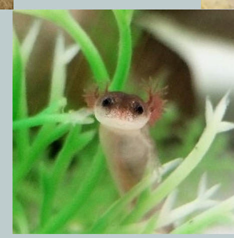

Edwards Aquifer Refugia Annual Report 2019

U.S. Fish and Wildlife Service

San Marcos Aquatic Resources Center
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Uvalde National Fish Hatchery
754 County Road 203, Uvalde, TX 78801

Implementation of the Aquifer Refugia Program under the Edwards Aquifer Habitat Conservation Plan

Annual Report 2019

Contract No. 16-822-HCP

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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ACKNOWLEDGEMENTS

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L to R: K. Kollus, L. Moon, K. Boren, C. Furl, L. Campbell

EXECUTIVE SUMMARY

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 (Covered Species) to the Edwards Aquifer 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 third annual report of the contract covering the calendar year of 2019. The third year of the contract slightly shifted to focus more on maintaining the existing standing stocks and conducting research, while still increasing standing stocks of Covered Species, and finishing construction of buildings that will house Refugia activities Uvalde National Fish Hatchery (UNFH).

Major objectives of the USFWS Refugia Program are to 1) develop and provide fully functioning refugia for the EAHCP 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 populations, in the event of a loss of species in their native environment, and monitor recovery; and 5) attend meetings and give oral presentations to Science Committee, Implementing Committee, and EAA Board of Directors as requested by the EAHCP Program Manager.

A Grand Opening was held for the Edwards Aquifer Refugia and Quarantine buildings at SMARC on April 25, 2019. Speakers at the event were Roland Ruiz, Edwards Aquifer Authority General Manager, and Amy Leuders, USFWS Region 2 Director. Chairman of the EAA Board Luana Buckner and Board member Ron Walton dedicated the buildings by breaking open a water drop piñata. Refugia staff gave tours to EAA Board members. This was a great celebration of the Aquifer Refugia Program as a whole and the long way come by many contributors to get to this point.

Construction, started in 2018, at UNFH on the Edwards Aquifer Refugia and Quarantine buildings finished in January with the final generator delivered and installed in April. Invertebrate species were moved into the Refugia space in March, and other species were moved in August. Modifications of the construction were required in fall of 2019 paid for by the USFWS.

The installation of a 35-ton chiller for incoming well water at SMARC was put out for bid, and a contract was awarded to AmeriVet Enterprises. Installation began in August, with the chiller on-line starting in October. Details of both construction projects can be found in their respective sections in the Building Construction segment of this report.

Field work and collections occurred in every month of 2019 with major increases in standing stock populations of San Marcos salamanders, Texas wild rice, and Texas blind salamanders. Details of collections for the Covered Species can be found in their corresponding sections of the report. At both SMARC and UNFH, staff maintained organisms in the existing and newly constructed systems, making modifications, fabrications, and updates as needed. At the SMARC Edwards Aquifer buildings, staff finished constructing systems and moved in all organisms. Staff at UNFH moved into their completed spaces and set up systems before moving in all their Covered Species by the end of September.

Three research projects carried out by USFWS staff in 2019 covered long-term tagging of three aquatic salamanders, reproduction of San Marcos salamanders, nutritional supplementation and requirements for adult Comal Springs riffle beetles. Two studies on the pupation and eclosion of Comal Springs riffle beetle larvae were contracted with BIO-WEST, Inc. and Texas State University under cooperative agreements with USFWS. Details of these projects can be found in the Research section.

The Aquifer Refugia Program did not exceed the allocated budget defined in the 2019 Refugia Work Plan previously approved by the EAA Board of Directors. The Refugia Program spent approximately $1.7M in 2019. Construction activities and related expenses totaled $519K. Research activities accounted for $372K, and approximately $826K was spent on staff, collections, husbandry and propagation, reporting, meetings, and presentations. The majority of 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.

Texas blind salamander

Field work collection set up. Photo by Scott Bauer, EAA

INTRODUCTION

BACKGROUND

The activities reported herein are in support of the Federal Fish and Wildlife Incidental Take Permit (ITP) for the Edwards Aquifer Authority (EAA) (TE-6366A-1, Section K) and fulfillment of Contract # 16-822-HCP between the EAA and the U.S. Fish and Wildlife Service (USFWS) as outlined within the 2019 Aquifer Refugia Work Plan. The overarching goal of the Aquifer Refugia Program conducted by the USFWS is to assist the EAA in compliance with its ITP and to meet its obligation within the Edwards Aquifer Habitat Conservation Plan (EAHCP) section 5.1.1. The refugia contract covers ten different species: seven endangered species, one threatened species, and three species currently proposed for listing (see Table 1 for list of the Covered Species). The idea of our 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 Rivers 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 at SMARC with back-up populations at UNFH. See the appropriate sections of this report for further details on each of the species collected and maintained, plus the section on research activities.

The EAA-USFWS contract awards 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, 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 (covered by funds in Task 1), 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 1 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). Color corresponds to the ESA status.

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* sp.	Petitioned
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.

OBJECTIVES

1. **Further Develop and provide fully functioning refugia for the EAHCP Covered Species.**
   USFWS will work towards fully functioning Refugia operations for all of 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 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 give 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 U.S. Fish & Wildlife Service (USFWS) manages the Edwards Aquifer Refugia program with dedicated staff at two facilities: The San Marcos Aquatic Resources Center (SMARC) and the Uvalde National Fish Hatchery (UNFH). Although both facilities are administratively under the direction of a single Center Director, Dr. Ken Ostrand, each facility is directed by its own project leader. Dr. David Britton, the Deputy Center Director at SMARC is responsible for the Edwards Aquifer Refugia Program in San Marcos. Dr. Patricia Duncan, the Project Leader at UNFH, is responsible for the Edwards Aquifer Refugia Program in Uvalde. Dr. Lindsay Campbell, the Managing Biologist for the Aquifer Refugia Program in San Marcos, coordinates the Program as the Lead and Point of Contact for EAA Refugia operations, in addition to the duties of supervisor listed below.

Table 2 USFWS Refugia Program Staff

San Marcos Aquatic Resources Center	
Lindsay Campbell, Ph.D.	Managing Biologist for the Aquifer Refugia Program San Marcos Program Supervisor
Kelsey Anderson, M.S.	Biotechnician
Amelia Everett Hunter, M.S.	Biotechnician
Linda Moon, B.S.	Biotechnician
Taylor McCrary	Temporary Biotechnician
Kevin Boren	Temporary Biotechnician
Uvalde National Fish Hatchery	
Mark Yost, B.S.	Uvalde Program Supervisor
Makayla Blake, M.S.	Biotechnician
Benjamin Whiting, M.Ed.	Biotechnician
Rachel Wirick, B.S.	Biotechnician

Day to day operations are 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 2 for staffing chart). The work they conducted involves resource management and affects the success and efficiency of the refugia programs at SMARC and UNFH. The supervisors managed and coordinated species husbandry, propagation, and field activities related to species covered under the reimbursable agreement. They also arranged purchases, oversaw facility maintenance repairs, developed and implemented budgets, and organized all activities that related to the reimbursable agreement. 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 coordinates efforts in San Marcos with those in Uvalde, but does not have direct authority over the UNFH staff. Dr. Campbell also, with input of supporting staff, prepared the Annual Report, 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, supervises the dedicated staff at UNFH and coordinates 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, with input into the yearly Work Plan.

Figure 1 SMARC Refugia staff and volunteers (blue shirts) summer 2019. L-R: Taylor McCrary, Amelia Hunter, Lindsay Campbell, Linda Moon, Kelsey Anderson, Melissa Wolter

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 (see Figure 1 and Figure 2 for pictures of Refugia Program staff). This included maintaining experimental and culture production systems, keeping records along with entering and filing 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 2019, including data analysis, writing, and presenting. All three with the addition of Makayla Blake, a biological technician at UNFH, helped to develop the 2020 research proposals along with Dr. Campbell.

Figure 2 UNFH Refugia staff 2019. L-R: Makayla Blake, Ben Whiting, Rachel Wirick, and Mark Yost

Figure 3 Temporary biotechnicians Taylor McCrary and Kevin Boren.

During the summer of 2019 temporary biological technicians, Taylor McCrary and KC Boren, were hired to help with refugia work at SMARC (Figure 3). These positions were limited to a maximum of 60-days. The technicians supported Refugia program efforts (husbandry and collections) and the practice salvage event, allowing other SMARC staff to focus more time on research.

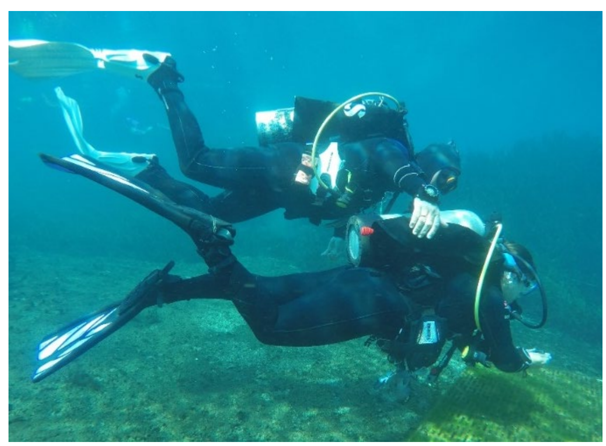


Figure 4 Linda Moon (front) during her SCUBA certification dives.

Linda Moon was certified as an USFWS SCUBA Diver in the fall of 2019. She will now dive for the Refugia program during collection activities (Figure 4).

Special note: U.S. Fish & Wildlife Service employees who are normally paid with 100% reimbursable funds were exempt from furlough during the Federal Government shutdown (December 21, 2018 through January 25, 2019) since the pay for these employees is not contingent on congressionally allocated funds. As exempt employees, the Refugia staff were able to continue work with our normal schedule and carry out much of our normal duties. Some tasks were limited because Service employees were not available to conduct administrative support such as purchasing or USFWS systems for invoicing could not be accessed during this time. The Refugia staff is highly appreciative that we were in the position through our unique contract to be able to continue to care for our organisms, conduct most of our normal duties, be able to go to work, and not have the burden of wondering about our financial situation.

Figure 5 USFWS Region 2 Director Amy Lueders with Refugia Program staff and Karst at Grand Opening.

BUILDING CONSTRUCTION

In 2019, construction and renovations proceeded at both locations according to plans. In San Marcos, construction finished in the fall of 2018 and, in 2019 all the systems had been set up and species moved into the spaces. USFWS, in conjunction with the EAA, held a grand opening of the new Edwards Aquifer Refugia and Quarantine buildings. (Figure 5). A 35-ton chiller was installed at the SMARC to chill incoming well water. In Uvalde, the original modifications and renovations of existing UNFH buildings were completed in January. Modifications to the Refugia spaces at UNFH were needed by the end of the year at the expense of USFWS. These projects created Edwards Aquifer Refugia and Quarantine work areas specifically for Covered Species activities necessary to meet our obligations under the EAA-USFWS contract.

GRAND OPENING OF REFUGIA BUILDINGS AT SMARC

The USFWS, in conjunction with the EAA, held a grand opening of the new Edwards Aquifer Refugia and Quarantine buildings on April 25, 2019 in San Marcos, Texas. The EAA board of Directors were extended a special invitation to attend the event that was open to the public. Many people who were integral in setting up the EAHCP and the Refugia contract attended. The event was chronicled by the San Marcos Record (local newspaper), an EAA reporter, and Austin television station KXAN. Scott Storment, EAHCP Program Director, convened the press conference, welcoming guests and introducing speakers and key guest in attendance.   Roland Ruiz, Edwards Aquifer Authority General Manager, spoke of all the work that led to this event and thanked key contributors.   Amy Leuders, USFWS

Southwest Regional Director, voiced appreciation for the valuable partnership between EAA and USFWS in the Refugia Program and thanked EAA staff, USFWS staff, and the Refugia staff. Chairman of the EAA Board Luana Buckner and Board member Ron Walton dedicated the buildings by breaking open a piñata shaped like a water drop (Figure 6). The educational and viewing hallway was full of pictures, informative posters, and display tanks (Figure 7 and Figure 8). Refugia staff gave tours to EAA Board members, highlighting features of the facilities, showcasing some of the research projects being conducted, and briefing them on the variety of work and activities of the Refugia Program (Figure 9, Figure 10, and Figure 11). Much preparation was put into the event by staff, and, it was worth the effort, as the event was very successful. The Refugia staff thank all of the EAA staff, especially Dr. Chad Furl, Kristy Kollus, Kristina Tolman, and Oliva Ybarra, for the preparation work done for this event. We also thank the many USFWS members that attended this event from around the area and our regional supervisors Amy Leuders (Figure 5) and Stewart Jacks, special guests that traveled from Albuquerque, New Mexico to attend the event. This was a great celebration of the Aquifer Refugia Program as a whole and the long way come by many contributors.

Figure 6 Ron Walton (L) and Luana Buckner dedicate the buildings with a raindrop piñata.

Figure 7 Dr. Campbell introduces the Refugia Program and building to EAA board members in the viewing and educational hallway.

Figure 8 Texas blind salamanders exploring habitat their display tank that made its debut at the Grand Opening.

Figure 9 Linda Moon speaks to EAA board members about the long-term tagging project and shows them the different tags in salamanders.

Figure 10 Kelsey Anderson explains the importance of quarantine and our procedures to the EAA board members.

Figure 11 Panoramic view of completed Refugia building at SMARC.

SAN MARCOS AQUATIC RESOURCES CENTER

The installation of a 35-ton chiller for incoming well water at SMARC was put out for bid and a contract was awarded in April to AmeriVet Enterprises. A long lead time was required for the manufacture and delivery of the chiller to our site. Once the chiller was delivered in August, installation began. First contractors removed the old, defunct chiller and cleaned old piping and wire connections. Copper piping was installed and wrapped in insulation (Figure 12). A new water meter was installed. Contractors tested the system, including a pressure test, and after final finishing items (labeling, thermometer replacement) the chiller was brought on-line in October.

Figure 12 Insulated wrapped piping and gauges on 35-ton chiller at SMARC.

UVALDE NATIONAL FISH HATCHERY

Construction and renovation of buildings at UNFH to create spaces dedicated to Refugia and Quarantine for the Covered Species was primarily completed by the end of January 2019 with a few finishing touches completed in February. Contractors and subcontractors adjusted plumbing hangers, painted plumbing to color code water and air supplies, installed and tested electrical services, labeled boxes and breakers, hung doors and hardware, sealed the concrete floors, painted the mezzanine floors, tested HVAC operations, load-tested the Refugia Building generator, and installed a lift station to drain the effluent from the Quarantine Building. The Quarantine Building's generator installation occurred in April, completing the work that was contracted. Although modifications to plumbing, electrical, and HVAC systems eventually became necessary to maintain optimal operations within these spaces.

Once the buildings were complete, UNFH Refugia staff placed tank systems, installed plumbing between tanks, pumps and heater-chiller units, and set up water lines. In November 2018, a purchase request was initiated for 20 350-gallon insulated tanks. The Government shutdown at the end of 2018 delayed this purchase until February 2019; the bid was not awarded until March. The bid was awarded to Water Management Technology, subcontracted through Hydro Composites LLC, and the tanks arrived in mid-July. Bulk utility racks were purchased in February to hold these tanks. Staff also purchased 12 100-gallon fiberglass tanks for hosing animals in the Quarantine Building and for sumps in the Invertebrate Room. In March, the racks and tanks arrived and installation began.

While waiting for tanks on order, UNFH staff set up the Invertebrate Room in the Refugia area using and modifying components of an old invertebrate rack system. This system, with an individual ¾ HP chiller unit, was operational by March and refugia invertebrates were moved in. This system initially had a packed column to degas the super-saturated well water, which can be harmful to aquatic organisms. However, this component elevated pH and calcification in the lines, pump casings, and sump tank, causing premature system failures. So, it was removed and replaced with an alternative solution. A valve was installed to restrict water flow to the recirculation pump, creating a vacuum degassing effect that reduces gas saturation levels in water from the well supply. This method has kept total dissolved gas saturation under 100%, with a target of around 95%, without impacting pH. Since this change, calcification rates and temperatures and other water quality parameters have been more stable, and system operations have not failed.

In August, the well water source was switched from the cooler Spurgeon Well (22.5–23.5 °C) to the warmer Wilson Well (24.5–25.5 °C) so that maintenance could be done on the Spurgeon Well's pump motor and casing. The invertebrate system was a straight flow-through system where water was conditioned and then left the system. The heater-chiller unit on this system could not cool the volume of the warmer well water required to meet the flow rates prescribed by the UNFH Project Leader. A 600-gallon tank was installed in the room where incoming well water was conditioned to temperature and total dissolved gas (TDG) by circulating water in a loop to a 1HP heater-chiller unit on the mezzanine level (Figure 13). Conditioned water was then pumped out of this tank to the invertebrate system and through a separate chiller to maintain optimum temperature and required flow for the invertebrates. During this modification, we discovered that one tank in the Refugia room was plumbed into the 1HP heater-chiller designated for the invertebrate room. The SMARC Refugia team provided UNFH with two 1 HP heater-chiller units to solve this problem and provide a backup unit. Two additional rack systems were approved to be set up in September.

Figure 13 Photo of the UNFH Invertebrate Room in the Refugia area with three rack systems set up.

When utility racks and 100-gallon tanks arrived in March, UNFH Refugia staff set up a few systems in the Quarantine Building. However, the station's well-water pressure was insufficient to push the water to the supply plumbing on the ceiling. In April, a 1.5-HP variable speed pump was installed to pressurize all of the well supply lines throughout the Quarantine Building, solving this issue. A flow meter was installed to monitor water usage into the building. Staff made supply manifolds for air, well, and conditioned water supplies. They also installed drain lines, plumbed a recirculation pump into each sump, and connected the manifolds to the overhead air, well water, and heater-chiller supply lines. Plumbing drops to and from the chillers were not constructed in a consistent manner, and modifications were needed to allow installation of rack systems in a mirrored image fashion at each paired drop. These modifications were made as each new system was added over the late summer.

Originally, 100-gallon tanks were purchased to house an entire collection of darters or salamanders in quarantine from a given river section. The UNFH Project Leader later decided to place all of newly collected organisms in smaller individual aquaria, as was the practice in the temporary quarantine building used before construction. Hence, 50 15-gallon glass aquaria were purchased and modified for bulkhead and drain fittings. These systems were organized into a rack design, containing a system sump (located under the rack), with two shelves to house aquaria, each with spigot valves on a well supply manifold and a chilled water supply manifold, and a drain line (Figure 14). Water in these systems is circulated with an inline suction pump from the sump to the heater-chiller units, then back to the sump. A submersible pump in the sump pressurizes the chilled water supply manifold. Each tank receives chilled water and non-chilled well water inputs. Water flowing out of the tanks does not go back into the system, but flows into the drain.

Figure 14 Quarantine Room at UNFH rack set up.

The 0.5 HP pumps purchased for the project in 2017 could supply more than the minimum 35 GPM flow required for the flow switch to operate the heater-chiller units if the flow was unrestricted. However, supersaturated well water, exceeding 115% TDG, since December of 2018, led to a design change. The UNFH Project Leader directed staff to use a valve to restrict flow through the pumps in order to degas the supersaturated water. This reduced flow to a level whereby the chillers would not operate properly. The problem was made worse by friction loss going through the pipes to each respective chiller. To address these problems, 1.0-HP Centrifugal pumps were purchased, tested, and were found sufficient to degas water to the target TDG saturation of 85–90% while maintaining a consistent flow to the chiller units of at least 35 GPM. Degassed water also buffered the non-conditioned well water inputs into each tank. The UNFH Project Leader later decided to try a system design with a single pump to degas, send water through the heater-chiller units, and then into the manifolds supplying the aquaria. A 1.5-HP variable speed centrifugal pump was purchased to try this design on a single system. This design added heat to the water and was difficult to balance the pump speed, vacuum degassing effect, and flows adequate for the heater-chiller units and aquaria. After much trial and error, we determined that this design was impractical. As a result, UNFH staff returned to the original approved design with two pumps: one for conditioning, degassing and chilling the water, and another for supplying conditioned flow directly from the sump to the manifold plumbing. Refugia staff were given permission by the UNFH Project Leader to build and install systems in the Quarantine Building in July.

To date, these rack systems have proven to be stable for temporarily housing newly collected organisms through their quarantine period (Figure 14). On a few occasions, a sump was found with low water in the morning, suggesting that input well flows had slowed overnight. We attributed this to an air lock in the plumbing outside and within the building that forms when there is very little pressure in the supply main at the Quarantine Building. This problem is exacerbated when flows are changed at other parts of the hatchery. Refugia staff asked to be informed in advance of all major water flow adjustments so we can take immediate corrective action. We also installed an air bleeder valve at the highest run of the well supply plumbing to help prevent air locks from occurring.

We installed residence tanks used to hold Comal fountain darters and Comal Springs salamanders in the Quarantine Building. These systems used the 350-gallon insulated tanks that arrived in July. The UNFH Project Leader did not require these systems to have as high amount of water exchange as quarantine rack systems, so the 0.5-HP centrifugal pumps that were pre-purchased for this project were used.

As the number of systems brought online increased during July, August, and September, it became increasingly clear that HVAC unit in the UNFH Quarantine Building was not keeping the room cooled to the desired temperature. Ambient temperature was 30 °C, when it should have been 20–21 °C. The problem was likely due an installation discrepancy: the approved design called for the chiller exhaust fans to be lined up with vent holes in the building walls, blowing hot air out; but most of the units were turned 90°, blowing the hot air into the room. The heat removed from the water combined with the heat generated by the heater-chiller units' compressors countered the output of the 5-ton HVAC specified in the design plans, which could not compensate for this increased heat load. USFWS Regional Mechanical Engineer, Mark Orton, visited the station in September and determined that an insulated partition wall constructed around the chiller units on the mezzanine level, and a large exhaust fan installed to dispel the heat produced from the chillers, could mitigate this problem. These costs will be covered by USFWS with an anticipated completion date in May of 2020.

Refugia staff moved 17 350-gallon insulated tanks, which arrived in July, into the Refugia Building and installed drain pipes. Staff fabricated and installed secondary drain boxes, pumps and degassing valves, built supply manifolds, and ran plumbing to the supply and return lines from the nine chillers on the mezzanine roof. All preinstalled plumbing drops from the chillers needed modifications to fit to the standardized spacing of the tank racks. Such modifications were necessary to avoid running plumbing in the middle of isles needed for accessing the systems. This was completed by early August (Figure 15). Thereafter, staff filled the systems to check the operations of the pumps, chillers, degassing valves, and for leaks at all the joints. Then, a small number of test organisms were moved into the systems to ensure the systems were operating as expected. Finally, on August 28, 2019, all of the refugia organisms were moved in to the space.

Figure 15 Residence tank systems in the UNFH Refugia building.

Even before moving into the new culture space, it was evident that the HVAC system in the Refugia Building was not performing well: the ambient temperature was generally over 28–30 °C, when it should have been around 20–21 °C. The USFWS Regional Mechanical Engineer, Mark Orton, visited the site in September and ran diagnostic tests. He determined the HVAC duct that was installed per engineering specifications was undersized and would not circulate enough airflow to cool the refugia space. Essentially, less than one-half of the target airflow of 1250 CFM was being achieved. The Refugia Building was still cooler than the temperatures in the larger Tank House; however, higher ambient temperatures prevented the use of more than half of the systems, which were originally designed to control water temperatures by the ambient temperatures from the HVAC unit, rather than having a dedicated chiller for each system. More chiller units were bought and installed on systems. USFWS contracted and paid for a company to enlarge the duct work, drops, and return grill. The contractor completed the work in early December, but is scheduled to come out and rebalance the system in the summer of 2020.

Lastly, UNFH Refugia staff purchased and installed structures for shade over the Texas wild rice tanks behind the UNFH Tank House (Figure 16). This project originally had been cut from the renovation plans, but staff were able to find cost-effective structures and use their own labor to install. During the summer months shade cloth will be applied to the structures to reduce algal growth in the wild rice tanks.

Figure 16 Shade structures in place of TWR tanks at UNFH.

Figure 17 One of the many very small juvenile Texas blind salamanders collected from our Diversion Spring net.

COVERED SPECIES ANALYSIS

Collections of the Covered Species continued this year to achieve to Standing Stock targets as outlined in the Contract and the 2019 Work Plan (Table 3 and Table 4). For many species, the accumulation to Standing Stock numbers can be achieved relatively quickly; this is particularly true for Texas wild rice, San Marcos fountain darters, and San Marcos salamanders. We have made large strides in increasing our populations of Texas blind salamanders through collections and increased survival of very small juveniles coming out of the Diversion Spring net; in particular, an unprecedented number of very small juveniles (Figure 17) were collected from Diversions Springs net during the spring, which more than doubled the Standing Stock numbers of Texas blind salamanders. After consultation with the Edwards Aquifer Authority staff, our other partners, and experts in the field, we decided to reduce the number of invertebrate collection events and numbers held in refugia to minimize any negative effects that collection events might have on wild populations in the Comal Spring system.

Husbandry of all the species continued to evolve, especially with moving into the new building spaces with tank set-ups that took the best concepts from old set-ups and combined with new designs that better fit our spaces, infrastructure, learned experiences, and relevant needs. Staff incorporated more habitat enrichment items into the species tanks. Invertebrate holding containers continued to progress to better fit specific needs of each species, such as adding artificial interstitial space for Peck's cave amphipods.

At both facilities, staff moved new tanks to the Refugia and Quarantine spaces (Figure 18), then spent considerable time and effort to design, plumb in the systems, set up water flow, arrange habitat, flush and test systems (Figure 19).

Figure 18 Unloading and moving in new tanks at UNFH.

Figure 19 Rachel Wirick glues plumbing to and from heater-chiller units.

After systems were set-up the transfer and movement of species previously held in different locations at the facilities began. There were some changes and iterations of the systems as the staff and organisms settled into the new spaces and learned the ins-and-outs of the new facilities. At SMARC, the invertebrate systems were converted to a partial re-circulation system to better maintain the optimum temperature for the invertebrates, which is slightly higher than some of the other species (Figure 20).

Figure 20. Dr. Lindsay Campbell and Juan Martinez wire a variable speed pump for the invertebrate system.

Figure 21 New, scaled up holding systems for very small juvenile salamanders. Left: Thirteen rows of groups of three individual tanks, plus room for two larger tanks on the end (two of these were built). Right: Larger tanks designed and completely built by Refugia staff out of Plexiglas to hold larger juveniles or groups. Most of the spring, summer, and fall all of these systems were completely full of salamanders

During the influx of Texas blind juveniles, SMARC staff quickly expanded upon the small holding container design for juveniles, but multiplied to accommodate seven times as many juveniles that we had ever previously held at one time (Figure 21).

Staff at UNFH had to manage elevated ambient and incoming water temperatures plus supersaturated levels of total dissolved gas (TDG) in their incoming well water in both their temporary spaces and newly constructed spaces. The first species to be allowed to move into the new spaces at UNFH were the invertebrates in March, where the ambient room temperature, while not optimal, was lower than the previous space. Staff built systems with large conditioning tanks for incoming water in addition to having water go through another heater-chiller unit on some systems to mitigate elevated temperatures and TDG (Figure 22). All systems at UNFH had higher water temperatures from August through November, while the station used the Wilson well during the maintenance of the Spurgeon Well. In addition to the warmer well water, the plumbing to the mezzanine level heater-chiller units (for the Refugia space) contributed heat to the systems. All of these factors could have contributed to lower survival rates of all organisms at UNFH.

Figure 22 Large conditioning tank (A &B) for incoming water. Invertebrate system where water is collected in the bottom sump before being pumped through a heater-chiller unit, then into the containers and out of the system.

In 2019, Dr. Lindsay Campbell traveled to the Southwest Fish Health Unit (SFHU) in Dexter, NM to receive training on the Fish and Wildlife approved DNA extraction and qPCR procedure for detecting the presence of *Batrachochytrium dendrobatidis* (Bd, commonly referred to as amphibian chytrid fungus) and *Batrachochytrium salamandrivorans* (Bsal). Our salamander species undergo swabbing for health screening of these fungi. Dr. Campbell had performed RNA/DNA extractions and qPCR procedures during her Ph.D. and post-doctoral projects, but had not run this specific procedure. After running many of the salamander swab samples while at SFHU, she returned to teach the technique to the refugia staff (Figure 23). The Refugia staff can now extract the DNA from the swabs and run a qPCR assay to detect the presence of the Bd/Bsal. In-house fungal assays provides a cost savings to the Refugia Program and a faster turnaround on the samples.

Figure 23 Dr. Campbell explains a part of the DNA extraction procedure to Rachel Wirick (L) and Makayla Blake (R).

The Refugia Program put out a request for bids for a water quality monitoring package for both facilities in May of 2019. The contract was awarded to DBA HydroTech ZS Consulting, a small local business in Texas, with the backing of HydrometOtt software. The system was installed at the beginning of August and the company provided hands on training at SMARC. We received four multiprobe data sondes that monitor temperature, dissolved oxygen, conductivity, and total dissolved gas (two per station). These sondes connect to hubs that upload real-time data to a secure account in the Hydromet Cloud. The system has capability for expansion of more data sensors in the future. The staff then programed different alert and alarm levels for the system to monitor and send messages if those thresholds were exceeded. Alerts were set to detect changes at a lower level and notify biologists by system so they could detect problems before values were at critical levels. Red-alert alarms were set to notify all refugia staff (per facility) when any critical thresholds were breached. At any time, staff can use a secure login to monitor real-time and past data via the web-portal or through a phone application. The system takes water quality readings every 15-minutes and logs these into the system. Staff learned of water quality trends within the first few weeks of using the system that would not otherwise have been detected by point water quality readings. Alerts have diverted potentially dangerous situations for organisms by allowing the staff to act before parameters become critical in tanks. SMARC Refugia staff purchased additional temperature monitoring probes to add to the system at the end of 2019. UNFH Refugia staff plan to add onto their system in 2020.

As in past years, Refugia staff at both facilities preformed their duties of animal care in addition to their duties of organismal collections, maintenance, data collection, reporting, and research (Figure 24).

Figure 24 Refugia staff collecting Covered Species, preforming husbandry activities, and conducting research.

Table 3 Number of organisms incorporated in the SMARC Refugia Standing Stock in 2019, the end of year census, and overall survival rate. 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, the number in parenthesis are those in quarantine period. 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.

Species		SMARC Incorporated into Refugia	SMARC End of Year Census	SMARC Survival Rate
Fountain darter-San Marcos *Etheostoma fonticola*		245	622	62.1%
Fountain darter-Comal *Etheostoma fonticola*		181	213	52.7%
Comal Springs riffle beetle *Heterelmis comalensis*		346	63 (30)	14.7%
Comal Springs dryopid beetle *Stygoparnus comalensis*		15	12	66.7%
Peck's cave amphipod *Stygobromus pecki*		220	206 (80)	48.1%
Edwards Aquifer diving beetle *Haideoporus texanus*		0	0	--
Texas troglobitic water slater *Lirceolus smithii*		*	*	*
Texas blind salamander *Eurycea rathbuni*		204	264	91.0%
San Marcos salamander *Eurycea nana*		269	343	77.1%
Comal Springs salamander *Eurycea* sp.		25	88	90.7%
Texas wild rice plants *Zizania texana*		35	211 (10)	85.1%

*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 4 Number of organisms incorporated in the UNFH Refugia Standing Stock in 2019, the end of year census, and overall survival rate.   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, the number in parenthesis are those in quarantine period.   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.

Species		UNFH Incorporated into Refugia	UNFH End of Year Census	UNFH Survival Rate
Fountain darter-San Marcos ***Etheostoma fonticola***		488	533	57.6%
Fountain darter-Comal ***Etheostoma fonticola***		5	36	67.9%
Comal Springs riffle beetle ***Heterelmis comalensis***		133	32	21.8%
Comal Springs dryopid beetle ***Stygoparnus comalensis***		4	1	25.0%
Peck's cave amphipod ***Stygobromus pecki***		229	157	54.7%
Edwards Aquifer diving beetle ***Haideoporus texanus***		0	0	--
Texas troglobitic water slater ***Lirceolus smithii***		0	0	--
Texas blind salamander ***Eurycea rathbuni***		38	31	81.6%
San Marcos salamander ***Eurycea nana***		177	305	74.6%
Comal Springs salamander ***Eurycea* sp.**		47	55	84.6%
Texas wild rice plants ***Zizania texana***		80	157 (14)	98.1%

Figure 25 Fountain darter in the Comal River (photo by Scott Bauer, EAA).

FOUNTAIN DARTER (*Etheostoma fonticola*), ENDANGERED

The Standing Stock goal for fountain darters (Figure 25) 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 at both facilities in 2019. High mortality rates for both incoming Comal fountain darters and those in refugia inhibit reaching target goals. Considering the on-going high mortality in newly collected Comal fountain darters the Managing Biologist, in concert with Refugia biologists and supervisors at SMARC, made the decision to not bring in additional fountain darters from Comal River until further studies investigate potential causes of these increased mortalities. We received approval from the Edwards Aquifer Authority (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 bolster refugia stocks. Numbers incorporated, end of the year census, and survival rates can be found in Table 5.

Table 5 Fountain darter refugia population figures.

		Beginning of Year Census	Incorporated 2019[1]	End of Year Census	In Quarantine End of Year	Target Goal 2019 Work Plan	Percent Survival
San Marcos River	SMARC	504	545	622	0	500	62.1%*
	UNFH	437	488	533	0	500	57.6%
Comal River	SMARC	233	181	213	0	300	52.7%
	UNFH	48	5	36	0	200	67.9%

[1]The number 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.

*Survival rate of treatment event losses were not included.

COLLECTIONS

Large collections for calendar year 2019 were conducted in April–May and October of 2019. Refugia staff partnered with BIO-WEST, Inc. employees during their fall and spring biological monitoring of the San Marcos and Comal Rivers to collect fountain darters. After BIO-WEST, Inc. employees trapped fountain darters via drop-netting, counted, and measured them for biomonitoring, they were transferred to Refugia staff for refugia populations. This partnership created efficiencies in collections and reduced disturbance to the fish and systems from both biomonitoring and refugia collections. The SMARC and UNFH coordinate to collect fountain darters to limit the number of fish that are sent to the Southwestern Fish Health Unit (SFHU) for analysis as our standard practice is to send 60 fish per river per collection event.

In 2019 the SMARC was notified of a Spring Lake Dam renovation project. Refugia staff worked with Zara Environmental, LLC (Figure 26) to use this opportunity as a practice salvage event and for staff to collect organisms in areas that were being disturbed. We wanted to mitigate any further disturbance after construction that our scheduled collections later in the year might cause to the area.

Figure 26 Mark Yost and Dr. Lindsay Campbell of USFWS consult with staff from Zara Environmental, LLC at the Spring Lake dam renovation site.

SMARC staff began further preparing quarantine spaces for incoming fountain darters and San Marcos salamanders. Several tanks are always open in the SMARC Refugia quarantine space in case the need arises to salvage organisms of any of the Covered Species. However, we were waiting on additional tanks that had been ordered but not yet delivered. As well, when not in salvage mode some quarantine areas are used for research activities (still leaving open tanks), thus in the event of a salvage these organisms are shifted to other spaces.

The original plans for the renovation called for dewatering the eastern spillway and in an area upstream of the dam in Spring Lake; but on the first day of the project, the plan was changed so that only sections at a time had reduced water flow. The area upstream of the dam was not dewatered, and consequently, fewer darters than originally expected were collected. On July 1 staff collected darters in areas around the renovation site. As SFHU had recently conducted assessments on fountain darters, it was deemed that we did not have to send additional samples for this collection.

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 (ANS). A standard fountain darter intake and quarantine procedure was used at both facilities starting in 2019 (see Appendix G). 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 50–60 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 SFHU for routine parasitology and health screening before the larger group of collected fish were incorporated into the refugia.

SURVIVAL RATES

At both SMARC and UNFH, survivorship of newly collected Comal River fountain darters was poor in comparison to San Marcos River fountain darters 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). Comal fountain darters collected at the end of 2018 were given approval from SFHU to be incorporated into refugia numbers in 2019; the clearance was delayed by heavy losses from unknown causes. At SMARC only 50 of the 483 collected in April/May survived past the quarantine period and none of the 308 Comal darters survived the quarantine period at UNFH. Comparatively, San Marcos fountain darters brought in during the same time period and exposed to the same conditions did not have this same mortality at either facility, with incorporation rates of 92.6% (SMARC) and 88.9% (UNFH). Both live and preserved Comal fountain darters were sent to SFHU for analysis. Extra tests targeting infectious pancreatic necrosis virus (IPNV), Infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia (VHSV), and Aquareovirus were conducted with negative results (Fish Health reports can be found in Appendix I). No conclusive cause of mortalities were found by the SFHU. The reports found spleen inflammation, liver degradation, atrophy of adipose tissue, moderate skin infection, mild ovarian infection, and gill hypertrophy. Histopathology and bacterial culture results agree upon absence of a bacteria pathogen as a possible cause for the mortality. Given these findings and the past history of low intake survival rates, we decided to suspend collections of Comal fountain darters for the fall of 2019. The potential exists for small collections in 2020. We will continue to research this phenomenon to determine the cause of high mortalities in these fish.

In contrast, LMBV negative Comal fountain darters, collected in 2016, have high survivorship and do not exhibit symptoms or mortalities of Comal fountain darters collected from 2017 to present (Table 6). The LMBV negative fountain darters have been in refugia for over three years and were brought in as adults, thus their age is over three. Mortalities may be natural senescence.

Table 6 Survival rates of Comal fountain darters at SMARC based on their LMBV status.

Comal fountain daters at SMARC				
LMBV Presence	***Census on Jan 1, 2019***	***Incorporated in 2019***	***Census on Dec 31, 2019***	***Survival***
Positive	172	181	168	46.1%
Negative	61	0	55	90.2%

The overall survival rates for San Marcos fountain darters in refugia at SMARC was 62% and the survival rate for Comal fountain darters was 52% for 2019. This survival rate excludes the impact of a treatment incident on San Marcos fountain darters. Fountain darters were given a routine treatment administered by an experienced staff member. A new staff member was instructed to turn on the input water on all the tanks at the end of the one hour treatment in order to flush the treatment. The staff member mistook the water flow from the recirculation loop as the fresh well water input. The system was not flushed until the next day, resulting in the death of 48 San Marcos stock fountain darters.

The overall survival of fountain darters at UNFH for the year was 57.6% for San Marcos fountain darters and 67.9% for Comal fountain darters. Low survivorship of San Marcos fountain darters was attributed primarily to a loss of 131 individuals succumbing to a faulty biofilter set-up in tanks. The inventory discrepancy was found in August when the fountain darters were hand-count inventoried and moved to new tanks in the Refugia Building. In further investigation, staff disassembled the biofilter barrels and observed a number of dead darters. Evidently, the darters had been swimming into the partially submerged biofilter barrels and dying. To prevent this from happening in the new residence tanks in the Refugia area, the biofilters were updated to using Matala® filter media, sealed in a fine-mesh filter bag. The Refugia Managing Biologist requested that hand-count inventories be conducted more frequently in order to discover such discrepancies and potential causes before losses climb in numbers.

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 (Figure 27), 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.

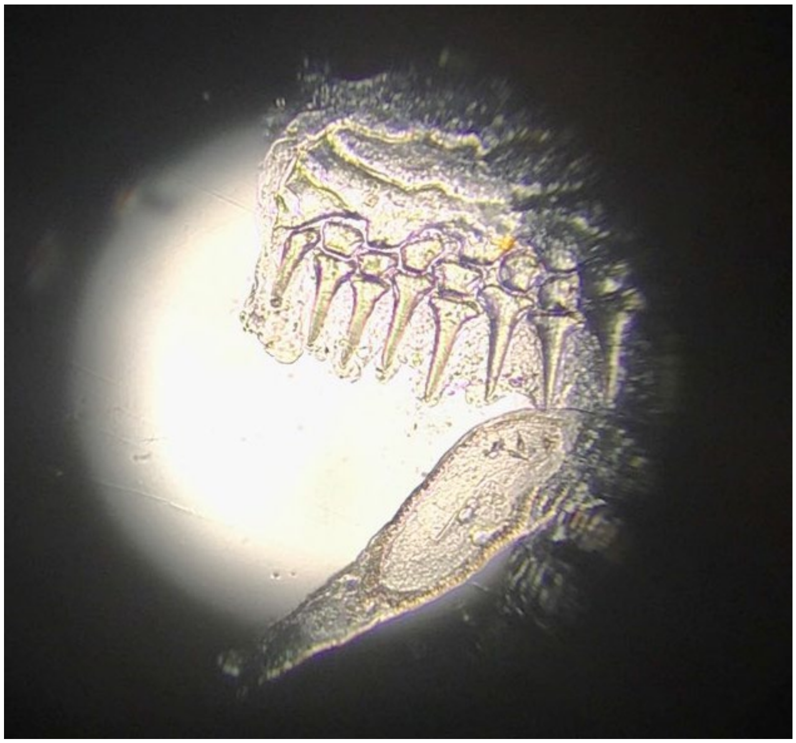


Figure 27 Monogenean parasite found on skin and gill clips of a fountain darter at UNFH.

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.

At SMARC, new tanks were a dark green color instead of the bright blue of the previous tanks. This noticeably increased the pigmentation displayed by the fountain darters. Studies of several fish species have reported that lighter colored tanks can increase cortisol levels in fish (Papoutsoglou *et al.* 2000; Rotllant *et al.* 2003; Merighe *et al*. 2004; Barcellos *et al.* 2009). In the case of Southern flounder (*Paralichthys lethostigma*) blue tanks increased sex reversal with increased cortisol levels (Mankiewicz *et al.* 2013). While we do not find sex reversal in fountain darters, darker colored tanks can reduce stress as darters are benthic and cryptic in nature; thus, light tanks could cause stress, as the darters try to blend into the background to avoid predators. Elevated stress levels in many fish species cause immunosuppression in response to elevated levels of corticosteroids, increasing mortality associated with common bacterial and fungal diseases to which fish might otherwise be resistant (Pickering and Pottinger 1989). Barcellos *et al*. (2009) found that tank color and the availability of shelter reduced chronic stress response in cortisol levels. Biologists added new habitat enrichment items, such as pebble mats, and additional shelter plants and structures to the tanks in order to further reduce stress. Biologists observed the fountain darters utilizing more of the tank space and routinely displaying natural behaviors (Figure 28 and Figure 29).

Figure 28 Male fountain darters displaying territorial behavior in new display tank located in the completed Edwards Aquifer Refugia building at SMARC.

Figure 29. Fountain darter display tank at SMARC with a variety of habitat enrichment items, including objects found in the San Marcos River during collections.

Maintenance of systems

Refugia systems were deep cleaned annually with 20–30% 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 be 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. SMARC currently has 56 F1 fountain darters on hand with plans to rear more F1 darters in 2020 to replace those that were provided to outside researchers in 2019. UNFH transferred 40 F1 fountain darters to SMARC in November of 2019 and also plans to rear F1 fountain darters in 2020.

Figure 30 Amelia Hunter takes water quality at Comal Springs during lure setting.

COMAL SPRINGS RIFFLE BEETLE (*Heterelmis comalensis*), ENDANGERED

Collections of Comal Springs riffle beetles (CSRB) were reduced in 2019 compared to years past to decrease potential impacts on the wild population and focus efforts on collections for research purposes (Figure 30). Research on this species focused on increasing survival of adults through the lens of nutrition. Two other groups, BIO-WEST, Inc. and Dr. Weston Nowlin's Lab from Texas State University (TXST), were contracted to investigate factors involved in increasing CSRB pupation and eclosion. More details on these research projects can be found in the Research section of this report. Numbers incorporated, end of the year census, and survival rates can be found in Table 7.

Table 7 Comal Springs riffle beetle refugia population figures.

	Beginning of Year Census	Incorporated 2019	End of Year Census	In Quarantine End of Year	Target Goal 2019 Work Plan	Percent Survival
SMARC	116	346	63	30	#	14.7%
UNFH	14	133	32	0	#	21.8%

 # for 2019 there was no net end of the year goal, as we planned on collecting CSRB mainly to support research, until survivability is increased in captivity

COLLECTIONS

CSRB were collected using poly-cotton cloth lures and wooden dowels placed together in spring orifices within the Comal Springs system in New Braunfels, Texas. Staff also hand collected adult CSRB from river wood found near upwellings for research purposes for Dr. Camila Carlos-Shanley at TXST. Cotton lure collections followed the standard protocol for cotton lures in the Comal River system. Wooden lures were historically made from poplar dowel rods, cut in half length-wise and sanded before being conditioned with biofilm growth from the springs. Not many organisms were found on these dowels. Refugia technicians substituted naturally decayed wood from the Comal Springs system at some sites to see if that would prove to be a better lure than dowels at Spring Island; the effectiveness of this will continue to be evaluated into 2020 to provide more collection events to reference. During lure collections, staff recorded water quality conditions at the sampling locations. Our Refugia invertebrate specialists examined the lures in shallow trays filled with spring water to identify and enumerate the invertebrates. Organisms not retained for refugia were carefully returned to the specific lure spring collection location.

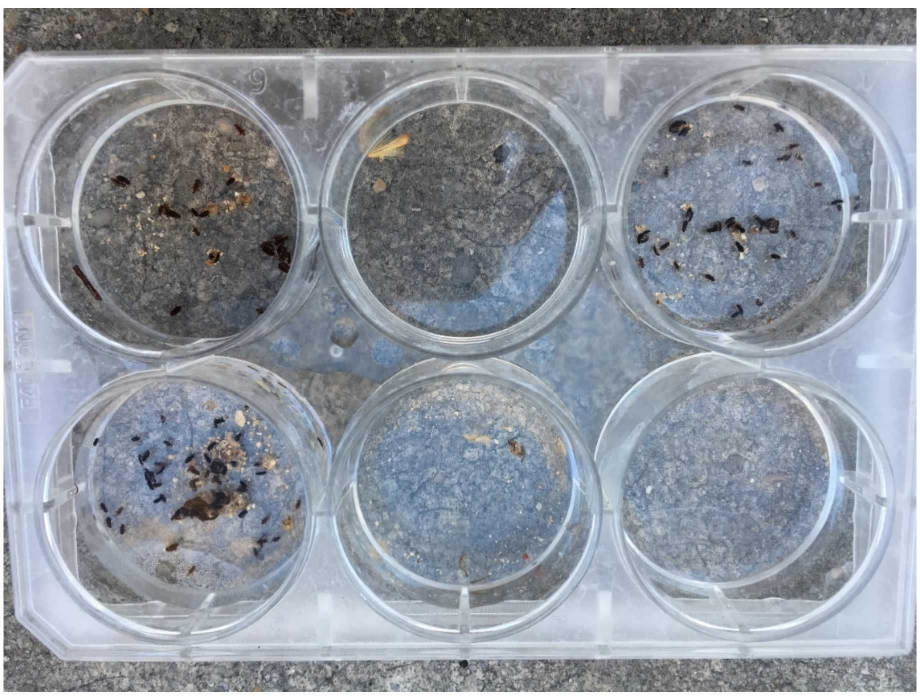


Figure 31 Comal spring riffle beetle collected from two different locations during biomonitoring activities.

Poly-cotton lure collections occurred in January (SMARC) and February (SMARC and UNFH) in 2019. In addition, CSRB were collected off the poly-cotton lures (Figure 31) set for the beetle species by BIO-WEST, Inc. contracted for biomonitoring of Comal Springs at a rate of 25%. SMARC staff collected the beetles from BIO-WEST staff at the spring sites during biomonitoring for refugia purposes; this type of sampling occurred in May and October 2019.

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 ANS 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.

SURVIVAL RATES

The riffle beetles at SMARC and UNFH were collected in 2018 and January–February 2019, with no additional collections. Because Comal Springs riffle beetles 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. Research into nutritional requirements of CSRB are on-going (see Research section), but aim to increase the survival rates and time in captivity. Currently about half of CSRB collected perish by the sixth month mark. 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.

HUSBANDRY

On a daily basis, all systems were checked 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, they do not have a traditional feeding schedule. Alternatively, leaves and cotton cloth containing biofilm are used in each system, providing food. Inventories were conducted every other month and new leaf and cotton material was added as needed (Figure 32). During the 2019 nutrition supplementation experiment conducted by SMARC staff, the addition of conditioned wood had a positive, but not statistically significant effect on adult survival rate. Most importantly, containers with wood doubled the amount of larvae produced/retained (see Research section for full details). Conditioned wood was not incorporated into refugia containers because 2019 research by our partners needed consistent diets for adults producing larvae that were used traditionally at SMARC: cotton cloth biofilm and leaves. After completion of partners' experiments, containers in refugia will be updated with conditioned wood pieces in addition to the traditional diet items.

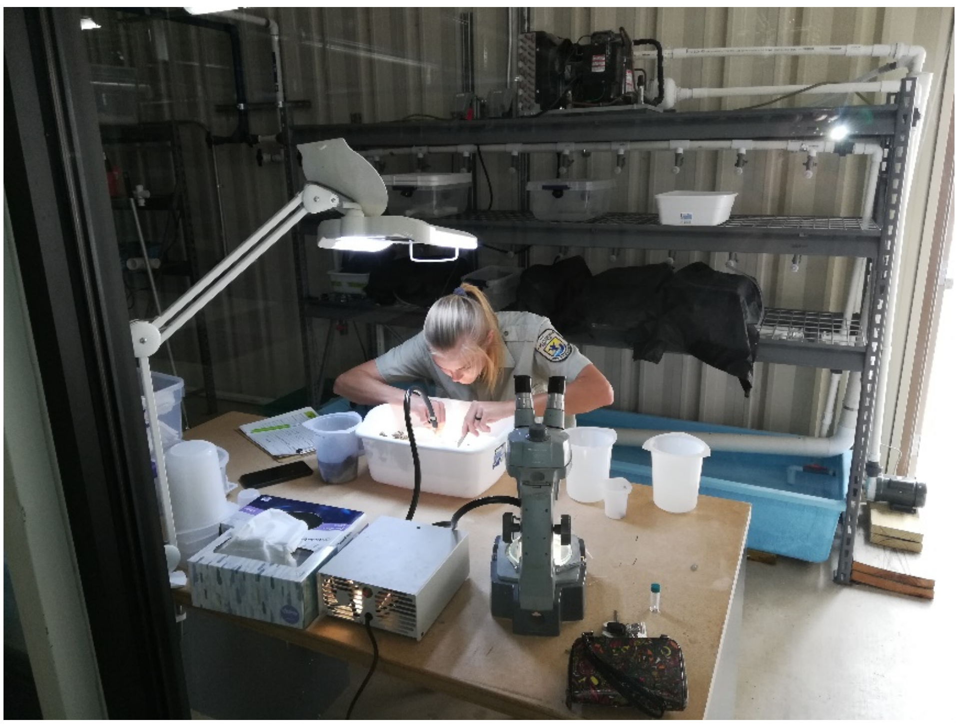


Figure 32 Makayla Blake performing a CSRB inventory at UNFH.

Maintenance of systems

Plastic totes were used for CSRB culture containers, with PVC piping that delivered water in a manner that mimicked upwellings. 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 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 (see Research section for details) and this information will be used in 2020 for CSRB PVC tube husbandry; historically they were aligned vertically. Air space and emergent structure is already given in box containers housing larvae. Our research found that larvae produced in the nutritional supplementation experiment burrowed through wood and used it as area of refuge; we will incorporate 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. Seventeen F1 generation adults successfully pupated and eclosed (i.e. pupa emerges into adult life history form) in 2019 at SMARC in the Refugia population (this number does not include any that pupated and eclosed from larvae provided to the two research groups). One F1 adult was observed at UNFH. This is an increase in comparison to the previous year; however, Refugia staff were not actively trying to propagate F1 beetles for refugia, rather produced larvae for two different contractors conducting research on pupation during 2019. Larvae production resulted in 1,023 larvae at SMARC and 795 larvae at UNFH in 2019; a majority of these larvae were provided to our research partners for their work.

Figure 33 Comal Springs dryopid beetles captured from poly-cotton lures in Comal Springs.

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. USFWS contracted BIO-WEST, Inc. for life history research on CSDB. Fourteen of the CSDB borrowed for research conducted by BIO-WEST, Inc. in 2018 were returned to refugia population at SMARC in February 2019. A summary of the dryopid life history research can be found in the Research section. 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 2019	End of Year Census	In Quarantine End of Year	Target Goal 2019 Work Plan	Percent Survival
SMARC	6	15	12	0	*	66.7%
UNFH	0	4	1	0	*	25.0%

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

COLLECTIONS

In 2019, 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 (Figure 33). While there were two sampling events, each station only took CSDB from one event each (SMARC January, UNFH February). Five adults were caught on poly-cotton lures and eight dowel samples successfully attracted dryopids in 2019 (Figure 34). The most reliable collection method was to hand pick from naturally decaying wood pieces found directly over spring sources, which we plan to utilize more in 2020.

Figure 34 CSDB found on conditioned wood in the field during the February collection event.

QUARANTINE

Incoming CSDB were quarantined in in the Invertebrate Refugia area at SMARC or the quarantine room at UNFH. 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 ANS 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 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

Culture boxes used to house CSDB are 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 to mimic underground environment. On a daily basis, all the systems were checked for 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, as CSDB have been known to become entrapped in the 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, they do not have a traditional feeding schedule. Alternatively, leaves, wooden dowels, and cotton cloth containing biofilm were placed in containers that provided them with a constant food source. A new source of habitat and food was introduced in 2019: conditioned wood pieces collected from 2017 that have been conditioning in SMARC well water for two years to monitor fungal growth and remove any ANS species (Figure 35). It was apparent the beetles utilized the new materials and will be kept for future culture containers. Inventories were conducted every other month and new food items were added as needed. Obtaining census numbers during monthly 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.

Figure 35 CSDB adults crawling on a conditioned piece of wood from Comal Springs utilized in refugia culture containers.

Maintenance of systems

Plastic totes were used as culture containers to house CSDB, with PVC piping that delivered water using a bar that sprays the side of the container and then flows down. 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

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. BIO-WEST, Inc. staff continued with their study of propagation techniques in 2019, which they will work on scaling up for refugia use in 2020.

During the February collection, dryopid beetles were collected off two small pieces of conditioned wood found under a specific rock dubbed "Randy's Rock" at Spring Island. The conditioned wood was retained by UNFH staff and added into the dryopid beetle culture box as a source of habitat and food from their natural habitat. Both large and small larvae were observed during the March and July inventories. Considering the timing, it is unlikely that these represent F_1 individuals, but rather these were likely, unknowingly retained in the wood and emerged in the culture box. Only larger larvae were observed in the September and December inventories. It is possible that these larvae represent F_1 individuals; however, their large size makes this is unlikely. Rather, they probably represent wild stock larvae. Considering the probability that the larvae represent wild stock individuals and the behavior of burrowing, all of the larvae observed are retained in the culture box with the adult dryopid beetles. Small larvae found were removed to a F1 larvae container.

Figure 36 Peck's cave amphipod collected from Comal Springs. Photo by Scott Bauer of EAA.

PECK'S CAVE AMPHIPOD (*Stygobromus pecki*), ENDANGERED

Peck's cave amphipods (PCA) were collected from Comal Springs during 2019 by four hand collection events and donated to the refugia from those caught in drift nets during biomonitoring activates. Changes in holding container habitat lead to increased survival rates and served a pilot information for a formal experiment testing different container habitat configurations in 2020. 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 2019	End of Year Census	In Quarantine End of Year	Target Goal 2019 Work Plan	Percent Survival
SMARC	208	220	206	80	250	48.1%
UNFH	58	229	157	0	160	54.7%

COLLECTIONS

Adult PCA were collected from the Comal River headwaters using drift nets, setting cotton cloth lures over upwellings, and by hand using dip nets. Specimens from the samples were sorted and brought to the SMARC Refugia invertebrate area and the quarantine area at UNFH (Figure 36). Biologists used small aquarium nets to scoop sand and gravel before carefully sifting through it in trays for PCA. Hand collections occurred in March, June, August, and November in 2019 (Figure 37). Occasionally PCA were collected off the poly-cotton lures set for the beetle species. PCA collected in driftnets during biomonitoring surveys by USFWS Biologist Randy Gibson and BIO-WEST, Inc. were transferred to SMARC for refugia purposes. These were collected in May and October 2019.

During 2019, Biological Technicians Linda Moon and Rachel Wirick worked together to devise a more effective method of collecting PCA around spring upwellings. Two people start on opposite sides of the upwelling out about a meter. Each person then works their way inward to the upwelling center sifting through the gravel and thus herding the PCA towards the center. When the two parties meet in the center and gravel is scooped up, many more PCA are collected in one or two net scoops than either person alone would have collected. A modification of this method was also applied to collecting salamanders; whereas, people work in teams with one person turning rocks and sifting sediment and the other person nets the salamanders.

Figure 37 Refugia staff sorting through sand and gravel for PCA.

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 ANS 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 the highest 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 estimated an optimum density (0.5–0.6 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 are 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 starting in March with different densities of Matala® biofilter media (Figure 38) to simulate interstitial spaces in PCA holding containers. She had great success (over 90% survival month-to-month, in some months individual containers had 100% survival) and will be further researched in 2020. For now, technicians at both stations have begun to incorporate the new media within all PCA holding containers at the end of 2019. Biologist also estimated an optimum density (0.5–0.6 per liter) for PCA in containers based on survival records and observations of cannibalism at higher densities.

Figure 38 Low-density Matala biofilter media material.

HUSBANDRY

On a daily basis, all the systems were checked for water temperature, adequate flow, and clear drain screens to maintain drainage and water level. PCA were fed small amounts of fish flake slurry 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 TXST 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, in 2020 Refugia biologist will conduct 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.

Eric Julius and Ely Kosnicki of BIO-WEST, Inc. suggested use of shredded PVC shavings to utilize the full container height and add surface area for the adult PCA to disperse. This was pilot tested at SMARC, but biologists determined the PVC shavings were not being used by the PCA refugia adults. The spaces between the shavings were too large and something with tighter weaves were needed. Matala® biofilter media (Figure 38) fit several parameters: created simulated interstitial spaces, had smaller spaces between fibers, could easily be taken out of containers and cleaned, did not trap or entangle organisms. In discussions with Ms. Blake and Ms. Hunter about PCA holding containers, Dr. Campbell suggested creating a wider spread configuration of water inflow at the bottom of containers (Figure 39) beyond the traditional two bar system to reduce any potential anoxic areas and add greater access to simulated spring flow to the PCA in the container so would not have to congregate in limited areas. These bars have been implemented at SMARC for pilot testing through the end of 2019 into 2020.

Figure 39 Serpentine water input bars with openings throughout the length create a more even flow pattern throughout the container.

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. Work in 2018 did not show success with flow-through tube rearing chambers so those designs were not used in 2019 at SMARC. Research in 2020 will focus on 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. At UNFH gravid females were isolated in flow-through tube brooding chambers. SMARC observed 19 brooding females in 2019 and UNFH had 16 brooding females during 2019; however, there were no F1 generation PCAs on station at either stations at the end of 2019.

Figure 40 Edwards Aquifer diving beetle (photo by Abbot).

EDWARDS AQUIFER DIVING BEETLE (*Haideoporus texnus*), UNDER REVIEW

No Edwards Aquifer diving beetles (Figure 40) were collected during 2019. 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 TXST to donate caught adults to the SMARC, at their discretion. Unfortunately, none were donated for this year.

Figure 41 Lirceolus spp. on a leaf curl (photo by Abbott).

TEXAS TROGLOBITIC WATER SLATER (*Lirceolus smithii*), PETITIONED

In 2019, *Lirceolus* spp. (Figure 41) were collected to better understand and determine if their needs could be met within captivity, and to study husbandry and rearing techniques. At the time we could not determine if our collected animals were the Texas troglobitic water slater, *Lirceolus smithii*, because the only known way to distinguish between the three co-occurring *Lirceolus* species was to dissect their mouth parts, a lethal procedure. At the SMARC, all species of *Lirceolus* found in the field were collected and labeled "*Lirceolus* sp." until a new method of distinguishing species non-lethally has been published.

Will Coleman, a doctoral student at TXST, discovered a non-lethal way to distinguish *L. smithii* from other species based on the characteristics of the pleotelson. This work is currently being prepared for publication by the researcher. Using his 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 decided to disband the *Lirceolus hardeni* we had in culture. 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.*

Numbers incorporated, end of the year census, and survival rates can be found in Table 10.

Table 10 *Lirceolus* spp. refugia population figures.

	Beginning of Year Census	Incorporated 2019	End of Year Census	In Quarantine End of Year	Target Goal 2019 Work Plan	Percent Survival
SMARC	509 (Fx)#	6	0	0	*	--
UNFH						

 # A mix of adults and offspring of Lirceolus spp.
**catch rates and hatchery survival are uncertain given the rarity of the species*

COLLECTIONS

After Mr. Coleman's research was brought to our attention in February of 2019 we ceased collections of *Lirceolus* spp. in March at Diversion Spring in San Marcos or Comal Springs resulting in only 6 wild stock individuals collected in 2019. UNFH did not house TTWS during 2019. This genus was collected primarily through incidental catch in the Diversion Spring driftnet at Spring Lake, in San Marcos, Texas and on cotton lures at Comal Springs, in New Braunfels, Texas. All of our refugia stock of *Lirceolus* spp. were from Diversion Spring or Spring Runs, not the Texas State Artesian Well, which is the only location the petitioned species has been found.

QUARANTINE

Incoming *Lirceolus* spp. were quarantined in the Invertebrate Refugia area at SMARC. *Lirceolus* spp. were acclimated to water conditions at a rate not to exceed one degree Celsius every half-hour. During the quarantine period staff monitored for potential ANS 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 *Lirceolus* spp. joined the Refugia population at the end of the 30 day quarantine period.

HUSBANDRY

On a daily basis, all systems were checked for water temperature, adequate flow, and drain screens cleared of debris to maintain drainage and water level. *Lirceolus* spp. were fed small amounts of fish flake once a week. Dried leaves from terrestrial sources were also used as potential supplemental food and to provide shelter within the systems.

Captive husbandry and propagation of *Lirceolus* spp. has shown proof of concept on SMARC's ability to maintain and rear this genus in captivity. Therefore, to better allocate staff time to other species, the remaining organisms in captivity were disbanded. Because of their unknown species identification, mixed origin, or any ability to cross-breed between species, all 509 adults were given as food for other species in captivity instead of releasing them into the wild. Gravid females were removed and euthanized to prevent the proliferation of these organisms in other systems.

Figure 42 A large number of *Lirceolus* sp. found during inventory. Water slaters are fragile; thus, it is important to delicately pick them up one by one.

Maintenance of systems

Plastic totes were used as culture containers to house *Lirceolus* spp., with PVC piping that delivers water in a manner to mimic upwellings. The systems did not have a traditional cleaning or siphoning schedule, but alternatively, were cleaned during inventory. *Lirceolus* spp. are cryptic and could easily be discarded along with debris during siphoning. During inventory, staff checked water lines, hoses, and valves for functionality and cleaned or replaced them as needed. An inventory was conducted every other month (Figure 42).

CAPTIVE PROPAGATION

Inventory was conducted once every two months to assess the suitability of food, shelter, and water quality provided to *Lirceolus* spp. in refugia. Reproduction was also noted. Brooding females released their young in each container, and offspring reached adult life stage quickly, making it difficult to identify offspring from parents. Therefore, we assumed all containers were a mix of several generations of *Lirceolus* spp. In March, inventory of those in refugia ceased after determining they were *Lirceolus hardeni*.

After inventory conducted in January 2019, over 500 individuals were recorded to have proliferated in captivity (brooding female example Figure 43) from collections in 2018. The extraordinary abundance of *Lirceolus* spp. bred in captivity provided evidence that the species had adjusted positively to the captive environment. The SMARC staff determined that husbandry techniques were appropriate and no further testing of habitat or nutritional needs would be conducted until *L. smithii* are held. The standard protocol was updated to reflect current techniques. Since this genus is not federally listed, and was most likely not the target species, captive propagation and monitoring of this species in captivity was determined to be complete and termination of the captive population at the SMARC was initiated.

Figure 43 Brooding *Lirceolus* spp. female can be seen at the top of the photo.

Figure 44 Texas blind salamander on habitat enrichment item.

TEXAS BLIND SALAMANDER (*Eurycea rathbuni*), ENDANGERED

The Standing Stock goal for Texas blind salamanders (Figure 44) is 500 individuals distributed between the two facilities. Historically, Texas blind salamander catches were infrequent, and we projected it would take up to 10 years to reach the standing stock goal. In 2019, there was a surge of small juvenile Texas blind salamanders collected in the Diversion Spring net in Spring Lake, San Marcos, TX.

When space was unavailable at the SMARC quarantine to house additional salamanders, juveniles were collected by UNFH Refugia staff and transferred directly to UNFH. At the end of the year, twelve additional sub-adult and adult Texas blind salamanders were transferred to UNFH to build and diversify their standing stock. Numbers incorporated, end of the year census, and survival rates can be found in Table 11.

Table 11 Texas blind salamander refugia population figures.

	Beginning of Year Census	Incorporated 2019	End of Year Census	In Quarantine End of Year	Target Goal 2019 Work Plan	Percent Survival
SMARC	95	204 (185/19)[1]	264[2]	0	110	91.0% * (98.0%/89.7 %)
UNFH	0	38 (26/12)[3]	31	0	15	81.6% (73.1%)[#]

[1] Incorporated broken down by those from Diversion Spring and those from all other locations combined
[2]This number represents the final number of wild stock on station after transferring 12 wild individuals to UNFH (not counted as mortalities). Three wild individuals of unknown origin location were also added to total refugia stock number.
[3]Incorporated broken down by those from Diversion Spring and those transferred from SMARC in December
*Percent survival for the overall refugium (91%), survival of sub and mature adults (96%) is higher than that of young-of-the-year juveniles (89.7%). Survival presented is only for those animals which surpass their 30-day quarantine period and are officially incorporated into the refugia.
[#]Percent survival for young-of-the-year juveniles at the end of the year at UNFH

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 weeks before being removed from the site. To avoid oversampling, only 1/3 of salamanders observed were retained for refugia from these sites (Figure 45).

Figure 45 Releasing a Texas blind salamander back into Primer's fissure.

Rattlesnake Cave and Well were not sampled in April, as efforts targeted an overabundance of salamanders still occurring in high-frequency from Diversion Springs. These sites were trapped for three weeks instead of two in both July 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. From February 22 through May 3 we retrieved 302 very small juveniles (average TL = 15 mm) from Diversion Springs net (Figure 46). Not all of these were retained or incorporated into the refugia.

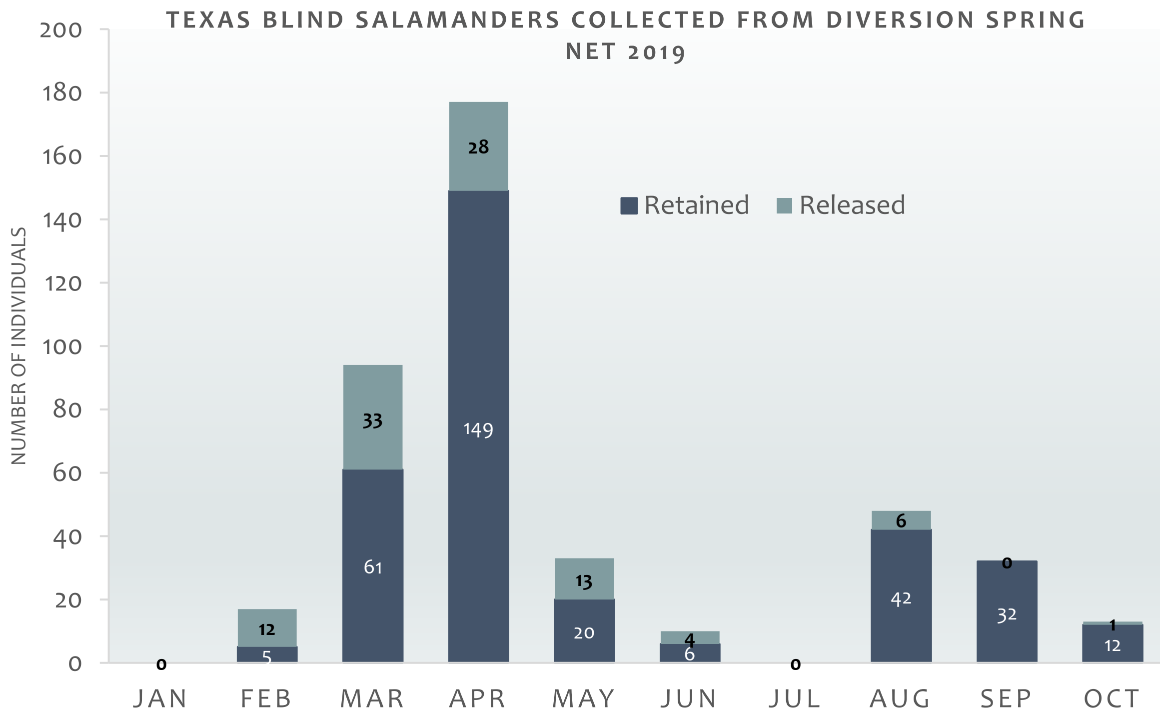


Figure 46 Numbers of Texas blind salamanders collected from Diversion Spring net in 2019 either retained or released. Net was not sampled in January, July, November, or December.

By the beginning of April SMARC did not have any more space in Quarantine for juvenile Texas blind salamanders, so staff from UFNH collected six times during a two-week period in April. Prompted by the high numbers of juveniles collected from the net, a detailed Juvenile *Eurycea* Collections SOP was written (see Appendix H) and followed by SMARC staff; the Project Leader at UNFH did not adopt this SOP at UNFH.

Texas State University personnel had nets on Sessom Creek and Artesian Well for their own uses during 2019, and donated three live Texas blind salamanders collected from those locations to SMARC in 2019.

QUARANTINE

Texas blind salamanders were transported directly to the quarantine space at SMARC or UNFH after collection. The quarantine area is a separate, biologically secure area away from the refugia systems, preventing the spread of disease and ANS. 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. Each tank received its own flow of fresh well water and habitat items. Animals remained in isolation for at least 30-days. During the influx of small juveniles, small groups of salamanders (3–4) collected on the same date were moved out of isolation at 30-days and into larger small tanks to make room for incoming individuals. Healthy individuals measuring 30 mm or greater were non-lethally cotton swabbed. 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 are taken for the group and tested for disease. Skin swabs were tested for presence of *Batrachochytrium dendrobatidis* (Bd, commonly referred to as amphibian chytrid fungus) and *Batrachochytrium salamandrivorans* (Bsal). In 2019 the SMARC began running these tests in-house, which will reduce costs and decrease 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 the 30-day quarantine period, organisms were incorporated into the refugia standing stock numbers; survival after the quarantine period for the small juvenile age class was 89.7% at SMARC (Figure 47). Juveniles collected from February to June had markedly higher survival during their quarantine period (91%) than those collected July to October (53%); the overall survival for juveniles in their first 30-days was 76.6% at SMARC. The reason for this difference in survival is unclear but could be a result of poor genetic stock, decreased spring flows coupled with increased temperatures, or flow dynamics in the net. Survival of these juveniles collected in 2019 that passed their 30-day quarantine period was 89.7%

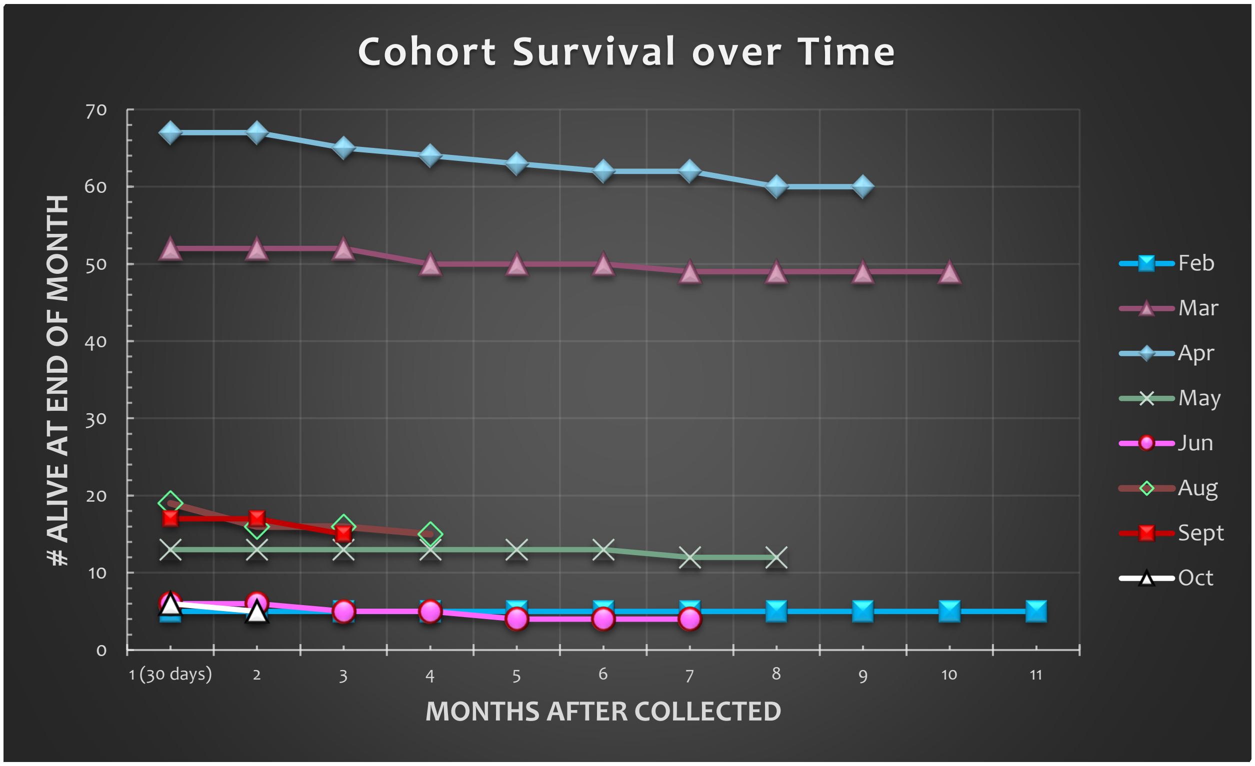


Figure 47 Survival of juvenile Texas blind salamanders cohorts (by month collected) at the SMARC over 2019.

(166 individuals), lower as compared to 98.0% (98 individuals) survival of established sub and mature adults. The overall survival rate of juveniles during their first 30-days was 52.1% at UNFH (73 were collected 26 made it to incorporation); survival of juveniles collected in April of 2019 that passed their quarantine period was 73.1%.

The UNFH Project Leader directed staff to follow protocols different than those established at SMARC for holding very young juveniles. At UNFH, juvenile salamanders were placed in group aquaria at densities of approximately one salamander per two liters. The quarantine aquaria were flow-through systems designed with two water supplies: an untreated well supply (23–25 °C) and a chilled-well-water supply (20–21 °C) so that the water temperature can be manipulated as needed. Air stones were placed in the aquaria holding salamanders. In December 2019, 12 individuals were transferred from SMARC and were incorporated into the UNFH refugia. This transfer inflated the incorporated survival and overall survival of Texas Blind salamanders at UNFH in 2019 to 81.63%.

Texas blind salamanders from all collection locations were housed together; however, individuals were tagged 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 SMARC with either flow-through or partial recirculation in Refugia tanks. Two of the three quarantine tanks at the SMARC were on partial recirculation and temperature control (maintained at 21 °C) in order to keep temperatures uniform and protect the very young juveniles from spikes in total dissolved gas. The system with larger juveniles and sub-adults was on flow-through water.

At UNFH salamanders were held in glass aquaria in Quarantine and large insulated fiberglass tanks in Refugia on partial recirculation through heater-chiller units to maintain the water temperature at 21 °C. Smaller glass tanks were placed on these systems when needed. 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. If a gas super-saturation event occurred at UNFH, adjustments were made to lower the gas saturation within the system by using a globe valve that restricts water flow to the pump, creating a vacuum degassing effect. The SMARC produced an SOP to describe how to reduce gas saturation in the various system setups (this SOP can be provided upon request). 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 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 30–40 mm in TL, juveniles were given one dot visible implant elastomer (VIE) tags (for individual identification) under sedation and 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 could then be moved out of their groups, retaining their individual data. 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 are culturing alternative food sources, including composting worms and daphnia. 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 Texas blind salamander daily care can be found in the USFWS Captive Propagation Manual for *Eurycea* spp., available upon request.

Those animals selected for use in the 2019 tagging study were observed monthly. The largest offspring Texas blinds were given passive integrated transponder (PIT) tags. Twenty mature adults were given visible implant alphanumeric (VIA) tags, twenty were given horizontal VIE tags, and another 20 smaller blinds were given 20 vertical VIE tags. Animals were removed from their tanks, placed under anesthesia, and had their tag checked for presence/absence and readability. All animals were returned to fresh well water and, once awake, were returned to original aquaria. Salamanders were observed for injuries or deleterious effects of initial tagging and tag checks. Mass as well as length were recorded every 3 months.

At the SMARC, we seized on the unprecedented opportunity of having high numbers of Texas blind salamander juveniles all at once and started collecting life history data; individuals were measured and monitored every 6 months from their date of collection (individual data has been retained for these organisms to track over time) to create a growth curve at this station for this species (Table 12).

Table 12 Average total length (TL), snout-to-vent length (SVL), and mass of monthly cohorts measured 6 months after their collection. August and September groups have not been in captivity for 6 months and are excluded from this table. Generally, juveniles arrive weighing <0.01 grams and measuring 10–15 mm total, 5–7 mm SVL. There may be more growth during summer (warmer) months for this species and our other aquatic Eurycea, which was observed in sub and mature adults used in the tagging study.

Texas blind 6-month Measurements				
Cohort	**# individuals at 6 months**	**average TL (mm)**	**average SVL (mm)**	**average mass (g)**
February	5	26	15	0.0725
March	49	29.4	16.5	0.1144
April	62	30.5	17.0	0.1192
May	12	29.2	17.1	0.1054
June	4	30.3	17.4	0.1025
AVERAGE	-	29.1	16.6	0.1028

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.

In October 2018, a large adult male salamander with two small back limbs was collected from Sessom Creek; it was not known at the time if the limbs were deformations or in the process of regeneration. Regeneration of full limbs had not be documented in any species of this genus. The two limbs were miniaturized in size, discolored, and lacking in strength. Photo documentation of the limbs began upon intake to the SMARC. By early 2019, biologists had documented the growth of these limbs as well as pigmentation of the tissue and signs of regained use. Dr. Campbell contacted Dr. Catherine McCusker of University of Massachusetts–Boston about the potential for regenerative studies in Texas blind salamanders using this case as a landmark for this species. Correspondence began between Dr. Campbell, Ms. Anderson and Dr. McCusker and her post-doctoral student Dr. Warren Vieria. Tail regrowth was already known for this species, though limb regeneration had not previously been noted or observed. One mature female Texas blind salamander captured in 2018 only had stump instead of a left arm at capture. This stump limited her ability to climb or grasp structures, and required more force for locomotion. At the opportunity to improve the well-being of this salamander, Dr. McCusker and Dr. Vieria suggested that a procedure they had previously done on axolotls could cause this salamander to regenerate her limb. Dr. Vieria traveled to the SMARC and performed surgery on her under anesthesia in hopes that this would encourage natural regeneration. The wound and regeneration were monitored weekly then monthly for progress (Figure 48). The salamander now has mobility and use of her new limb. A paper on Texas blind salamander regeneration is forthcoming with this partnership.

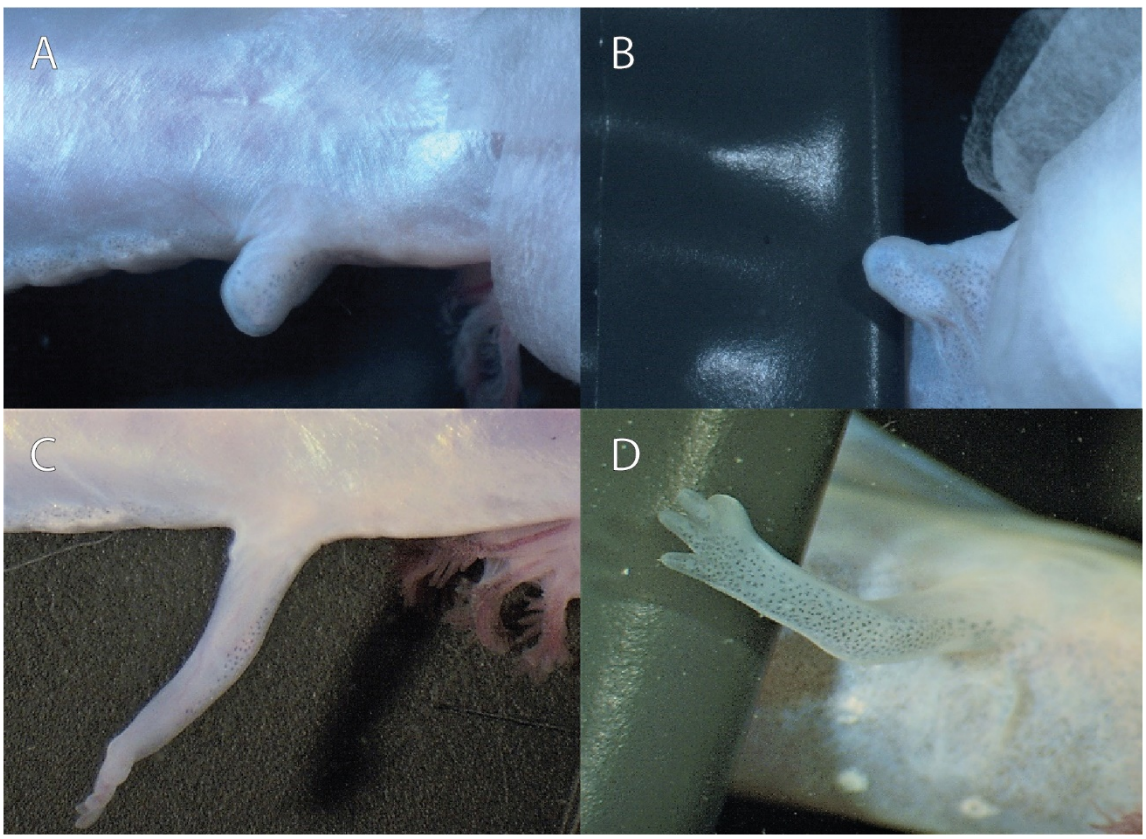


Figure 48 The front limb of a female Texas blind salamander before and after regeneration. A) Ventral view of stump that salamander came into the facility with prior to surgery, August B) side view of stump prior to surgery, August, C) Ventral view of regenerating limb, Jan 2020, D) side view of regenerating limb, Jan 2020.

Maintenance of Systems

Salamander refugia systems were deep cleaned annually with 20–30% 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

To encourage production of offspring for future research, male and female salamanders were tagged so that collection location is known, and were housed in group systems (Figure 49). Individuals were checked periodically for presence of visible eggs in females. Offspring produced during this combination can be identified by maternal origin but not paternal; thus, these offspring will 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.

Texas blind salamanders produced one clutch of eggs in 2019. In December, a female oviposited a clutch of 8 eggs and 2 survived to hatch. Several wild stock individuals of previously unknown sex became identified as they aged and animals were tagged accordingly. Newly mature females began putting on visible egg mass in the fall, including known-age 2 year olds. At the end of 2019 SMARC held 23 F1 individuals.

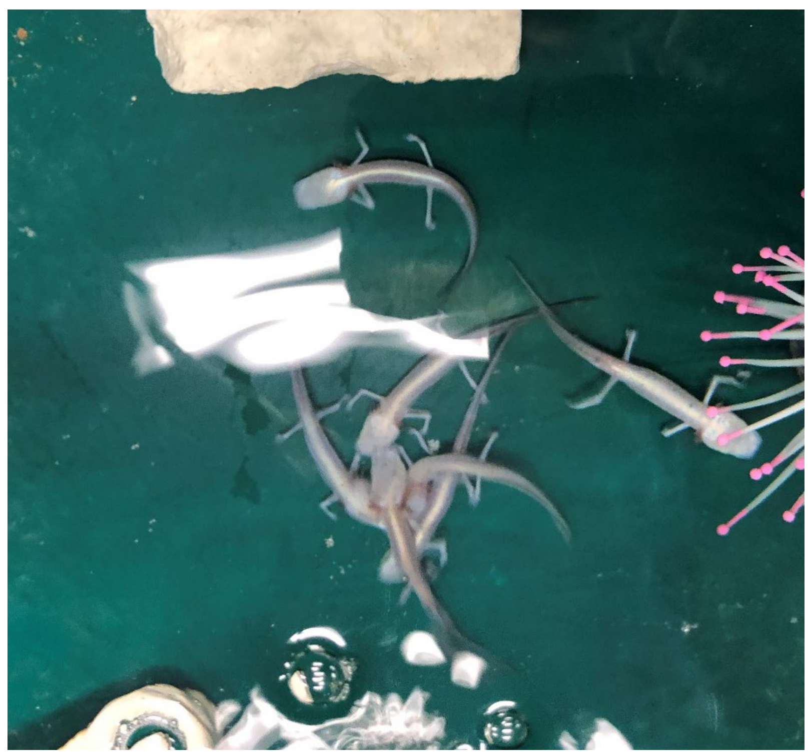


Figure 49 A spout of courting behavior seen in Texas blind salamanders, all rubbing mental glands on each other and initiating tail-straddle-walk.

Figure 50 San Marcos salamander on rock in a tank.

SAN MARCOS SALAMANDER (*Eurycea nana*), THREATENED

The Standing Stock goal for the San Marcos salamander (Figure 50) is 500 individuals divided between the two facilities. In 2019 we met our standing stock goal, divided between the two facilities. One large collection event in Spring Lake took place in March, with additional salamanders being collected from the falls below Spring Lake dam and from Diversion Spring net. We participated in a practice salvage event in June and July during the renovation of Spring Lake Dam, where we collected sufficient numbers of San Marcos salamanders that no additional collections were needed during the rest of the year. We also continued research into producing San Marcos salamander reproduction on demand (see Research section for more details). Numbers incorporated, end of the year census, and survival rates can be found in Table 13.

Table 13 San Marcos salamander refugia population figures.

	Beginning of Year Census	Incorporated 2019	End of Year Census	In Quarantine End of Year	Target Goal 2019 Work Plan	Percent Survival
SMARC	261	269	343*	0	250	77.1% [2] (85.5%, 42.2%)
UNFH	232	177[1]	305	0	250	74.6%

[1] *Incorporated number includes 25 salamanders collected in 2018 and the 50 salamanders transferred from SMARC in October*

[2] *Animals that are not part of the heritage population represent the bulk of the population and experienced a survival of 85.5% whereas the heritage population experienced 42.2% survival in 2019.*

* *End of year census and percent survival reported excludes 50 salamanders transferred to UNFH and 35 salamanders sent to the Washington Animal Disease Diagnostic Laboratory.*

COLLECTIONS

USFWS SCUBA divers collected adult San Marcos salamanders using dip nets from Spring Lake near spring orifices in March (see Figure 51 for locations).

Once a salamander was captured, the dip net was brought to the surface where support staff processed each individual. Staff inspected each salamander for abnormalities, injuries, or lesions. Any abnormal individuals were noted, enumerated, and returned to where they were found. During these collections salamanders smaller than 30 mm TL were returned. 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 its 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). In 2019, the SMARC began running these analyses in-house, saving time of animals spent in quarantine and cost of the analysis. Salamanders were placed into transport coolers with soft mesh for the salamanders to hold onto. Gravid females were kept separately in a small transport cooler. Before coolers were loaded for transport, water was refreshed and the temperature was recorded.

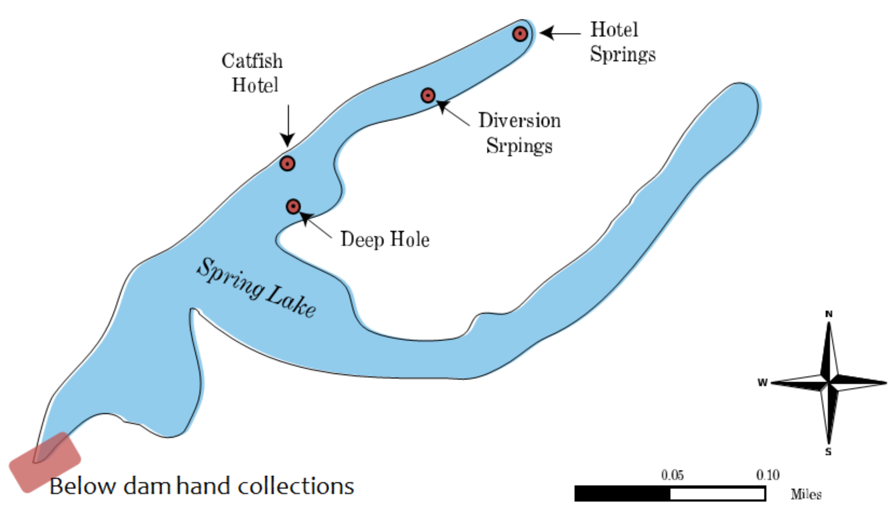


Figure 51 Locations of San Marcos salamander collections in Spring Lake, San Marcos, TX.

Throughout the year, larval, juvenile, sub-adult, and adult San Marcos salamanders were collected in the Diversion Spring net. When quarantine space was available at SMARC, a portion of these were retained for Refugia.

Special collection

In 2019 the SMARC was notified of a Spring Lake Dam renovation project (see Fountain darter section for full details). Refugia staff worked with Zara Environmental, LLC to use this opportunity as a practice salvage event and for staff to collect San Marcos salamanders and fountain darters in areas that were being disturbed.

Figure 52 Staff collecting San Marcos salamanders during Spring Lake dam renovations. Renovation activities can be seen in the top right of the photo.

The original plans for the renovation called for dewatering of the eastern spillway of the dam, but on the first day of the project the plan was changed so that only sections at a time had reduced water flow. Staff went to the dam three times during the project (June–July) to collect animals in areas that had reduced flows or were scheduled to be disturbed Figure 52). Refugia staff used a modification of the Moon-Wirick PCA collection method to more successfully collect San Marcos salamanders–staff worked as teams with one person moving rocks and sifting through substrate and the other person collecting the salamanders with a dip net. Small salamanders (<30 mm TL) were relocated either downstream of the construction area behind the turbidity curtain (still within their natural habitat range) or into appropriate habitat in Spring Lake. Refugia staff from both facilities collected 270 animals as part of this practice salvage event. These animals were incorporated into the refugia after 30-days, negating the need to collect additional salamanders in fall of 2019 and spring of 2020. At the time of the event 61 San Marcos salamanders were taken to UNFH, and in October 50 more of these animals were transferred to UNFH from SMARC.

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 ANS. Salamanders were acclimated to quarantine water conditions over the course of several hours after arrival. San Marcos salamanders collected by SCUBA divers and/or snorkelers were swabbed in the field before being transported back to their respective facilities. Healthy individuals collected from Diversion Spring net were transported back to SMARC where they were measured and those with a TL of 30 mm or greater were non-lethally cotton swabbed. Weak, injured, or very small individuals were not swabbed until they had recovered and/or reached 30 mm TL. 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 continues to decline, but is still found in refugia populations at both facilities. Primarily this has occurred with San Marcos salamanders that are of the SMARC "heritage group" of unknown older ages rather than newly collected and presumably younger individuals. At SMARC, there was a marked difference in survivor rates between San Marcos salamanders that were collected fall 2017 to present compared to those collected before the fall of 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. A portion of salamanders collected in spring 2017 were co-mingled with the heritage salamanders before the procedure of tagging by year collected started, so we cannot differentiate them from heritage salamanders. Salamanders collected in the fall of 2017 to present have not been mixed with the heritage salamanders in tanks or shared water systems. Survival rates of the heritage group of salamanders was 42.2% in 2019 as the older population dies out; we do not expect to have any of this group left at the end of 2020. Whereas survival of the more recently collected salamanders was 85.5%. The makeup of the San Marcos refugium population is shown by year collected in Figure 53.

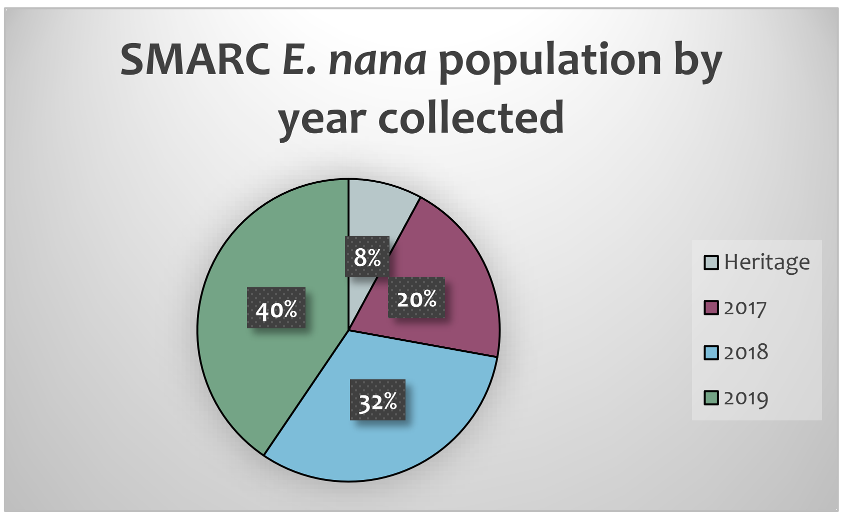


Figure 53 Refugia population make up by year collected at SMARC.

At UNFH during the December hand-count inventory, staff found a loose standpipe screen in a refugia system, which allowed salamanders to escape the via the outflow standpipe. A total of 39 salamanders were missing, lowering the overall survival rate to 74.6%. If these individuals were retained within the system, the survival rate of the San Marcos salamander refugia population at UNFH would increase to 84.1%. Mark Yost discussed proper expectations of staff when setting up systems and when monitoring the security of screens from day to day. All other systems were subsequently checked. Hand-count inventories will be conducted more frequently at UNFH.

Refugia research activities in 2019 found high barium levels in an analysis of captive and wild individuals, with high doses 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 it can cause muscular weakening and can impact reproduction.

HUSBANDRY

Genetic analysis (Lucas *et al*. 2009) determined that there is 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. At UNFH, we plan to institute this type of marking in 2020.

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 °C (ranging between 19 – 22 °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 begin showing symptoms of gas bubble disease, including the presence of trapped air bubbles underneath the skin, bloating, or an inability to stay submerged. If a gas super saturation event occurred, at UNFH adjustments were made to lower the gas saturation within the system by using a globe valve that restricts water flow to the pump, creating a vacuum degassing effect. At SMARC a SOP was written to describe how to reduce gas saturation in the various system setup (SOP can be provided upon request). 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. Feeding occurred Monday, Wednesday, and Friday, varying between live amphipods and live black worms. Juveniles were fed *Artemia* spp. nauplii or chopped blackworms as they increased in size. Once high barium levels were detected in San Marcos salamanders (with the source being blackworms), blackworms were phased out for salamander feeding at SMARC in 2019. Staff rotated in red composting worms fed whole or chopped with amphipods. Staff are culturing alternative food sources, including composting worms and daphnia. At the UNFH Project Leader's discretion, Blackworms were not phased out of feeding 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 San Marcos salamander daily care can be found in the USFWS Captive Propagation Manual for *Eurycea* spp., available upon request.

Those salamanders selected for use in the 2019 tagging study were tagged and observed monthly. Twenty adults were given VIA tags and twenty were given vertical VIE tags. Animals were removed from their tanks, placed under anesthesia, and had their tag presence/absence and readability checked. All animals were returned to fresh well water and, once awake, were returned to original aquaria. Salamanders were observed for injuries or deleterious effects of initial tagging and tag checks. Mass as well as length were recorded every 3 months.

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 20–30% 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 2019 female and male salamanders were housed in mixed groups to encourage reproduction in refugia systems at both facilities. Reproduction can occur year-round as females come in and out of gravidity. Details on the 2019 San Marcos salamander reproduction research can be found in the Research section and in the full report document in the Appendix D.

At SMARC in 2019, wild stock salamanders produced three clutches, all of which were viable (February: 22 deposited, 14 hatched; November: 26 deposited, 17 hatched; and December: 31 deposited, not hatched by end of year). Offspring San Marcos (SMARC) produced one clutch of 36 eggs, 20 of which hatched in November. At UNFH, a wild stock salamander deposited a clutch of 29 eggs, 21 hatched. At the end of 2019, SMARC had 35 San Marcos salamander offspring of varying generations and ages, UNFH held 19 F1 salamanders.

Figure 54 Comal Springs salamanders in collection dish at Spring Island. Photo by Scott Bauer of EAA.

COMAL SPRINGS SALAMANDER (*Eurycea* sp.), PETITIONED

The Standing Stock goal for the Comal Springs salamander (Figure 54) is 500 individuals divided between the two facilities. Staff prioritized efforts in 2019 to collect Texas blind salamanders, as these salamanders are more rarely collected than are Comal salamanders or San Marcos salamanders, and their ESA listing is higher priority. Collections to build the refugia population numbers of Comal salamanders have been limited by lower historical densities of Comal Springs salamanders in the currently utilized sampling locations (compared to sampling locations of San Marcos salamanders). 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 2019	End of Year Census	In Quarantine End of Year	Target Goal 2019 Work Plan	Percent Survival
SMARC	72	25	88	0	80	90.7%
UNFH	18	47	55	0	50	84.6%

COLLECTIONS

USFWS staff snorkeled to collect adult Comal Springs salamanders using dip nets around the Spring Island area of Landa Lake in May and August 2019. 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. 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 (Figure 55). 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 refreshed and temperature was recorded.

Figure 55 Swabbing a Comal Springs salamander for chytrid testing. Photo by Scott Bauer of EAA.

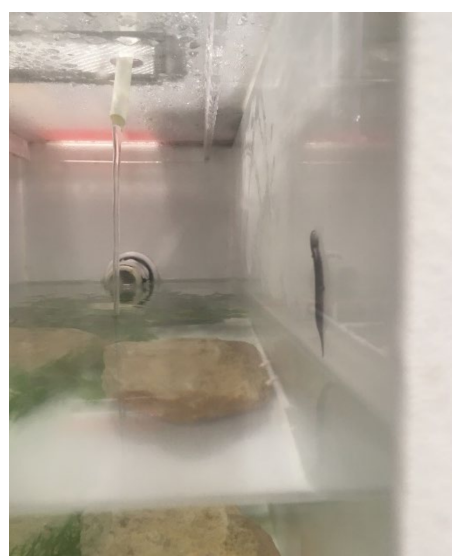
QUARANTINE

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 ANS. 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 high, with few individuals succumbing to sickness. Survival rates in 2019 have improved from previous years. Mortalities of this species tend to be attributed to escapement from their tanks. Refugia staff continue to iteratively modify the Comal Springs salamander tanks to prevent escapement, as the salamanders find different ways to escape tanks. Many designs were tried in 2019 and the current best-method containment unit is made of an acrylic frame covered in mesh and secured around the tank perimeter 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 for extensive periods of time (Figure 56). This occurs more often at night with more than one animal engaging in the explorative behavior simultaneously. One individual, during its quarantine period, escaped its tank twice during the day (after morning checks) into a fountain darter tank below through the water hose input space and then through a narrow bowing of the tank lid. Both were further secured after each incident. The fountain darters were startled and schooled at one end of the tank (alerting staff), but neither species was harmed.

Figure 56 Comal Springs salamander exploring the vertical space in its tank.



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. 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 19–22 °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 begin showing symptoms of gas bubble disease, including the presence of trapped air bubbles underneath the skin, bloating, or an inability to stay submerged. If a gas super saturation event occurred, at UNFH adjustments were made to lower the gas saturation within the system by using a globe valve that restricts water flow to the pump, creating a vacuum degassing effect. At SMARC a SOP was written to describe how to reduce gas saturation in the various system setup (SOP can be provided upon request). 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 seek refuge in. 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 two to three times a week varying between live amphipods and live black worms. Juveniles were fed *Artemia* spp. nauplii or chopped blackworms as they increased in size. Once high barium levels were detected in San Marcos salamanders (with the source being blackworms), blackworms were phased out for salamander feeding at SMARC in 2019. Staff rotated in red composting worms fed whole or chopped with amphipods. Staff are culturing alternative food sources, including composting worms and daphnia. Blackworms were not phased out of feeding 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 San Marcos salamander daily care can be found in the USFWS Captive Propagation Manual for *Eurycea* spp., available upon request.

Those salamanders selected for use in the 2019 tagging study were tagged and observed monthly. Forty adults were given VIA tags and twenty were given vertical VIE tags. Animals were removed from their tanks, placed under anesthesia, and were checked for tag presence/absence and readability. All animals were returned to fresh well water and, once awake, were returned to original aquaria. Salamanders were observed for injuries or deleterious effects of initial tagging and tag checks. Mass as well as length were recorded every three months.

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 20–30% 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 2019 female and male salamanders were housed in mixed groups to encourage reproduction in refugia systems at both facilities. Reproduction can occur year-round as females come in and out of gravidity. Wild salamanders produced three clutches at the SMARC during 2019. In May, a clutch of 14 was produced and six hatched. Salamanders laid a small clutch of 11 eggs in November, 10 of which hatched. Another clutch of 10 eggs was deposited in December and were developing at the end of the calendar year but not yet hatched (Figure 57). At the end of 2019 SMARC held 15 F1 salamanders and UNFH held 10.

Figure 57 Developing Comal Springs salamander in egg.

Figure 58 Diver collecting Texas wild rice tillers.

TEXAS WILD RICE (*Zizania texana*), ENDANGERED

The Standing Stock goal for Texas wild rice (TWR) is 430 plants divided between the two facilities (Figure 58). 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 59). 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 annual Texas Wild Rice Survey. UNFH staff are building their refugia population numbers and representative locations. Numbers incorporated, end of the year census, and survival rates can be found in Table 15.

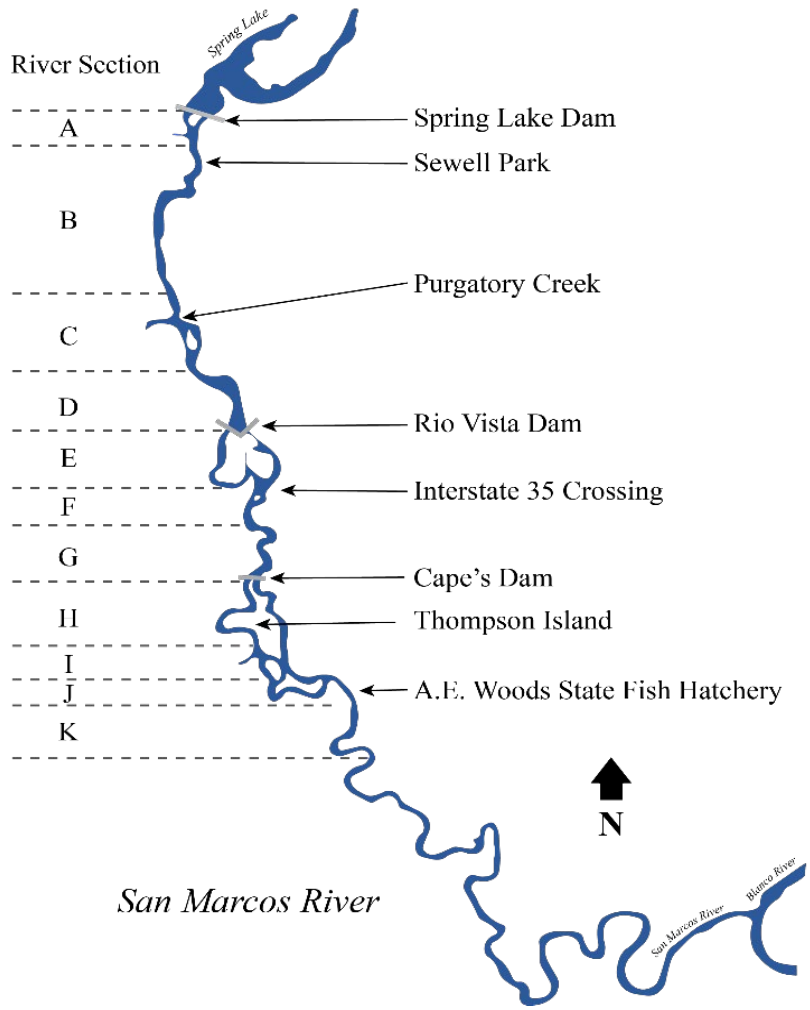


Figure 59 Lettered sections of the San Marcos River designating Texas wild rice habitat established by Texas Parks and Wildlife Department

Table 15 Texas wild rice refugia population figures.

	Beginning of Year Census	Incorporated 2019	End of Year Census	In Quarantine End of Year	Target Goal 2019 Work Plan	Percent Survival
SMARC	213	35	211	10	215	85.1%
UNFH	80	80	157	14	150	98.1%

COLLECTIONS

Tiller collections in the San Marcos River occurred in February, March, April, May, November, and December. Plant tiller collections were suspended during the summer months because heat stress negatively affects survivorship. 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 (WAAS-enabled with 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.

Collections in the San Marcos River occurred in April and December of 2019. UNFH transferred four GPS duplicate plants to incorporate into the SMARC refugia stock.

For UNFH during 2019, four San Marcos River collections of Texas Wild Rice were made. All Texas wild rice plants in refugia were tagged and have an associated field location obtained by Global Positioning System (GPS) and river section.

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 ANS. Salt treatments of incoming tillers (2% salt dip) have been discontinued at SMARC, but continue at UNFH. After consulting with an invertebrate specialist, the SMARC staff concluded that the 2% salt treatments of the TWR was not any more effective at removing *Melanoides* than visual inspection and removal. This consultation was sought after finding that tillers treated with a salt dip had lower survival rates. Staff at UNFH have not reported negative effects from salt treatments. 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 ANS, specifically the invasive snail *Melanoides tuberculata.* After 30-

days, plants at SMARC were un-potted and the full plant visually inspected for ANS, before the tillers were re-potted and incorporated into the standing stock population. In a change from previous quarantine potting techniques at 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 in the tank (Figure 60). 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 period, and once the tillers have taken firm root in the pots, plants were visually inspected again for ANS. The plants were not repotted before being incorporated into the standing stock population and combined into refugia tanks from the same river section.

Figure 60 TWR plants after completing 30 day quarantine at SMARC. Tillers on the left were grown in mesh cylinders show health roots. Tillers on the right grown in traditional pots show signs of anoxia in the root system.

SURVIVAL RATES

Overall survival rate of Texas wild rice plants at the SMARC was 85%, with older plants more likely to succumb to mortality. 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, based on records, is 5.4 years from Section A of the San Marcos River. The overall survival rate of TWR plants at UNFH was 98.1%

HUSBANDRY

We continued to investigate different soil and potting techniques for Texas wild rice plants at 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.

Figure 61 Plecostomus cleaning algal build up from TWR tanks at SMARC.

During September 2019 SMARC refugia staff began a study using phycophagous fish species found in the San Marcos River to reduce algae in Texas wild rice tanks. The goal was to reduce algal growth in the raceways allowing staff to spend fewer hours scrubbing, reallocating that time to other refugia duties. Staff collected Central Stonerollers (*Campostoma anomalum*) and Suckermouth Catfish (*Hypostomus Plecostomus)* to be used in randomly assigned treatments in four raceways: control of no fish, Suckermouth only, Stonerollers only, and mix of Suckermouth and Stonerollers. Data revealed that having only *Hypostomus plecostomus* reduced cleaning time by 4 hours over the month compared to the control (Figure 61). In treatment groups where Stonerollers were included, cleaning times increased.

At UNFH, a photographic, along with a narrative, descriptive system of health assessment was begun to better assess the health and condition of plants in individual pots; a full SOP is expected in 2020. These health categories are listed as "*Healthy,*" "*Marginal,*" and "*Sub-Marginal*" (Figure 62). Plants were photographed to show these condition categories and matched to a narrative description. Plant maps and data sheets were implemented to record plant conditions and pot locations, on a quarterly or bi-annual basis. Pot rotation occurred as necessary, based upon the assessment of the condition of the plant and the best location within each tank for renewed growth.

Figure 62 TWR plants at UNFH categorized as "Healthy" (left) and "Sub-Marginal" (right) with the new system they were developing.

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. SMARC TWR tanks had individual heater-chiller units on tanks with 2HP pumps to circulate water through units and produce flow throughout the tanks. During 2019 three new 2HP pumps were bought and installed to replace older pumps set up prior to the start of the Refugia contract.

At UNFH, the Project Leader preferred a different set up. Pump bags are used on the quarantine pumps to prevent debris from clogging intakes; pumps and pump bags are cleaned regularly of debris. Recirculation manifolds were maintained to facilitate flow throughout the tank, driven by 1/5 to 3/4 HP submersible pumps. Additional 3/4–1 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. TWR leaves were routinely trimmed to approximately 30" 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 both facilities during the summer months to control algal growth in tanks (only over Quarantine tanks at UNFH) (Figure 63). In December 2019, structures were installed above the UNFH refugia tanks that shade cloth will be affixed to during summer months starting in 2020.

Figure 63 Shade cloth being put up over TWR quarantine tanks at UNFH.

CAPTIVE PROPAGATION

The Refugia Program was not engaged in propagation of TWR by sexual reproduction, seeds, in 2019 (Figure 64). The Plant Ecology and Restoration Program at the SMARC was engaged in plant production and continues to study and refine techniques.

Figure 64 New TWR plant produced from a tiller off-shoot found during repotting of TWR plants.

Figure 65 Amelia Hunter installing flow through tubes for the CSRB nutrition experiment.

RESEARCH

The majority of research activities for the Refugia program (USFWS and sub-contractors) focused on invertebrate species in 2019, specifically on Comal Springs riffle beetle adult nutrition and increasing larval pupation and eclosion rates.

CAPTIVE POPULATION NUTRITION AND LONGEVITY OF THE COMAL SPRINGS RIFFLE BEETLE

The full report can be found in Appendix B, below is a summary:

At the SMARC, Comal Springs riffle beetles have always been offered decaying leaves and cloth with biofilm as food items in their holding containers, though no targeted study has investigated their nutritional requirements. Based on a sharp decline in survival of captive beetles around the 5–6 month mark, we south to investigate if there were nutritional deficiencies in the food items offered to the captive beetle populations. In the first line of investigation we offered additional food times to groups of beetles and tracked their survival and larval production over time (Figure 65). For our second line of investigation we partnered with Dr. Camila Carlos-Shanley at TXST to compare the microbiome and bacterial diversity of Comal Springs riffle beetles' guts between wild versus those held in Refugia.

We found slightly elevated survival rates for beetles offered conditioned wood, but the values were not statistically significantly different from controls. However, twice the number of larvae were produced or survived in the wood treatments than in the other two treatments. We plan to add conditioned wood to our adult and larval riffle beetle containers going forward.

Microbiome analysis found differences in the number of unique genera of bacteria between wild and captive beetles. In total, 30 genera belonging to four phyla have been identified. Of these, 11 were found only in wild beetles, 23 only in captive beetles and 8 were found in both groups. The diversity of culturable gut bacteria in wild beetle microbiomes was lower than their captive counterparts at the genus level. The percent relative abundance of each major genera (n >1) in wild and captive beetles demonstrates the difference of major and minor genera in each microbiome (Figure 66). The largest percentage of the captive microbiome is made of 14 low abundance genera that are not present in the wild microbiome. Alternatively, the wild microbiome contained three low abundance genera that are not present in the captive beetle microbiome. Gene sequencing of the bacteria isolates will continue into 2020.

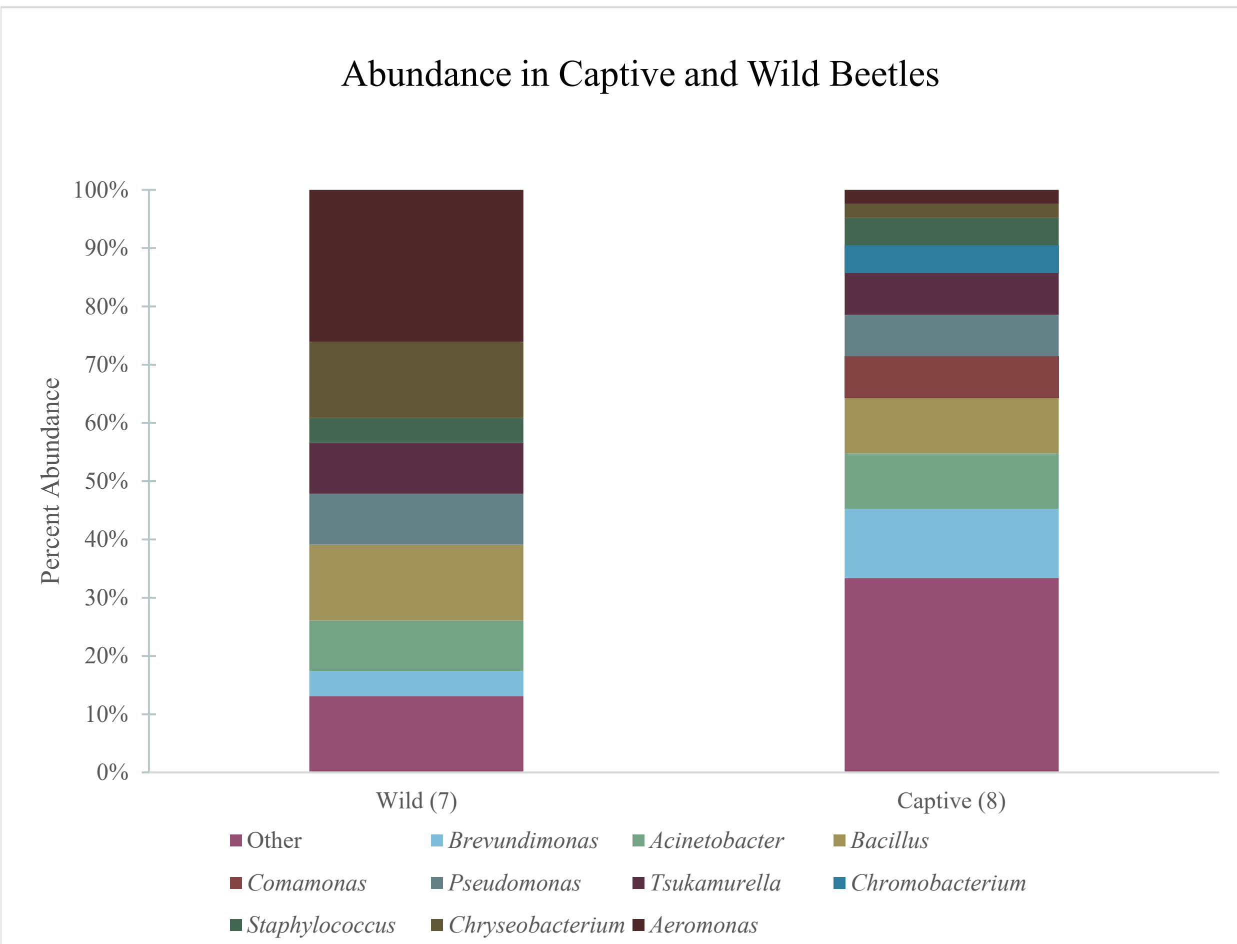


Figure 66 Percent relative abundance of the culturable bacteria within the wild and captive beetle microbiome.

INTERIM REPORT ON EVALUATING THREE DIFFERENT LONG-TERM TAGGING METHODS IN AQUATIC SALAMANDER SPECIES

The full report can be found in Appendix C, below is a summary:

The ability to individually mark captive salamanders increases specificity of record keeping and allows us to follow information of an individual over its lifetime. Additionally, it allows for consolidation of specimens, increasing the probability of mating success, and simultaneously affords efficiencies in refugia operations by reducing the number of systems needed to maintain. Identifying individuals and knowing the parentage of offspring is vital in many conservation, propagation, and genetic management plans. This research is the first to evaluate the efficacy of three tagging methods in fully aquatic salamander species and compare them side by side for readability and retention. We used Visible Implant Elastomer (VIE), Visible Implant Alphanumeric (VIA), and Passive Integrated Transponders (PIT) in Texas blind (*Eurycea rathbuni*), San Marcos (*Eurycea nana*), and Comal Springs (*Eurycea species*) salamanders. In each of the three species we selected 20 individuals to test each tagging type, with exceptions for animal safety, and collected photographic, total length, snout to vent length, and weight data. PIT tagging (Figure 67) was only completed in the larger (Texas blind salamander) species, as we had safety concerns for the other two smaller species. VIA tags were not successful in the San Marcos and Comal salamanders with only one tag retained after 2 months. Overall, individually marking salamanders with vertical line VIE color combinations had the highest readability and retention scores of all three species of aquatic salamanders verses VIA and PIT tags. These results are only from the first six months of the study. A full year is planned to study the tag types on the species. A final report will include all findings from following individuals for a full year. Our goal in the future is to implement the most successful tagging method to our salamander refugia programs.

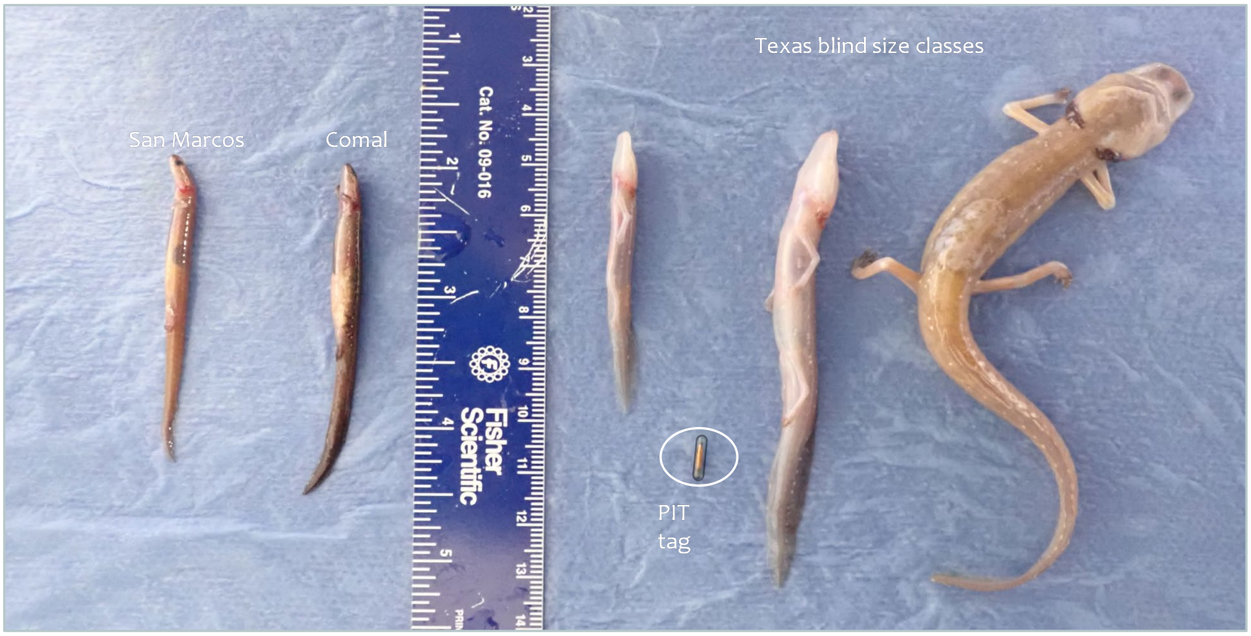


Figure 67 Species and size range of salamanders tagged for the study, with a PIT tag for size comparison.

SAN MARCOS SALAMANDER REPRODUCTIVE DYSFUNCTION

The full report can be found in Appendix D, below is a summary:

San Marcos salamanders (*Eurycea nana*) are a federally threatened aquatic species endemic to San Marcos, TX and to the Edwards Aquifer. While this species has been held on station for years as part of a captive assurance colony, we cannot predictably produce offspring. We continue to investigate potential causes for this perceived disrupted reproductive behavior in captivity with three lines of investigation. Based on recommendations from the 2018 reproduction study, we conducted a pilot study in 2019 using only group mating and removal of males after 72 hours. Aside from this, we filmed one pair and one group from three angles to capture spermatophore deposition on video. Overall, engagement in courtship for this trial was low with animals engaging in more exploration than mating. Females were more engaged than males and courted without male participation. When males were involved, they walked the tank for over 45 minutes (an unusually long time) without deposition. This lack of courtship overall produced no ovipositions. Additionally, we tested our water quality for endocrine disrupting compounds and other deleterious compounds. For the third line of investigation, we sacrificed female individuals from wild and captive populations for toxology and histopathology to assess potential reproduction inhibitors, such as vitamin deficiencies, heavy metals, toxins, and/or disease. Initial findings show increasing levels of barium in captive individuals. Micropsoridial infection rates were much lower and less acute in wild populations. Micropsoridial infections tended to be concentrated in the ovaries and reproductive organs of the salamanders. Further studies are needed to investigate, or remedy problems discovered with these findings, and to improve the health of captive populations. Initial consultation with a salamander reproductive specialist, Dr. Ruth Marcec-Greaves, suggested hormonal studies and altered reproduction trials for the future.

INTERIM REPORT ON COMAL SPRINGS RIFFLE BEETLE (HETERELMIS COMALENSIS) PUPATION ENHANCEMENT – BIO-WEST, INC.

The full report can be found in Appendix E, below is a summary:

Project Goals and Objectives

The goal of this project is to better understand the conditions that lead to successful pupation of the riffle beetle *Heterelmis comalensis*.

Study objectives:

a. Reexamine the utility of flow-through tubes (Figure 68) specifically for enhancing successful metamorphosis.
b. Determine if starvation may encourage pupation after a period of being well fed.
c. Test the effectiveness of exposing mature larvae to terrestrial conditions.
d. Construct and test the efficacy of an apparatus that emulates bubble stream conditions seen at Comal Springs.
e. Assess beetle fitness resulting from different pupation methods.
f. Implement adaptive management considerations to enhance outcomes.

Figure 68 Example of a flow-through tube with resource/habitat packing materials.

INTERIM REPORT ON FACTORS AFFECTING PUPATION IN THE ENDANGERED COMAL SPRINGS RIFFLE BEETLE – TEXAS STATE UNIVERSITY, DR. NOWLIN LAB

The full report can be found in Appendix F, below is a summary:

As a part of the Edwards Aquifer Habitat Conservation Plan (EAHCP), the USFWS is tasked with maintaining off-site populations of endangered and imperiled species covered under the HCP. These populations primarily serve as "refuge" populations, but the USFWS and collaborators have also performed research in the lab examining the life history and ecology of these organisms. Despite these efforts, there are still substantial questions and issues associated with many of these taxa which currently impede the ability to maintain captive populations. Given the requirement to maintain sustainable captive populations, there is clearly a need to examine factors which may contribute to the successful pupation of CSRBs in captivity. The number of studies examining the CSRB has intensified over the last several years and have provided insights regarding this organism's life cycle and trophic ecology. Recent experiments conducted by the Nowlin Lab at Texas State University and collaborators (USFWS personnel and BIO-WEST, Inc.) have provided information on the life history, trophic ecology, and environmental tolerances of the CSRB. These research efforts identified quantitative and non-invasive methods for sexing adult beetles, quantified the number and head capsule widths of CSRB larval instars, determined egg laying preferences, adult and larval food preferences, and conducted preliminary experiments on factors affecting pupation. Those research efforts found that CSRB are particularly sensitive to changes in water temperature and dissolved oxygen (DO) concentrations and that there were likely substantial nutritional constraints to larval growth and development that may also affect pupation rates in captivity. We proposed to examine several factors which may contribute to successful pupation and emergence of adult CSRB in a captive setting. Specifically, we propose to examine several factors in captivity and has two main research goals:

1) How does the origin (wild or lab-grown biofilms) and nutritional and microbial composition of biofilms utilized by CSRB larvae affect pupation and adult eclosion rates in captivity?

2) Does the presence of conspecifics (CSRBs) affect the quality (i.e, microbial composition and nutritional value) of biofilms utilized by CSRB larvae prior to pupation?

2) Does the presence of conspecifics (CSRBs) affect the quality (i.e, microbial composition and nutritional value) of biofilms utilized by CSRB larvae prior to pupation?

Progress Summary

(1) Examination of the role of microbial composition and origin of OM types on CSRB pupation rates

Growth chambers and experimental set-ups (including the "conditioning" of food materials) were constructed starting in February 2019. Experiments were conducted starting in April 2019 and are now concluding (December 2019). At this point, the last group of larvae are in housing chambers and we are tracking their development.

All samples for biofilm have been collected and sent off for sequencing and we have refined methods for nutritional quality analysis.

Preliminary results indicate that we have had considerable success rearing larvae and getting them to pupate (~40 pupation events); however, there is substantial mortality associated with pupae in captivity. At this point only 4 adults have eclosed from pupal cases. We are in the process of conducting data analysis on rates of larval mortality, pupation, pupal mortality, and successful adult emergence for both "wild" and "lab" grown biofilms. The last group of larvae are being tracked, but we expect to conclude these experiments in the next ~2 months. Biofilm analysis and data analyses are expected to continue for the remainder of the project.

(2) Determination of the role of presence CSRB grazing on the microbial composition and nutritional composition of OM biofilms

Experimental set-ups and "conditioning" of organic matter sources started in October 2019. Will begin larval experiments in December 2019. Expect the experiments to conclude in April or May 2020, depending on rates of larval development and pupation.

BUDGET

Task	U.S. Fish and Wildlife Service 2019	Budget Spent	Total Task Budget Spent
1	**Refugia Operations**		**$1,270,034**
	SMARC Refugia & Quarantine Bldg.		
	Construction	$137,245	
	Equipment	$37,442	
	Utilities	$2,807	
	UNFH Renovation Refugia & Quarantine Bldg.		
	Construction	$239,968	
	Equipment	$104,828	
	Utilities	$13,413	
	SMARC Species Husbandry and Collection	$173,595	
	UNFH Species Husbandry and Collection	$219,023	
	Water Quality Monitoring System	$52,123	
	Fish Health Unit	$8,219	
	SMARC Reimbursibles	$37,489	
	UNFH Reimbursibles	$59,346	
	Subtotal	*$1,085,499*	
	Admin Cost	*$184,535*	
2	**Research**		**$271,428**
	BIO-WEST: CSRB pupation	$73,740	
	BIO-WEST: Peck's Cave amphipod life history	$16,962	
	BIO-WEST: Dryopid life history	$20,856	
	TxSt: CSRB pupation	$45,766	
	USFWS Research Projects	$120,431	
	Subtotal	*$231,990*	
	Admin Cost	*$39,438*	
3	**Species Propagation and Husbandry**	$0	**$0**
4	**Species Reintroduction**	$0	**$0**
5	**Reporting**		**$67,047**
	SMARC Staff	$39,932	
	UNFH Staff	$17,373	
	Subtotal	*$57,305*	
	Admin Cost	*$9,742*	
6	**Meetings and Presentations**		**$8,436**
	SMARC Staff	$4,688	
	UNFH Staff	$2,522	
	Subtotal	*$7,210*	
	Admin Cost	*$1,226*	
		TOTAL	**$1,616,945**

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APPENDICES

A. 2019 Refugia Work Plan
B. Captive population nutrition and longevity of the Comal Springs riffle beetle
C. Evaluating three different long-term tagging methods in aquatic salamander species
D. San Marcos salamander reproductive dysfunction
E. Comal Springs riffle beetle (Heterelmis comalensis) pupation enhancement – BIO-WEST, Inc.
F. Factors affecting pupation in the Endangered Comal Springs riffle beetle – Texas State University, Dr. Nowlin Lab
G. Fountain darter Quarantine Standard Operating Procedures
H. Juvenile Eurycea Collection Standard Operating Procedures
I. Fish Health Unit Reports
J. Monthly Reports