for full details.

Table 5 Number of organisms incorporated in the UNFH Refugia Standing Stock in 2020, 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*		0	480	90%
Fountain darter – Comal *Etheostoma fonticola*		0	27	75%
Comal Springs riffle beetle *Heterelmis comalensis*		96	14	11%
Comal Springs dryopid beetle *Stygoparnus comalensis*		0	0	-
Peck's cave amphipod *Stygobromus pecki*		274	322	75%
Edwards Aquifer diving beetle *Haideoporus texanus*		0	0	--
Texas troglobitic water slater *Lirceolus smithii*		0	0	--
Texas blind salamander *Eurycea rathbuni*		0	29	94%
San Marcos salamander *Eurycea nana*		0	246	81%
Comal Springs salamander *Eurycea* sp.		0	49	89%
Texas wild rice plants *Zizania texana*		14	174	94%

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 does not include any organisms 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.

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 2020. High mortality rates for both incoming Comal fountain darters and those in refugia inhibited reaching target goals for Comal 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 Comal River until further studies investigate potential causes of these increased mortalities. We received approval from the EAA to suspend target goals for the Comal fountain darters in the interim. If drought or flow conditions reach critical levels, we will collect Comal darters to increase refugia stocks. Numbers incorporated, end of the year census, and survival rates can be found in Table 6.

Table 6 Fountain darter refugia population figures

		Beginning of Year Census	Incorporated 2020[1]	End of Year Census	Target Goal 2020 Work Plan	Percent Survival
San Marcos River	SMARC	622	417	601	500	58%
	UNFH	533	0	480	500	90%
Comal River	SMARC	213	0	172	*	81%
	UNFH	36	0	27	*	75%

* 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.

COLLECTIONS

SMARC staff collected fountain darters from the San Marcos River and the Comal River in February 2020 for routine testing for *Centrocestus* sp., a trematode parasite. These fountain darters were not included in the counts for our refugia. SMARC staff collected fountain darters from the San Marcos River in August and October, 2020 for the SMARC refugia. In April and October 2020, BIO-WEST Incorporated donated fountain darters from the San

Marcos River, collected during their biomonitoring activities.

Figure 4. Linda Moon, Kelsey Anderson, and Lindsay Campbell collecting fountain darters in the San Marcos River

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 2020. 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 our Southwestern Fish Health Unit (SFHU), in Dexter, New Mexico, for routine parasitology and health screening before the larger group of collected fish were incorporated into the refugia.

SURVIVAL RATES

Last year, 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 fountain darters since collections were restarted in 2017 after Comal fountain darters were found to test positive for Large Mouth Bass Virus (LMBV). Given the past history of low intake survival rates, we suspended collections of Comal fountain darters in the fall of 2019. We only collected fountain darters from the Comal River in 2020 for parasitic testing.

LMBV negative Comal fountain darters, collected in 2016, have high survivorship and did not exhibit symptoms or mortalities of Comal 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.

In 2020, SMARC staff conducted a pilot LMBV exposure study. To investigate if LMBV could be causing the health issues, we proposed to compare mortality between groups of healthy captive bred fountain darters exposed to fish positive for LMBV (LMBV+) with groups of healthy captive bred fountain darters exposed to healthy fish (LMBV-). We hypothesize that otherwise healthy darters, when exposed to LMBV, will show higher mortality rates compared to control fish not exposed to LMBV. This pilot study could provide evidence for LMBV as the causative agent for increased mortality seen in fountain darters from the Comal River. We hope that this will be the first step in investigation of the high mortality rates. In addition to this research, we will also consult with USFWS veterinarians on potential treatments (not already tried) to reduce incoming mortality rates.

The EAA approved this line of investigation to be housed under Task 1 in our 2020 Work Plan. This research is ongoing and a final report will be prepared in 2021.

The overall survival rates for San Marcos fountain darters in refugia at SMARC was 58% and the survival rate for Comal fountain darters was 81% for 2020. The overall survival of fountain darters at UNFH for the year was 90% for San Marcos fountain darters and 75% for Comal fountain darters.

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, then 24-hour static baths of 0.5% sea salt and/or 15–20 ppm formalin were 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 °C (ranging between 19–22 °C). 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 and live black worms.

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. Water lines, hoses, valves, and restrictors were frequently checked for wear and clogs and were cleared, rebuilt, or replaced as needed.

CAPTIVE PROPAGATION

Limited space and activities surrounding setting up the new facilities prevented efforts to produce captive offspring of either San Marcos River or Comal River fountain darters at either facility during 2019. Generally, fountain darters in captivity lay eggs on the undersides of PVC and other habitat structures placed in the tanks. If offspring were not desired, staff removed the structures and disposed of the eggs. F1 generations were separated based on the river system from which their parents originated. Egg production was opportunistic and not controlled or directed by staff during periods when offspring were not needed for research or for reintroduction. A captive propagation plan is on file and available upon request for fountain darters.

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

Comal Spring riffle beetle collections for standing and refugia stocks occurred four times in 2020 (January, February, July, and December) from a variety of 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). No specific spring orifice was sampled two times in a row. All riffle beetle adults and larvae were collected from the lures (Table 7). Standing stock numbers were reduced to 75 per station until propagation methods are refined and better knowledge of population numbers and meaningful standing stock numbers are derived. 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 2020	End of Year Census	In Quarantine End of Year	Target Goal 2020 Work Plan	Percent Survival
SMARC	87	345	0	133	#	0%
UNFH	100	96	14	41	#	7%

for 2020 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 15, 2020, staff collected lures with Comal Springs riffle beetles (CSRB) by hand at Spring Run 3. A total of 198 CSRB were collected, with 100 returned to UNFH and 98 returned to the SMARC for the refugia populations. On February 13, 2020, SMARC staff collected 275 CSRB on cotton lures at Spring Run 3 and retain 274 for the SMARC refugia. Some of these (72) were used for nutrition experiments. In June, SMARC staff placed additional cotton lures on the western shoreline of Landa Lake. These lures were retrieved in July, 2020 and 17 CSRB were retained for the SMARC refugia. The last collection event for CSRB was on December 3, 2020. A total of 104 CSRB were collected. Sixty-three of these were retained for the SMARC and 41 were sent to UNFH for their refugia population.

QUARANTINE

Incoming CSRB were quarantined in separate systems than the existing refugia population in the Invertebrate Refugia area at SMARC or the quarantine room at 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 in order to observe survival rates over time.

Figure 5. Lindsay Campbell and Adam Daw sorting Comal Springs riffle beetles

SURVIVAL RATES

The riffle beetles at SMARC and UNFH were collected in 2019 and January, February, July, and December 2020. Because CSRB have an average life span of approximately a year, and adults of unknown age are collected from the field, high mortality rates are expected, as it is possible the high mortality rate is driven by natural senescence. Historically, about half of CSRB collected perish by sixth months in captivity. The small size of CSRB makes it difficult to assess for mortality on a day-to-day basis. Mortalities are therefore calculated as inventories were conducted, where the number of dead or missing CSRB equates to the number of mortalities for that time-period. We observed greater than typical mortality between April and August 2020. A careful assessment of our holding chambers revealed high levels of calcification restricting flow. These systems were cleaned and reset in August.

HUSBANDRY

All systems were evaluated daily for water temperature, adequate flow, and clear drain screens to maintain drainage and water level. CSRB refugia systems were not siphoned because adults, larvae, or eggs could easily be discarded along with debris. As CSRB feed predominantly on biofilm, we did not follow a traditional feeding schedule. Alternatively, leaves and cotton cloth containing biofilm were used in each system, providing food. Inventories were conducted every other month and new leaf and cotton material was added as needed. Conditioned wood was incorporated into refugia containers.

Culture boxes used to house CSRB were square 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 were kept dark through painting the outsides of the boxes, wrapping in shade cloth, or transitioning to new boxes constructed of opaque black plastic. Vertical flow through tubes were also used; these consisted of clear PVC that made up a viewing chamber, and threaded PVC couplings and reducers. Both types of 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

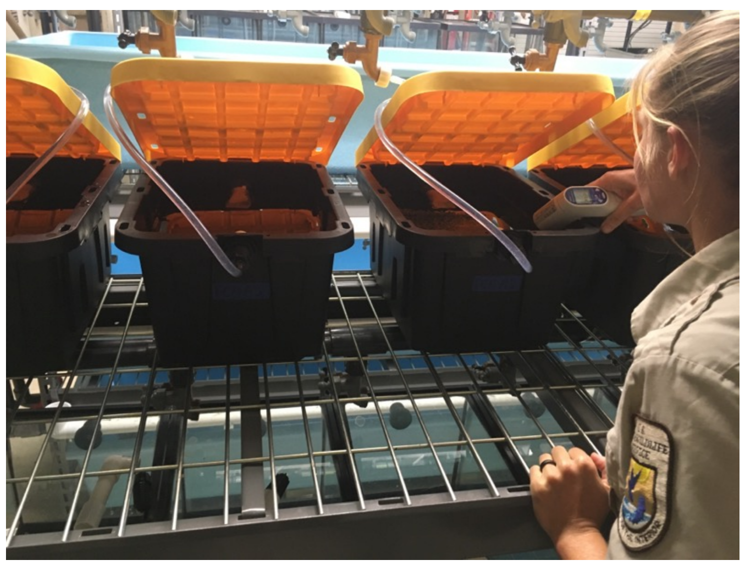


Figure 6. Makayla Blake checking temperature in an invertebrate holding chamber

cleaned during inventory. At this time, staff checked water lines, hoses, and valves for functionality and cleaned or replaced them as needed. Horizontal PVC tubes with air space worked best for producing adults from larvae; historically they were aligned vertically. Air space and emergent structure is already given 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 that were found were placed into a separate container from wild stock adults. These larvae were provided to our research partners for their research.

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 2020	End of Year Census	In Quarantine End of Year	Target Goal 2020 Work Plan	Percent Survival
SMARC	12	4	12	0	*	75%
UNFH	1	0	0	0	*	0%

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

COLLECTIONS

In 2020, sampling events occurred for CSDB in the Comal Springs system at Spring Runs 1 and 3 and Landa Lake by setting poplar wooden dowels adjacent to poly-cotton lures near spring orifices in order to attract CSDB. We collected six CSDB in September, two in October, and one in December 2020. All of these were retained for the SMARC refugia population.

Figure 7. Comal Springs dryopid beetle

QUARANTINE

Incoming CSDB were quarantined in the Invertebrate Refugia area at the SMARC. CSDB were acclimated to quarantine water conditions at a rate not to exceed 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, 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

We used square plastic containers as culture boxes for CSDB. We fitted each of these 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 underground environment. All the systems were checked daily for appropriate water temperature, adequate flow, and clear drain screens to maintain drainage and water level. Conditioned wooden dowels in the containers were checked for fungal growth, and if found were removed; CSDB may become entrapped in fungus and parish. 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

To encourage production of offspring, male and female wild stock were housed together. As per our container design, each container had a portion of rock, leaf, and wood habitat above the waterline because dryopid beetles are known to lay eggs and have larvae that need moist, terrestrial habitat.

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

We collected Peck's cave amphipods (PCA) from Comal Springs by five hand collection events in 2020 and received PCA caught in drift nets during biomonitoring activities. We conducted formal experiment testing different container habitat configurations, which led to increased survival in 2020, especially at UNFH. 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	241	277	265	250	51%
UNFH	157	274	322	250	75%

COLLECTIONS

We conducted five collection events in 2020 for Peck's cave amphipods (PCA). SMARC staff collected PCA in January 2020 around Spring Island in the Comal River, New Braunfels, TX. A total of 97 PCA were returned to UNFH for a habitat density study. In May

2020 SMARC biologist Randy Gibson donated 52 PCA to the SMARC refugia program. These were collected during biomonitoring work conducted in Comal Springs, New Braunfels, TX. Additional PCA were collected in June 2020, by the refugia staff for the habitat density study at UNFH, and in July for the refugia populations at the SMARC and UNFH. UNFH staff collected PCA from the Comal River in November 2020. UNFH and SMARC together collected PCA in December 2020 from Spring Run 3 in the Comal River.

Figure 8. Makayla Blake sorting Peck's cave amphipods at Comal Springs

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 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 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 of the 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 potentially 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.

Biological technician Makayla Blake of UNFH conducted a pilot study in March 2019 with different densities of Matala® biofilter media to simulate interstitial spaces in PCA holding containers. A full study was implemented in 2020. This study provided evidence that the use of medium density biofilter material resulted in an approximately 90% survival of captive PCA. Technicians at both stations incorporated the new media within all PCA holding containers in 2020.

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). With this knowledge, Refugia biologist conducted research on PCA preference of different live food items and the feasibility of scaling up these items to holding containers.

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.

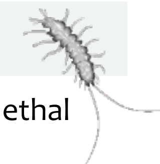
CAPTIVE PROPAGATION

When counting PCA from refugia containers during inventory, each amphipod was carefully observed for brooding. PCA females hold their eggs and young in a brood pouch under the body. Research in 2020 evaluated new designs for PCA brooding chambers. At SMARC, gravid females when observed, were noted and placed back into refugia wild stock. PCA juveniles would easily be 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 2020. 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 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 for over a month. This evidence suggests that *L. smithii* are a deep aquifer species, like the Edwards Aquifer diving beetle, that are rarely found in surface waters; those that are found have likely suffered physical damage during the distance traveled to the surface.

We held no *L. smithii* in refugia in 2020. In the future, if *L. smithii* are collected from Texas Sate Artesian Well, we have documented husbandry procedures to use 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. We propose genetic testing and transferring some of this age class to UNFH to discourage inbreeding in the refugia.

No Texas blind salamanders were transported or donated during 2020. Three individuals previously excluded and from unknown collection origin were included in our overall refugia count. Numbers incorporated, end of the year census, and survival rates can be found in Table 10.

Table 10 Texas blind salamander refugia population figures

	Beginning of Year Census	Incorporated 2020	End of Year Census	In Quarantine End of Year	Target Goal 2020 Work Plan	Percent Survival
SMARC	264	20	269	0	250	94%
UNFH	31	0	29	0	40	94%

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 oversampling, only one third of salamanders observed were retained for refugia from these sites. Any gravid females were retained due to their rarity; and three gravid females were collected while trapping in 2020. Sampling was largely discontinued in mid-March due to COVID-related closures and safety precautions.

Rattlesnake Cave and Well were sampled in January 2020 and not again until October 2020, when only the well was sampled due to the confined nature of cave-work and very low water levels. In January, a ring-tailed cat entered the covered well and perished within. Staff were sampling at the time and removed the animal. In August, a squirrel had drowned in the well before sampling started, increasing ammonia to toxic levels that did not decrease during sampling. In January, Rattlesnake Cave had abundant water for sampling. Many more crayfish were collected than has been typical, and one large crayfish was captured in a trap with a salamander that was lethally injured.

Figure 9. Kelsey Anderson holding a Texas blind salamander

Primers Fissure and Johnson's Well were both sampled in February, August, and November 2020, as these sites can be accessed while easily maintaining social distancing procedures. Video was collected in August from both sites showing high counts of Texas blind salamanders present (four or more individuals each visit) and troglobitic leeches present in the well. Both sites continued to be highly productive even as water levels decreased throughout the year. These sites were trapped for three weeks instead of two in both January and October to compensate for missed trapping opportunities. 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 2020, we retrieved one live juvenile from (TL = 15 mm) from Diversion Springs net. The collection cup was removed in mid-March as Texas State University, Spring Lake, and the Meadows Center closed for safety concerns related to the Covid-19 pandemic. As installing and checking this net and collection cup requires multiple individuals working in close proximity, the site was not sampled again in 2020. In November, the entire netting was removed by divers and returned to station where it was deep-cleaned and repaired for future deployment.

Texas State University personnel had nets on Sessom Creek and Artesian Well for their own uses during 2020, but they collected no animals. The net and collection cup for Artesian Well was redesigned in February to increase salamander survival during times of high velocity. Our collection site at Sessom Creek ceased flow during 2020 and could not be sampled.

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 larval 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 reside 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 *Euycea* species (*E. wilderae*) (Martel et al 2014). In 2019, the SMARC began running in-house tests for chytrid fungi, which reduces costs and decreases the time that animals remain in quarantine before joining refugia systems. Texas blind salamanders were housed in quarantine according to their collection location, collection date, and size. Individuals remained in quarantine for 30 days under observation before being incorporated towards Standing Stock numbers.

SURVIVAL RATES

After their 30 day quarantine period, organisms were incorporated into the refugia standing stock numbers. Overall survival of all Texas blind salamanders at SMARC and UNFH was 94% in 2020. 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 VIE tags so that collection origin was known. We are awaiting a report of the population genetic structure from the USFWS Genetics Unit and will separate salamanders by location if differences are found. Texas blind salamanders were housed in large insulated fiberglass systems at the SMARC with either flow-through or partial recirculation tanks.

At UNFH, salamanders were held in large insulated fiberglass tanks in Refugia on partial recirculation through heater-chiller units to maintain the water temperature at 21 °C. Water temperature and flow were checked multiple times daily. Total gas pressure was checked immediately if salamanders begin showing symptoms of gas bubble disease, including the presence of trapped air bubbles underneath the skin, bloating, or an inability to stay submerged. Water quality parameters including, but not limited to, dissolved oxygen, pH, and total gas pressure, were checked weekly.

Habitat enrichment items, including natural and artificial rock, plastic plants, and mesh were placed throughout the tanks for salamanders to explore and in which to seek refuge. Staff routinely siphoned tanks to remove waste and other debris and replaced habitat items with clean ones. Each tank system had dedicated equipment (nets, cleaning supplies) to prevent the potential spread of pathogens from system to system. If equipment was ever shared, it was cleaned and disinfected between systems. Upon reaching 30 to 40 mm in TL, juveniles were marked with visible implant elastomer (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 us to quickly identify individuals so that we can access sex, collection location, and year of collection.

Adult salamanders were fed twice weekly and received either live amphipods or blackworms. Juveniles were fed *Artemia* spp. nauplii or chopped blackworms as they increased in size. Blackworms were phased out for salamander feeding at the SMARC in 2019 after discovering high barium levels in this food item. Potential deleterious impacts of high barium levels are being investigated. Staff cultured alternative food sources, including composting worms and daphnia. Composting worms were fed whole or chopped to sub and mature adults while daphnia were fed to all size classes. Blackworms were not phased out at UNFH. Starting in November at UNFH, frozen bloodworms (midge larvae) and enriched adult *Artemia* spp. were also fed three times weekly (Monday, Wednesday, Friday) as a supplemental food source to train the salamanders to eat frozen feed in case blackworms become unavailable. A detailed description of Texas blind salamander daily care can be found in the USFWS Captive Propagation Manual for *Eurycea* spp., available upon request.

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; thus, these offspring may not be used for restocking purposes. If future genetic analysis shows that collection locations are part of one panmictic population, then these offspring could be used should a restocking event occur.

As the 2019 tagging study was completed, staff noticed several newly mature and older females all were increasing with egg masses. Dr. Campbell and Kelsey Anderson combined these females with unfamiliar males into a new system on recirculating water to retain hormones. Three clutches were produced within one month of strategic combination. Following this success and the presence of still late-stage gravid females, we tested the efficacy of luteinizing hormone releasing hormone (LHRH) on the species. With the advice of veterinarian and amphibian reproduction expert Dr. Ruth Marcec-Greaves, both male and female salamanders were held in a damp dipnet while LHRH was topically applied to the nose, face, and down the upper back. Following this application, 11 more clutches were produced within 7 months. Further, we noted that female salamanders that had deposited eggs began generating new eggs faster than previously documented. After 6 months, male salamanders were removed from the system. In total, Texas blind salamanders at the SMARC produced 16 clutches of eggs in 2020, 14 of which were produced by those females selected for the reproduction trial and 11 of those produced after hormone application. Clutch data is reported in (Table 11). As we are over-capacity with juvenile F1 salamanders, the two clutches produced in December 2020 were donated to Ruben Tovar and Dr. Tom Devitt at University of Texas for ongoing ocular development research. At the end of 2020, SMARC held 152 F1 individuals, 129 of which were produced during 2020.

Figure 10. Texas blind salamander embryo

Table 11. Individual clutches produced by Texas blind salamanders at the SMARC during 2020

Date	Parent Generation	Offspring Generation	# Deposited	# Hatched	(%) Survival	Comments	# Survived 3 months post-hatch
3/20/20	WS	F1	28	10	35.7		8
3/25/20	WS	F1	52	23	44.2		14
3/27/20	WS	F1	11	2	18.2		2
April 2020 *	WS	F1	35	0	0	None developed	-
May 2020 *	WS	F1	36	17	47.2		16
June 2020 *	WS	F1	32	7	21.9		7
7/11/2020 *	WS	F1	43	23	53.5		17
7/14/20 *	WS	F1	24	5	20.8		4
7/20/20 *	WS	F1	28	2	7.1	slow growth	1
7/26/20*	WS	F1	23	10	43.5		8
7/28/20*	WS	F1	28	12	42.9		10
8/15/20*	WS	F1	17	11	64.5		10
8/24/2020*	WS	F1	26	22	84.6		22
9/7/2020*	WS	F1	21	13	61.9		13
12/29/20	WS	F1	64	+	+		+
12/30/20	WS	F1	17	+	+		+

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 produced from the LHRH trial within 7-months of administration
+Clutches have not yet hatched

SAN MARCOS SALAMANDER (*EURYCEA NANA*), THREATENED

The Standing Stock goal for the San Marcos salamander is 500 individuals, divided between the two facilities. We remained above the goal for nearly all of 2020. Very few San Marcos salamanders were collected in 2020. Typically, we collect San Marcos salamanders twice each year in amounts sufficient to cover the expected loss given average mortality. Without new animals incorporated, mortality rates were lower in 2020 (60%) than the 20-year average (73%). Staff collected 33 adults from below Spring Lake Dam in December, but these animals will not be incorporated until January 2021; thus, these are excluded from overall count and survival calculations. We also continued research on San Marcos salamander reproduction with the aim to produce offspring on demand (see Research section for more details). Numbers incorporated, end of the year census, and survival rates can be found in Table 12.

Table 12 San Marcos salamander refugia population figures

	Beginning of Year Census	Incorporated 2020	End of Year Census	In Quarantine End of Year	Target Goal 2020 Work Plan	Percent Survival
SMARC	343	38	226	33	250	60%*
UNFH	305	0	246	0	250	81%

**Survival reported excludes 6 salamanders transferred to Washington Animal Disease Diagnostic Laboratory.*

COLLECTIONS

San Marcos salamanders were collected in March from Diversion Springs and by snorkelers below the falls in December. No sampling was conducted by USFWS SCUBA divers in Spring Lake during 2020 (see Figure 11 for locations).

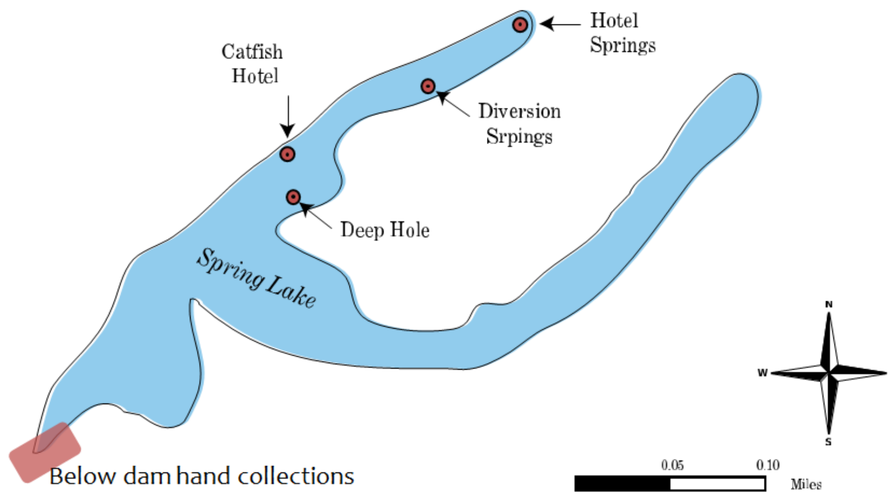


Figure 11 Locations of San Marcos salamander collections in Spring Lake, San Marcos, TX.

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 Diversion Spring net were transported back to 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 counted towards Standing Stock numbers.

SURVIVAL RATES

The cases of egg-related mortality continue to decline, but were still found in refugia populations at both facilities. At SMARC, there was a marked difference in survivor rates between San Marcos salamanders that were collected between fall 2017 to present compared to those collected before fall 2017 (what we call the "heritage group"). Most of these older salamanders were already at the facility before the new Refugia Program started in 2017. Salamanders collected between fall 2017 to present have not been mixed with the heritage salamanders in tanks or shared water systems. As of December 2020, only six individuals from this heritage group remain at the SMARC. Individuals from other younger populations (2017 and 2018) are beginning to show similar issues as the heritage group; therefore, it may be likely that reproductive-related death increases in probability with increasing animal age and/or time in captivity.

HUSBANDRY

Genetic analysis (Lucas *et al*. 2009) determined that there was no population structure within this species between the sites sampled in the wild, so individuals from all collection locations were combined. At SMARC, individuals were marked with a VIE tag on the right side, posterior to the hip to indicate the year collected and on the left side, posterior to the hip to indicate the sex of the individual.

San Marcos salamanders at both facilities were housed in large insulated fiberglass systems with either flow-through chilled well water (SMARC) or partial recirculation through heater-chiller units (UNFH) to maintain water temperature at 21 ±1 °C. Smaller glass tanks were placed on these systems if needed. 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 or blackworms. Juveniles were fed *Artemia* spp. nauplii or chopped blackworms as they increased in size. Blackworms were phased out for salamander feeding at SMARC in 2019 after discovering high barium levels in this food item. Potential deleterious impacts of high barium levels are being investigated. Staff cultured alternative food sources, including composting worms and daphnia. Composting worms were fed whole or chopped to sub and mature adults while daphnia were fed to all size classes. Blackworms were not phased out at UNFH. Starting in November at UNFH, frozen bloodworms (Chironomid midge larvae) and enriched adult *Artemia* spp. were also fed three times weekly (Monday, Wednesday, Friday) as a supplemental food source to train the salamanders to eat frozen feed in case blackworms become unavailable. A detailed description of salamander care can be found in the USFWS Captive Propagation Manual for *Eurycea* spp., available upon request.

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

During 2020, we scaled up the San Marcos salamander reproduction trials at the SMARC. With the advice and assistance of licensed veterinarian and amphibian reproduction expert Dr. Marcec-Greaves, Director of the National Amphibian Conservation Center of the Detroit Zoological Society, we also tested the safeness and efficacy of LHRH hormone. During the first trial, males and females from both sex-segregated groups and mixed groups were tested to see if sex-segregation would increase production. Concurrently, groups had either both sexes or just the males dosed with LHRH. It was seen from past courtship footage that males were not engaging in courtship as readily as females and, if courting, were having difficulty dropping spermatophores. Finally, the heritage population was tested against the nonheritage group. From this trial, four clutches were produced, all to the nonheritage population and all from groups where the males and females had been in sex-segregated systems for one month prior. One of these clutches was nonviable and all

Figure 12. Rachel Wirick applying a reproductive hormone to a salamander held by Linda Moon, as veterinarian scientist Dr. Mercec-Greaves observes

were small in number. Ultimately from this trial we concluded that recirculation systems, segregating the sexes, and age were vital for encouraging San Marcos salamander reproductive behavior and the production of offspring. Unfortunately, the amount of reproductive behavior and egg production resulting from these practices was still below the level we desired for large-scale production.

After this trial ended, all available adult San Marcos salamander wildstock were moved into four male or female-only raceways in the SMARC refugia. They remained isolated for three months before being combined again. In two of the four raceways, males received hormone while the other two did not. Raceways contained 50 animals at a 1:1 sex ratio and remained in these systems for six months (February 2021). As of December, six clutches were produced: three to groups that had not received hormone and three with the aid of LHRH. These offspring were all donated to Ruben Tovar and Dr. Tom Devitt at University of Texas for ongoing ocular developmental research. More testing and work with veterinarians is needed in the future to aid in the low production. For example, hormone dose may need to be examined. Females may need to receive hormone as well. A three-month total isolation phase may be too long. In this second trial there was no (phase II) shared water stage. As a result, it remains unclear if this stage plays an essential role in preparing the sexes for courtship.

Ultimately, San Marcos salamander egg production may remain low regardless of the methods used. It may be part of the species' biology. Unlike with Texas blind salamanders, it is more difficult to see and observe individual female San Marcos salamanders for peak gravidity, which may help us improve timing reproductive stimulation. This species appears to become stressed when handled or working in and around them. As a result, normal reproductive behaviors may be disrupted. Presumptively, reproduction efforts should be tried once or twice per year when offspring have been observed in an attempt to produce eggs in captivity (winter to early spring). Production from this second trial is promising in that it resulted in above-normal production, utilizing all available adults. However, more research is necessary to further define stages or processes that are crucial in maximizing reproductive output.

Refugia research activities in 2019 found high barium levels in an analysis of captive and wild individuals, with higher barium concentrations increasing with duration in captivity (see Research section for full details of report). While the exact impact of high barium levels is still being investigated for this species, in other species (toads) it can cause muscular weakening and can impact reproduction (Hopkins et al. 1997). Animals were re-tested in 2020. We sent six gravid females from both heritage and non-heritage populations for testing. Barium levels had not decreased even after phasing out barium-rich blackworms from the diet.

At the SMARC in 2020, wild stock salamanders produced thirteen clutches. At UNFH, a wild stock salamander deposited a clutch of 29 eggs in May, 21 hatched. At the end of 2020, SMARC had 88 San Marcos salamander offspring, almost all of which were small juveniles. UNFH held 19 F1 salamanders.

Table 13. Clutches of San Marcos salamanders

Date	Parent Generation	Offspring Generation	Eggs Deposited	# Hatched	(%) Survival
1/13/20	WS (Show tank)	F1	24	23	95.8
3/2/2020	WS (show tank)	F1	27	20	74.1
3/4/20	WS	F1	8	4	50
3/10/20	WS	F1	20	2	10
4/15/20	WS	F1	18	8	44.4
4/16/20	WS	F1	28	21	75
4/24/20	WS	F1	10	7	70
10/19/20 *	WS	F1	12	+	+
10/21/20	WS	F1	12	+	+
10/31/20 *	WS	F1	16	+	+
12/15/20 *	WS	F1	11	+	+
12/19/20	WS	F1	33	+	+
12/21/20	WS	F1	20	+	+

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 produced by groups where at least some individuals received LHRH
+Clutches donated to University of Texas.

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

The Comal Springs salamander is one of the species covered in the Edwards Aquifer Habitat Conservation Plan (EAHCP), when it was known 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. (2017) evaluated genetic markers and considered *Euycea* 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 our contract with the EAA, we will consider the Comal Springs population of *E. pterophila* as the Comal Springs salamander, and 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. Collections to augment the refugia population of Comal 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 our 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 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 2020	End of Year Census	In Quarantine End of Year	Target Goal 2020 Work Plan	Percent Survival
SMARC	88	60	122	0	115	82%
UNFH	55	5	49	0	80	82%

COLLECTIONS

USFWS staff snorkeled to collect adult Comal Springs salamanders using dip nets around the Spring Island area of Landa Lake in January, May, and October 2020. Four juveniles were collected by Randy Gibson while driftnetting and transferred to the SMARC. 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. Each salamander's TL was recorded and gravidity noted, if present. Staff then ran a cotton swab (in duplicate) down the ventral side of the salamander and around the limbs to collect material for Chytrid fungus testing. The swab tips were placed into pre-labeled centrifuge vials and were stored in a freezer until they were processed to test for two types of Chytrid fungus, *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal). Salamanders were placed into transport coolers with mesh onto which the salamanders could hold. Gravid females were kept separately in a small transport cooler. Before coolers were loaded for transport, water was replaced, and temperature was recorded.

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. 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

Overall, survival rates of Comal Springs salamanders were similar in 2020 to past years. Mortalities of this species tend to be attributed to escape from their tanks. Refugia staff continue to iteratively modify the Comal Springs salamander tanks to prevent escape. Many designs were tried in 2019 and 2020 with the current best-method containment unit being made of heavy channel supporting one-half hinge lid of heavy plexiglass and one-half very fine mesh to allow gas exchange. All edges are adhered to the tank with Velcro. Ample habitat enrichment, coupled with lower water depth than in the other salamander species, reduced escapement. Water trails are avoided; however, the animals have been seen on video monitoring climbing on and up dry surfaces.

HUSBANDRY

At the SMARC, individuals were marked with a VIE tag on the right-side, posterior to the hip, to indicate the year collected and on the left side posterior to the hip to indicate the sex of the individual. Comal Springs salamanders at both facilities were housed in large insulated fiberglass systems with partial recirculation through heater-chiller units to maintain the water temperature at 21 °C (ranging between 19 to 22 °C). Smaller glass tanks were placed on these systems as needed. 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 or blackworms. Juveniles were fed *Artemia* spp. nauplii or chopped blackworms as they increased in size. Blackworms were phased out for salamander feeding at SMARC in 2019 after discovering high barium levels in this food item. Potential deleterious impacts of high barium levels are being investigated. Staff cultured alternative food sources, including composting worms and daphnia. Composting worms were fed whole or chopped to sub and mature adults while daphnia were fed to all size classes. Blackworms were not phased out at UNFH. A detailed description of salamander care can be found in the USFWS Captive Propagation Manual for *Eurycea* spp., available upon request.

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 2020, 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. Wild salamanders produced nine clutches at the SMARC during 2020 and offspring produced one clutch (Table 15). At the end of 2020, the SMARC held 47 F1 salamanders and UNFH held 10 F1 salamanders.

Table 15. Propagation of Comal Springs salamanders

Date	*Parent Generation*	*Offspring Generation*	*# Deposited*	*# Hatched*	*(%) Survival*	*Comments*
1/5/2020	WS	F1	10	1	10%	
1/20/2020	WS	F1	7	6	86%	
4/5/2020	WS	F1	13	4	31%	
4/9/2020	F1	F2	4	0	-	Eggs were Non viable
5/9/2020	WS	F1	16	16	100%	
7/21/20	WS	F1	18	5	28%	
8/18/20	WS	F1	22	2	9%	
8/24/20	WS	F1	17	13	77%	
9/16/20	WS	F1	12	9	75%	September clutches
9/23/20	WS	F1	10	6	60%	Combined
11/28/2020	F1	F2	10	0	-	Eggs were Non-viable

TEXAS WILD RICE (*ZIZANIA TEXANA*), ENDANGERED

The Standing Stock goal for Texas wild rice (TWR) is 430 plants divided between the two facilities. Native habitat for Texas wild rice is divided into alphabetical sections of the San Marcos River, determined by Texas Parks and Wildlife. Texas Parks and Wildlife categorizes TWR in alphabetical (A–K) sections 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 plus 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 separate plants are genetically identical, thus calling for the combining or removal of one of the clones and collection from a genetically different wild plant. The Refugia Program wishes 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. SMARC staff specifically targeted plant stands that were not currently represented in the refugia population. Plant stands were selected after overlaying refugia plant locations (determined with GPS) onto GIS maps produced by the SMARC Plant Ecology Program during their last (2019) annual Texas Wild Rice Survey. UNFH staff concentrated on maintaining their refugia population numbers and representative locations. Numbers incorporated, end of the year census, and survival rates can be found in Table 16.

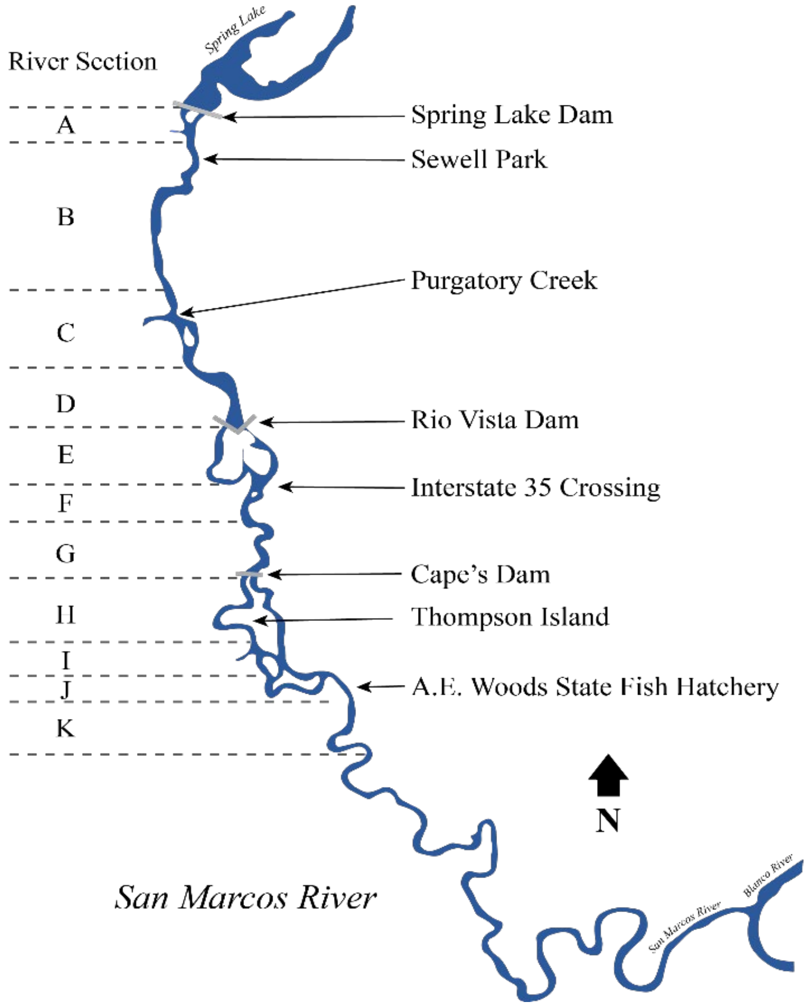


Figure 13. Lettered sections of the San Marcos River designating Texas wild rice habitat established by Texas Parks and Wildlife Department

Table 16 Texas wild rice refugia population figures

	Beginning of Year Census	Incorporated 2020	End of Year Census	In Quarantine End of Year	Target Goal 2020 Work Plan	Percent Survival
SMARC	211	20	206	13	215	89%
UNFH	157	28	174	0	215	94%

COLLECTIONS

Tiller collections in the San Marcos River occurred in February, October, and December 2020. USFWS SCUBA divers or snorkelers 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.

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 at SMARC, but continue at UNFH. Tillers from each plant were potted together in a tagged pot and placed in a quarantine raceway tank for 30-days. During this time, they were routinely checked for aquatic nuisance species, specifically the invasive snail *Melanoides tuberculata.* After 30 days, plants at SMARC 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. At the SMARC, 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 fitted with a small chiller and pump that increases flow velocity. This method reduced the chances of anoxia to roots while in quarantine and the amount of soil discarded after the quarantine period (soil was not reused). At UNFH, after the 30 day quarantine, and once the tillers have taken firm root in the pots, plants were visually inspected again for aquatic nuisance species. The plants were not repotted before being incorporated into the standing stock population and combined into refugia tanks from the same river section.

SURVIVAL RATES

Overall survival rate of TWR plants at the SMARC was 89%, with older plants more likely to succumb to mortality. The overall survival rate of TWR plants at UNFH was 94%. 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. The oldest living plant on station (SMARC), based on records, is 5.4 years from Section A of the San Marcos River.

HUSBANDRY

We continued to investigate different soil and potting techniques for TWR plants at the SMARC. 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. Additional ¾ to 1 HP submersible pumps were added to the UNFH Refugia and flow bars were removed.

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 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 structures at UNFH were installed, and welded to prevent displacement by wind, above the UNFH refugia tanks. This construction was delayed by the Covid-19 pandemic but has been completed. Shade cloth will be affixed to during summer months starting in 2021.

CAPTIVE PROPAGATION

The Refugia Program was not engaged in propagation of TWR by sexual reproduction through seed production in 2020. However, the Plant Ecology and Restoration Program at the SMARC engaged in TWR plant propagation and continues to study and refine techniques.

Figure 14. 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 in 2020. Much of this research was built on knowledge gained in previous studies. We also continued to investigate reproductive dysfunction in San Marcos Salamanders. Below are summaries for each project approved within the 2020 Work Plan.

INCREASING SURVIVAL RATES OF PECK'S CAVE AMPHIPOD ADULTS AND F1 OFFSPRING

This research had three parts. The first part evaulated survival under three different denisties of filtration media as habitat substrate for PCA. The second part of this research evaluated whether PCA would feed on three different prey items, including *Daphnia* sp., *Lirceolus* sp., and *Hyalella* sp. as alternatives to their standard diet. The third part of this research evaluated brooding chamber protoypes designed to separate adult PCA from their offspring to prevent canabalism.

Historically, captive PCA have suffered from low survival rates in captivity. Increasing survival rates of captive PCA is fundamental to building a fully functional refugium for the species. A pilot study in 2019 revealed that adding filtration material as habitat substrate improved PCA survival (from 35% to approximately 90% survival). This research expanded on that study by comparing PCA survival in three different densities of filtration material: low, medium, and mixed (50% low and 50% medium density). PCA held with medium-density Matala® filtration media were associated with the highest survival in captivity (80%). Amphipods held in mixed-density media (half low-density media and half medium-density media) had intermediate survival (77%), and survival of amphipods held in low-density was lowest (62%). We have updated our standard operating procedures to include medium density filtration material in all PCA holding chambers.

Our small-scale diet experiment provided evidence that PCA will consume *Daphnia* sp., *Lirceolus* sp., and *Hyalella* sp. However, we found that *Daphnia* sp. is problematic as a food source for PCA because of its own nutritional requirements. We were unable to keep *Daphnia* sp. alive for more than 5 days after they were introduced into PCA holding chambers. A full-scale experiment comparing PCA survival under diets of *Lirceolus* sp. and *Hyalella* sp. versus a control of standard PCA is warranted as a follow up to this study. Since studies by Nair (2019) indicate PCA are carnivorous. We would suggest adding in *Lirceolus* sp. and *Hyalella* sp. (once a month on different weeks) as a supplement to the normal flake slurry.

We had insufficient gravid female PCA to fully test the two prototype brooding chambers. The limited testing that we were able to perform resulted in no surviving PCA offspring. PCA will eat their young if given the opportunity. Our results suggest that offspring were consumed by adults. We were able to learn that the adult exclusion barrier was unsuccessful and needs to be improved. A full redesign of this experiment is warranted if this research is to be further pursued.

CONTINUATION OF INCREASING SURVIVAL RATES OF COMAL SPRINGS DRYOPID BEETLES IN CAPTIVITY – DR. ELY KOSNICKI, BIO-WEST, INCORPORATED

Due to the long life cycle of Comal Springs dryopid beetles, BIO-WEST, Incorporated proposed to continued following larvae, adult fecundity, and life cycle of this species. This also will further explore holding containers that are optimized so that different life stages will not have to be moved by refugia staff. Disturbance is thought to inhibit survival in this species. This research is ongoing. At the end of 2020, BIO-WEST, Incorporated were still in the phase of designing and building holding chambers for Comal Springs dryopid beetles.

CONTINUATION OF SAN MARCOS SALAMANDER REPRODUCTION

Our objective in 2020 was to investigate questions raised from past years' research on reproduction and to continue to evaluate different techniques to induce reproduction in San Marcos salamanders. 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. We tested the use of Luteinizing Hormone Releasing Hormone (LHRH) in a pilot study, to be scaled-up to full experiment if successful. We offered food sources, other than blackworms (known to have high levels of barium) to reduce barium levels in salamanders. Lastly, we consulted our regional veterinarian and other animal health professionals on a broad spectrum of health tests.

No deleterious effects from LHRH were observed in a pilot experiment, so we proceeded to a large-scale experiment using all mature captive wildstock *E. nana*. LHRH was applied at 50 µg/g only to males, as they initiate courtship in this species by producing pheromones in their mental glands and rubbing them on the females to induce receptivity. The treatment groups were randomly assigned to each of the four tanks: two tanks where males were dosed with LHRH and two control tanks where males were not dosed. Groups of 25 females were selected without bias and distributed into the four tanks. As of November 2020, we have not observed any increase in clutches laid after the application of LHRH. We will continue to monitor these salamanders into 2021.

Figure 15. Mark Yost (right) administering a dose of hormone to a San Marcos salamander held by Rachel Wirick (left) while Dr. Mercec-Greaves (center) observes

After one year without blackworms as a food source, we sent an additional three female individuals from our heritage population of 3+ year-old captives and three females from the standing stock population (that were not in the scaled-up LHRH experiment) for heavy metal testing. There was no decrease in barium levels in captive salamanders tested in 2020 versus those tested in 2019, despite the elimination of blackworms as a food source.

Dr. Allen Pessier noted Mycobacteriosis in both short- and long-term captive salamanders. Dr. Pessier informed us that these lesions could be ascending infections from the environment via the cloaca/reproductive tract or by introduction of environmental bacteria via skin punctures.

CONTINUATION OF COMAL SPRINGS RIFFLE BEETLE NUTRITION AND SURVIVORSHIP RESEARCH

Our goal was to increase survival rates of adult CRSB through nutrition studies. We suspected that the standard food items offered in captivity may not be adequate in macro- or micro- nutrients that could affect CSRB long-term survival. These deficiencies could be potentially supplemented through manufactured feed. With this research, we compared pellet types manufactured by Bozeman Fish Technology Center (USFWS) for use in our refugia. We used stable isotope analysis to determine which pellets were consumed and utilized within the guts of wild caught adults in captivity. We attempted to determine which, if any, pellet in contrast to current diet given in captivity improved longevity and colonial fecundity of adults.

Four diet formulations were utilized to assess the ingredient preference of CSRB: a single-cell, protein based diet, a plant-based diet, an animal-based diet, a bacteria/yeast-based diet, and log-shaped diet utilized to provide a substrate for natural biofilm growth on which CSRB could graze. These artificial diets were compared with standard diets of biofilm covered leaves and cloth. We used ^{13}C:^{15}N isotopic analysis, conducted at the University of California–Davis's Stable Isotope Facility, to assess diet signatures. We used a Bayesian mixing model to analyze stable isotope data from the food items and the CSRB, allowing estimation of the proportion of each food item eaten by CSRB. Our Bayesian mixing model suggested that leaves were the main source of the CSRB diet. Of all tested, manufactured diets, the plant pellet was consumed the most. However, our results suggested that none of the manufactured diets were equivalent or better than a leaf-based diet, our standard food offering for CSRB.

CONTINUATION OF INCREASING PUPATION SUCCESS IN THE COMAL SPRINGS RIFFLE BEETLE IN A CAPTIVE SETTING – DR. ELY KOSNICKI, BIO-WEST, INCORPORATED

This study sought to examine factors that may enhance captive pupation success in Comal Springs riffle beetles and track the fecundity of first generation captive reared females. This information is useful for estimating the number of adults necessary to maintain a fully functional refugium. BIO-WEST, Incorporated tested flow-through tubes to test whether access to more air would improve pupation success end eclosion of late-instar larvae. BIO-WEST also tested small flow-through tubes, starvation, terrestrial, and modified flow-through container habitats. Twenty adult females reared from these experiments, and four additional F1 females from other studies, were paired with males and tracked for number of viable larvae produced over time. Two additional trials, one with small and one with medium-sized larvae, were also implemented to assess of larval survivorship. BIO-WEST found that more access to air was associated with improved pupation and eclosion rates of late-instar larvae. They also found that pupation of late instar larvae was better with leaves alone compared to a habitat of conditioned cotton, wood, and leaves.

Post-trials were mainly observational. BIO-WEST found 16 out of 75 larvae pupated. They tracked 24 females until death. These produced 703 larvae with an average of 29.3 ± 37.1 larvae per female. No evidence was found that female size affected the number of larvae produced. Five out of 22 larvae pupated and eclosed to adult from the medium-sized trial after 183 days and 15 out of 63 larvae pupated (14 eclosing to adult) from the small-sized larvae trial after 211 days. A total of 74 adults eclosed during this study and the sex ratio of F1 adults produced was not different from a 50:50 ratio ($X^2 = 1.515$, p-value = 0.218). From this study, it can be estimated that a colony consisting of 10 females surviving 60 days with unlimited access to mates would produce ca. 185 larvae. Conservatively using a 12% survival rate (half that of the observations from the small-size trial), 22 larvae would be expected to become adults. With a 50/50 sex ratio demonstrated in this study, 11 would be F2 females. If F2 females have the same fecundity and survivorship as F1 females, a perpetual captive colony could be expected.

CONTINUATION OF 2020 STUDY: FACTORS AFFECTING PUPATION RATES IN THE COMAL SPRINGS RIFFLE BEETLE – DR. WESTON NOWLIN, TEXAS STATE UNIVERSITY

The overall goal of this research is to examine how captive holding conditions and methods affect pupation rates and the successful eclosion of adult CSRBs. The research addresses two main questions: 1) Do CSRB pupae need access to air-water interface areas to successfully pupate in captivity and, 2) does frequent handling of larvae and more specifically pupae lead to lower adult eclosion rates? At the end of 2020, Dr. Nowlin was finalizing his report with an anticipated completion date of February 28, 2021.

FUNCTIONAL GENOMICS OF BACTERIA ASSOCIATED WITH WILD AND CAPTIVE-REARED COMAL SPRINGS RIFFLE BEETLE – DR. CAMILA CARLOS-SHANELY, TEXAS STATE UNIVERSITY

Texas State University (TXST) isolated 300 bacteria from adult Comal Springs riffle beetles, water, and wood biofilms collected in the wild and in captivity. At the end of 2020, 142 isolates were successfully identified using partial 16S rRNA gene sequencing. In total, TXST have identified 30 genera belonging to four phyla. Of these, 23 were found only in wild beetles, 41 only in captive beetles and eight were found in both groups. The diversity of culturable bacteria was higher in the field water samples than those from the refugium environment, but the wild beetles displayed lower diversity in their microbiome than their captive counterparts at the genus level. Dr. Carlos-Shanely sent 87 bacteria isolates for genomic sequencing to the Joint Genome Institute (JGI). She received the completed genomes of 58 isolates and are expecting the remaining 29 after JGI continues processing samples, which were delayed because of the Covid-19 pandemic. This work will continue into 2021. TXST is currently working on phylogenetic and functional analyses of these genomes.

LONG TERM TAGGING IN COVERED SALAMANDERS

Long term tagging allows for effective species management by monitoring biological data over a period of years and possibly through an individual's lifespan. For this study, we evaluated the readability and retention of three tagging techniques used in monitoring salamanders, including visible implant elastomer tags (VIE), visible implant alphanumeric tags (VIA), and passive integrated transponder tags (PIT). We found that individually marking salamanders with vertical VIE color combinations resulted in the highest readability and retention scores in all three species of aquatic salamanders verses VIA tags. Skin texture and thickness of each species affected the retention and readability scores of the three different tagging methods used. PIT tags were not injected on any of the smaller Texas blind salamanders and were not injected into San Marcos or Comal springs salamanders due to the animals' small size. In general, we found that un-shed VIE tags and VIA tags could easily and accurately be identified by both expert and novel readers.

BUDGET

Task	U.S. Fish and Wildlife Service 2020		Budget Spent	Total Task Budget Spent
1	**Refugia Operations**			**$454,759.27**
	SMARC Refugia & Quarantine Bldg.			
	Construction		-	
	Equipment		$8,526.04	
	Utilities		$6,428.09	
	UNFH Renovation Refugia & Quarantine Bldg.			
	Construction		-	
	Equipment		$11,450.42	
	Utilities		$19,924.86	
	SMARC Species Husbandry and Collection		$135,699.15	
	UNFH Species Husbandry and Collection		$147,174.04	
	Water Quality Monitoring System		$709.52	
	Fish Health Unit		$7,821.43	
	SMARC Reimbursables		$23,594.82	
	UNFH Reimbursables		$26640.75	
		Subtotal	*$388,683.12*	
		Admin Cost	*$66,076.15*	
2	**Research**			**$365,604.16**
	BIO-WEST: CSRB pupation		$91,473.74	
	BIO-WEST: Dryopid life history		$15,351.77	
	TXST: CSRB pupation		$46,330.80	
	USFWS Research Projects		$154,506.62	
		Subtotal	*$312,482.18*	
		Admin Cost	*$53,121.98*	
3	**Species Propagation and Husbandry**		-	-
4	**Species Reintroduction**		-	-
5	**Reporting**			**$77,360.65**
	SMARC Staff		$40,994.03	
	UNFH Staff		$25,126.19	
		Subtotal	*$66,120.22*	
		Admin Cost	*$11,240.42*	
6	**Meetings and Presentations**			**$10,167.06**
	SMARC Staff		$5,680.41	
	UNFH Staff		$3,009.38	
		Subtotal	*$8,689.79*	
		Admin Cost	*$1,477.27*	
			TOTAL	**$907,891.14**

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
GIS	Geographic Information System
GPS	Global Positioning System
HP	Horse Power
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. 2020 Refugia Work Plan
B. Research Report: Increasing survival rate of Peck's cave amphipods (*Stygobromus pecki*) – USFWS
C. Research Report: Continuation of Captive Reproduction Techniques in San Marcos Salamanders (*Eurycea nana*) – USFWS
D. Research Report: Increasing Pupation Success in the Comal Springs Riffle Beetle in a Captive Setting – Dr. Ely Kosnicki, BIO-WEST, Incorporated
E. Research Report: Functional Genomics of Bacteria Associated with Wild and Captive-Reared Comal Springs Riffle Beetles – Dr. Camila Carlos-Shanely, Texas State University
F. Research Report: Evaluating Three Different Long-Term Tagging Methods in Aquatic Salamander Species – USFWS
G. Fish Health Unit Reports
H. Monthly Reports