Standard Operation Procedures for the Comal Springs dryopid beetle, *Stygoparnus comalensis*

Updated 2017

Daily Activities

1. Log water temperature and pressure
   1.1. Find datasheet on a clipboard above the desk and take the thermometer to the sink.
   1.2. Run the water for about 20 sec and then fill any beaker greater than 400mL and let it overflow.
   1.3. While water is overflowing and still running, take a temp reading and record the value on the datasheet.
   1.4. Pressure gauge will be on the same nozzle in the sink. Make sure water is on full before taking a reading, if pressure reading deviates from normal check the pressure gauge outside the invert room in the greenhouse. Change in pressure can be the first indication that there may be problems with flow in the containers.
2. Check for inflow in all containers. If flow is either too high or too low first refer back to the water pressure reading to see if it is abnormal before adjusting any valves. See also the Container Issues and Resolutions section for further trouble-shooting.
3. Check the outflow standpipes in all containers and remove debris with designated toothbrush for the container.
4. Check the poplar wood dowels in the containers. Remove any fungal growth as fungus can cause entrapment and death of Comal Springs dryopid beetles (CSDB).
5. If mortalities are seen preserve individual in 95% EtOH in small glass vial with appropriate labeling on Rite-In-The-Rain paper slip inserted into vial.
6. Check for flooding, water puddles, and/or drips.
7. Clean floors, desks, equipment, and tools.
8. Work spaces must be neat and tidy. Make sure walkways are clear. Dry containers and desk with a rag. Put away tools in proper place and make sure the light is turned off at the end of the day.

Weekly Activities

1. Wear the correct PPE. Replace disinfectant (e.g. Virkon®) in mats by the door. Mix chemicals outside of the invertebrate room (see Virkon Biosecurity section for details) and carefully fill the mats making sure not to overfill. Virkon is highly toxic to invertebrates so be sure not to mix chemicals inside room and that it does not spill out into the room or come into contact with containers.
2. Check for $CaCO_3$ deposits interfering with valve functionality and/or water flow. Obstructions should be removed or valves replaced as needed.
3. Check all water quality parameters using the one of the water quality meters available. Use the same method as Daily Activities #1 but with a beaker large enough to fully immerse the probes. Record on the data sheet on same clipboard as the daily log. Check a random flow through container weekly for water quality parameters using test strips or meter if box is deep enough.
4. Check chlorination system.

Monthly Activities

1. Record all inventories on data sheets, keep track of Daily Log datasheet, and transfer both to appropriate files using the folders on the local drive. Make sure to scan the hard copies of daily and weekly logs (and all data sheets) and save these files.
    1.1. Keep a copy of these records on another computer. This prevents loss of data in case of a system failure and serves as a backup to paper documents.
2. The Deputy Director requires a monthly total of each species completed by close of business on the Wednesday of the last week of the month:
    2.1. Report total wild stock individuals in refugia, total in quarantine, total number of individuals that were captive bred, and the number of mortalities in refugia.
    2.2. Other numbers and data not required by the monthly report still need to be collected and reported, just not for this particular monthly report.
3. Update documents
    3.1. Online documents
        3.1.1. HACCP plans and logs
        3.1.2. Field collections

3.1.3. EAA species records

3.1.4. Outside take records

3.1.5. Inventory

3.1.6. Daily Water Quality and Feed logs

3.2. Paper copies are to be scanned and stored on the local drive or transferred to the correct filing excel sheet system in place for EAA contract. Keep backups of all documents on a separate computer or drive.

4. Check A/C unit
   4.1. Clean/replace the air filter
   4.2. Clear debris. Remove the filter and clean with a water hose if necessary.
5. Clean walls
   5.1. Wear the correct PPE. Fill a bucket with a bleach solution and use a mop to cleanse and rinse the walls to prevent molding. Focus attention to the doors and ceiling near windows. Avoid areas around refugia containers to prevent drips or spills into containers.

Yearly Activities

1. Keep good records of monthly collections into the Federal and TPWD collections permit log. Submission is based on the calendar year for both, but with different start and endpoints. For example the Federal permit is due in December while the TPWD permit is due in June.
2. Keep good records of monthly files to utilize and compile for SMARC station annual reports; submission is for full fiscal year.
3. Same files are used for the EAA annual reports; submission is for the calendar year.

COLLECTION AND INITIAL QUARANTINE

Comal Springs dryopid beetles (CSDB) are collected using poly cotton cloth lures (Figure 1a), conditioned hard wood dowels (Figure 1b), and by hand collection off rocks and larger conditioned logs in vicinity of upwellings at Comal Springs, New Braunfels, Texas and sampling with drift nets at Sessom Creek in San Marcos, Texas. Cotton lures and wood dowels are to be placed directly on an upwelling- less than half of the height of the lure buried with several rocks and/or identifiers to weigh down the lure(s) and prevent escape and predation from outside sources. Lures that are placed in a terrestrial margin are to be placed as far back, closest

to the source of flow, as possible. It is acceptable for it to be half buried within the stream bottom substrate and/or touching roots, but exposed area must be covered with rocks. Do not bury the lure(s) if the substrate is primarily silt, this will cause anoxic conditions over time. Sand grain size or larger is preferable.

Cotton lures primarily attract Comal Spring riffle beetles, but may attract dryopid beetles, depending on location set. CSDB have been found in areas where roots or woody debris are present and vary in depth from lake floor to as shallow as the riparian divide. Currently, up-wellings are the most effective location to collect adults.

The cotton lure assemblage specifications can be found on the station's shared hard drive ("local drive") under SOPs folder named the "Comal Springs Riffle Beetle Cotton Lure SOP". Blank data sheets for each cotton and wood lure can also be found on the local drive.

Upon collection of a retrieved lure, open the wire cage and unfold the lure (Fig. 1a) to count and separate species and life stages. SMARC has modified the data sheet from the Comal Springs Riffle Beetle Cotton Lure SOP to fit our needs as a station for data collection. Use small plastic pipettes for larvae or soft forceps for adults when separating/counting. After species identification is verified, move individuals to a target container (e.g. Polypropylene jar with lid ranging in sizes from 200 ml – 1 L). Separate each species and place organisms for collection in different holding containers. The holding containers are then moved to a transport container (small drink coolers filled with spring water to maintain temperature during transport).

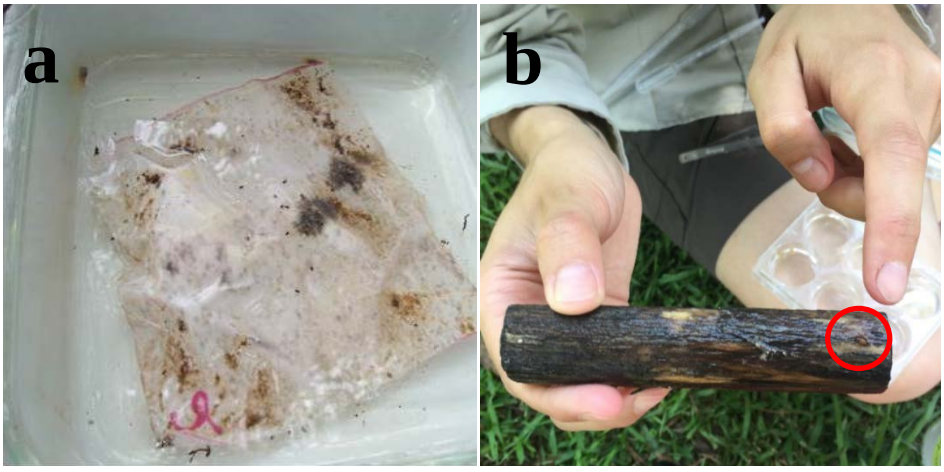


Figure 1 Cotton cloth lure with riffle beetles near center (a) and a wood lure with a dryopid beetle (b).

After arrival back at the station, compare the water temperature inside the transport container that is holding the invertebrates from the field to the water temperature within the appropriate destination container; you may use the invertebrate room sink water temperature as a proxy if needed. Acclimate the water temperature as necessary before introducing the newly collected species into their designated quarantine container within the SMARC invertebrate room. A rule of thumb would be changing the water temperature 1 °C every half-hour; however, no studies have been done parameterizing acclimation speeds for these invertebrates, more research is warranted. Collections in refugia must be separated by spring systems (i.e., Comal Springs system vs. Sessom Creek system). This applies to quarantine, wild stock, and F_1 containers.

Organisms are kept in containers marked "Quarantine" for 30 days before joining containers of Refugia population. During this time we observe for other potential ANS species that might have come in with the collection. Observe the general health of the organisms and watch for large die-offs that might indicate a disease. If none of these occur, then they can be moved to the Refugia population at the end of the quarantine period.

CULTURE SYSTEMS

Easy-Spring Culture Systems

Culture containers currently favored at the SMARC for CSDB are Easy-Spring model systems. This is a spray bar type system (Figure 2) uses a single spray bar and one or two horizontal outflow standpipes. Designed and specially constructed by SMARC staff in order to better replicate the hydrology of Comal Springs, the Easy-Spring systems have thus far provided success in survival and potential reproduction. These systems have been run completely on flow-through Edwards Aquifer well water, but could easily be modified to accept re-circulating water.

Easy-Spring culture containers are constructed from plastic tubs/totes (Figure 2). Easy-Spring containers have holes drilled in the sides to allow for adaptors/parts to be inserted for water inflow and outflow. Containers should be assembled using PVC glue and/or aquarium

safe silicone, allowed to cure-dry, and water run through them for a period of time to flush out any potentially harmful residual chemicals before organisms or habitat are added (this is also a good time to check and fix leaks).

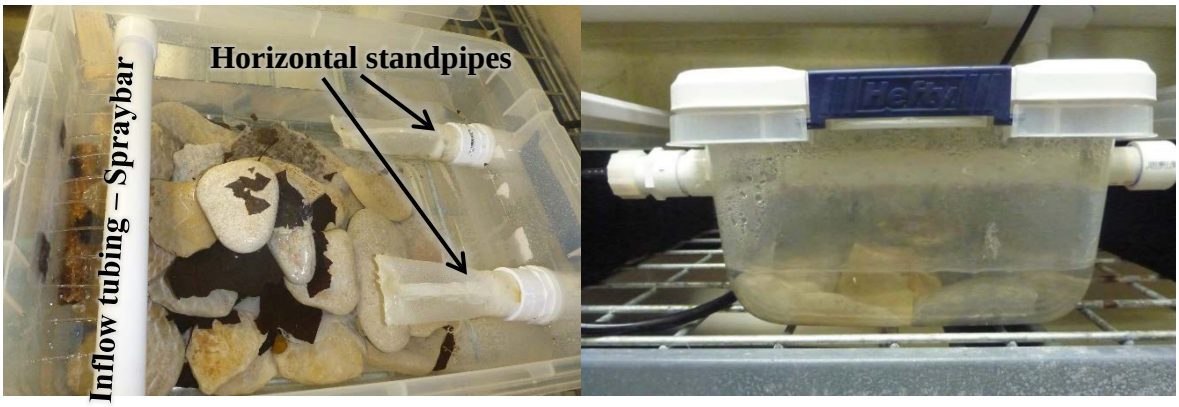


Figure 2 Easy-Spring Culture Container (Spray bar model).

Holes in the front side near the top allow piping to provide water into the system via a spray bar. The spray bar has 1/16 inch (1.6 mm) holes drilled evenly spaced along a horizontal line. The spray bar is pointed so that the water sprays onto the side of the container, not down into the container or out over the surface. The intention of the inflow spray bar design is to simulate the hydrology of spring orifices at Comal Springs in which water moving down the side of the container will move in a laminar fashion through the culture medium into the standpipe. Water level should be as low as the container will allow (this is determined by the position of the outflow standpipes). Ideally, the water line should be less than half the height of the container in order to allow sufficient terrestrial space for potential egg deposition and larval development. This is necessary because the CSDB life cycle likely includes terrestrial stages, which is consistent for all other beetles in the family Dropidae. One or two short outflow standpipes used for drainage are attached horizontally and low in the container (Figure 2). The outflow standpipes are 3/4 inch Schedule 40 PVC. The standpipes are capped with 300-400 µm mesh, held by hot glue, to prevent escape of specimens and smaller life cycle stages.

After containers are made, habitat items can be assembled in the container. It is best to put the container in its place on the rack system and get the water lines hooked up (but not filling) before adding rocks, leave, and dowels, then fill with water. This prevents items from shifting while the container is being moved. A more detailed description of contents is detailed in the "Refugia Container Contents" section. In addition, organisms should only be placed in the

container after it is secured on its shelf, habitat is in place, and water has filled the container.

Culture containers should be kept dark to mimic the underground environment. This may be accomplished by partially covering containers with black plastic or shade cloth. Bottoms and sides of containers may require shading as well to prevent light from causing distress.

Refugia Container Contents

Container habitat includes rocks with pebble legs and/or limestone rocks, a conditioned poplar wood dowel directly below the spray bar, new and conditioned leaves, and new and conditioned poly cotton-cloth.

- Limestone rocks (and all rocks) should be cleaned and fully dried before using in containers to prevent the potential transfer of other species.
- To condition a dowel, place several dowels in a container with running water (or a sump) to soak and eventually become negatively buoyant (it must sink). The dowel must not float before it is put in the container, this can take several month to achieve.
- The leaves must come from a terrestrial source (not an aquatic source) so that accidental introduction of non-target aquatic organisms is minimized. These leaves should be collected from trees around the river system that the organisms inhabit and can include, but are not limited to: Anaqua, Sycamore, Pecan, or Black Walnut. Leaves should be quarantined for at least 2 weeks (following corresponding HACCPs). Leaves should be dried in the drying oven and then can be stored in a dry, sealed container until they need to be used. Leaves may be used by the organisms as cover or as a potential source of food. When conducting inventory, save some of the leaves from a container to use when placing habitat back into that container.
- Cotton cloth is used in the field to attract CSDB for monitoring efforts. It has also been found to be a useful food source in culture for CSDB. Conditioned cloth, that is cloth that has been colonized by biofilm, can be added to the container. You can save conditioned cloth from a container as you are conducting inventory

and add back when re-assembling the habitat. Alternatively, condition cloth can be added by placing new strips in a container with flow-thru well water, but no organisms, for several weeks to allow biofilm to colonize. Additionally, new cotton cloth is added for cover and so that it can start the process of colonization by biofilm.

To begin habitat assembly (after container is in designated location) put a layer of smooth rocks with pebble legs on the bottom of the container. The pebble legs allow for water flow underneath the habitat, reducing anoxic spaces. Then begin layering leaves between limestone rocks. Leaves should not be stacked too densely in one spot as they can reduce the flow of water and create anoxic areas. Cotton cloth strips (both conditioned and un-conditioned) can be placed near, but not stacked with, the leaves as to avoid reduction of water flow and anoxia. The conditioned dowel should be placed under the spray bar; if needed, a small rock could be placed on top of the dowel to hold it in place. Once habitat is constructed, turn on the water flow and allow the container to fill. Observe for any problems or adjustments that need to be made. Again make sure there is some habitat above the waterline in case terrestrial space is needed for parts of the lifecycle. Add in the organisms. Containers may also be completed and water running weeks before invertebrates are added so the further conditioning and colonization by biofilm can occur.

Suggested Maximum Capacity per Container (these numbers have not been experimentally tested, just a basic guideline, mainly make sure there is adequate flow).

Container	CSDB adults
Small (9 qt)	15
Medium (15 qt)	40
Large (34 qt)	100

WATER QUALITY

Water quality values (see table below) should be checked weekly, unless problems arise

and then should be checked more frequently. These values given here are a rule-of-thumb based on observational and anecdotal evidence, and are not necessarily backed by scientific analysis or experimentation. If water quality deviates from normal see "Container Issues and Resolutions" section for potential trouble-shooting or seek help from other biologists as to ways to correct the situation and be sure to inform your supervisor of changes and potential problems.

Metric	"Safe Range"
Temperature	18-24 °C
Dissolved Oxygen	6-8 mg/L
pH	7-8.2
Total dissolved gas	≤ 100%
Ammonia	< 0.05 mg/L

Temperature should be monitored daily. Temperatures above 24 °C might cause stress and possible death to dryopid beetles and chilled well water should be added in addition to non-chilled well water as sources for these systems in the event temperatures begin to reach these danger zones. Typically in late summer these actions will need to be implemented for all invertebrate species. It's important to have a backup recirculation system with a heater/chiller and a pump that can recirculate water from the sumps in the event flow-through water quantity or quality is insufficient for Edwards Aquifer invertebrates.

FEEDING-RELATED PROCEDURES

It is assumed that CSDB eat the leaves and biofilm on cotton cloth placed in the container during habitat construction. Cotton cloth should always be used in combination with leaves and both replenished with new sources within a two month period after completion of the inventory or the last container clean-out. Ratio of leaves to cloth should be about 4:1. At this time no additional food, such as fish flake food, is added to dryopid beetle containers.

PRODUCTION

Production has been seen rarely in SMARC refugia so the following instructions are preliminary in the event of early stages are found during inspection or inventory. If larvae or

eggs are found they should be noted whether they were above or below the water line and moved on the substrate they are found (if they are not free floating) to their own container for further monitoring and grow-out. It is assumed in comparison to other dryopids that part of the life cycle requires the larvae to crawl out of the water in order to pupate. Whether this is true for CSDB is unknown. In the event larvae are found, monitor them weekly or every other week, depending on how sensitive they are to disturbance. Record all notes.

CLEANING SYSTEMS

Containers with dryopid beetles generally are deep cleaned whenever the container must be disturbed (e.g., bi-monthly inventory or removing young). This is cleaning that goes beyond the routine daily care and cleaning of flow bars, outflow standpipes, and checking for fungus on dowels. Since the debris on the bottom may contain offspring, it must be carefully examined by pouring into a sieve before discarding the debris. Several stages of CSDB can cling to the underside of small leaves, cloth, out-flow cover mesh, or rock pieces and need to be checked before cleaning or discarding these pieces.

In order to remove $CaCO_3$ deposits without toxic chemicals, rinse empty containers with well water in the sink and scrape or wipe the containers and/or spray bars using brushes and sponges. If $CaCO_3$ deposits cannot be removed or are particularly bad, a replacement container or parts may be required. Any valves or tubing needs to be checked for $CaCO_3$ deposits and functionality and should be replaced if damage or obstructions are found. *Do not acid-wash*: most chemicals on station are not safe for aquatic invertebrates.

- Do not use bleach
- Do not use acid (e.g., HCl)
- Do not use Virkon

If a container or components have particularly stubborn $CaCO_3$ deposits they can be soaked in a vinegar and water solution to loosen up the deposits. The containers should be triple rinsed and allowed to completely air dry before using again for invertebrates.

VIRKON® BIOSECURITY

A blue disinfection mat is located at each entrance to the invertebrate room. These mats

help reduce the spread of nuisance species both into and out of the invertebrate room. Both mats should be refilled weekly with 1% Virkon® Aquatic solution. Each mat holds approximately 2.65 gallons of liquid. Record date Virkon® solution changed on data sheets located in invertebrate room notebook. If mats becomes dry during the week, add water as needed.

CONTAINER ISSUES AND RESOLUTIONS

1. If the floor is wet, there is probably a slow drip leak or water gushing somewhere. Find the source and adjust to cease.
    a. A nozzle may need to be tightened on either the inflow spray bar or upwelling connector or from the water inline on the back wall.
    b. Check outside the greenhouse door to see if a Texas wild rice refugia outflow pipe has been dislodged or moved away from the drainage system on the floor. The invertebrate room is not level and water from outside the room can travel under the greenhouse door inside the invertebrate room. Sand bags are in place outside the greenhouse door as a precaution, but are not a fail-safe.
    c. Check with other technicians and supervisors to see if any large alterations in water usage have occurred: such as starting or draining a pond, well location switchover, immense well water pressure change, power was down and came back on, water turned off intentionally, break in the water line, etc. Any minor adjustments or changes in water usage at SMARC have the possibility to affect the invert rooms well water pressure. The installation of a water pressure regulator valve on the water line into the invertebrate room (after the area where non-chilled and chilled water are mixed) is designed to reduce high pressure spike issues.
2. Water levels in containers
    a. If the water line is above the outflow bar or stand pipe:
        i. Check for clogging and use a toothbrush or a pipette to remove the debris. Debris larger than a quarter can be placed under a rock or on top of rocks in the “terrestrial” portion of the container. Do not under any circumstance siphon the container for debris as various life stages could be in this debris and accidentally discarded. If you see a pocket of fungus covered or anoxic debris you can remove it with a 3 ml pipette, but be sure to check the material on a petri dish for organisms before it is disposed of.

ii. Flow into the container may also be too strong and turn the nozzle down and adjust the flow to a slower rate and see if that reduces the water line back down to the correct level.

b. If container has water overflowing outside the lid:
    i. Check the outflow bar or stand pipe for same conditions as 2a. Another reason could be that the flow bar is rotated to a position that causes it to spray water on the lid or out of the lid instead of in its proper stream alignment on the inner wall of the container or slightly downward hitting the water line directly.

c. If there are slow drips:
    i. Make sure the thread seal tape is not damaged or nonexistent and replace with new tape. Make sure gaskets are in place and functional. Also, check the PVC fittings are flush to the PVC pipe on the outside the container may need to be glued to prevent drips from occurring.

3. Change in water quality
    a. Increased/ Decreased Temperature:
        i. If temperature has decreased under 18 °C, levers outside the invertebrate room in the greenhouse need to be adjusted to allow more non-chilled well water to stabilize the temperature. Check the pressure before and after making adjustments, the pressure should be between 10-13 psi, over 13 psi is too high. First, have a hammer or rubber mallet to gently raise well water lever (yellow) and gently lower the chilled well water lever (blue) on. No water source lever will be on at full capacity; it is a mix of both levers to gently adjust the temperature. Changes can be tested in the sink with a temperature gun relatively soon after alterations. Drastic changes from hot to cold can stun CSDB and possibly result in death, so be careful and use gentle taps to adjust the amount of each water source.
        ii. If temperature has increased to over 24 °C, levers outside the invertebrate room in the greenhouse need to be adjusted to allow chilled well water to stabilize the temperature. Check the pressure before and after making adjustments. The pressure should be between 10-13 psi, over 13 psi is too high. First, have a hammer or rubber mallet to gently lower/turn off well water lever (yellow) and gently raise the chilled well water lever (blue) on. No water source lever will be on at full capacity; it is a mix of both levers to gently adjust the temperature. Changes can be tested in the sink with a temperature gun relatively soon after alterations. Drastic changes from hot to cold can stun CSDB and possibly result

in death. So be careful and use gentle taps to adjust the amount of each water source.

iii. If these changes do not cause a great enough effect to change the water temperature to within the safe range, the recirculation system connected to the heater/chiller unit and the sumps may need to be initiated.

b. Increased Dissolved Oxygen:

i. Refer to the Supersaturation SOP on the local drive to address this immediate issue. Bubbles will be visible in all containers and a change in pressure will occur. Refer to the supersaturation diagrams above the sink in the invertebrate room to know which valves to turn off and/or on. Have the heater/chiller and pump ready to recirculate water from the sump if the supersaturation event is prolonged over 8 hrs. There is not much else you can do to prevent the bubbles from appearing. Use a toothbrush to disturb the water to release some bubbles to the surface. Lids on containers can be left ajar to allow gas exchange. Because dryopid beetles respire using a plastron (external bubble film) and not gills, the effects of supersaturation are likely not as dangerous as to animals with gills.

Standard Operation Procedures for the Texas troglobitic water slater, *Lirceolus smithii*

Updated 2017

Daily Activities

1. Log water temperature and pressure
   1.1. Find datasheet on a clipboard above the desk and take the thermometer to the sink.
   1.2. Run the water for about 20 sec and then fill any beaker greater than 400mL and let it overflow.
   1.3. While water is overflowing and still running, take a temp reading and record the value on the datasheet.
   1.4. Pressure gauge will be on the same nozzle in the sink. Make sure water is on full before taking a reading, if pressure reading deviates from normal check the pressure gauge outside the invert room in the greenhouse. Change in pressure can be the first indication that there may be problems with flow in the containers.
2. Log feedings
   2.1. Find datasheet on a clipboard above the desk and fill in the appropriate boxes for the type of feed and which species were fed. Use the abbreviations listed in the legend.
3. Check for inflow in all containers. If flow is either too high or too low first refer back to the water pressure reading to see if it is abnormal before adjusting any valves. See also the Container Issues and Resolutions section for further trouble-shooting. With upwelling containers, check that the flow is not so high it is looks turbulent. Flow should be a balance of steady flow without too much disturbance to the species and container contents.
4. Check the outflow standpipes in all containers and remove debris with designated toothbrush for the container.
5. For vertical flow through brooding chambers, identify that there is a steady thin stream of water exiting the outflow portion of the flexible tubing. Adjust flow as necessary.
6. If mortalities are seen preserve individual in 95% EtOH in small glass vial with appropriate labeling on Rite-In-The-Rain paper slip inserted into vial.
7. Check for flooding, water puddles, and/or drips.
8. Clean floors, desks, equipment, and tools.

9. Work spaces must be neat and tidy. Make sure walkways are clear. Dry containers and desk with a rag. Put away tools in proper place and make sure the light is turned off at the end of the day.

Weekly Activities

1. Wear the correct PPE. Replace disinfectant (e.g. Virkon®) in mats by the door. Mix chemicals outside of the invertebrate room (see Virkon® Biosecurity section for details) and carefully fill the mats making sure not to overfill. Virkon® is highly toxic to invertebrates so be sure not to mix chemicals inside room and that it does not spill out into the room or come into contact with containers.
2. Check for $CaCO_3$ deposits interfering with valve functionality and/or water flow. Obstructions should be removed or valves replaced as needed.
3. Check all water quality parameters using the YSI water quality meter. Use the same method as Daily Activities #1 but with a beaker large enough to fully immerse the probes. Record on the data sheet on same clipboard as the daily log.
4. Check chlorination system.

Monthly Activities

5. Record all inventories on data sheets, keep track of daily metrics datasheet, and transfer both to appropriate files using the folders on the local drive.
    5.1. Keep a copy of these records on another computer. This prevents loss of data in case of a system failure and serves as a backup to paper documents.
6. The Deputy Director requires a monthly total of each species completed by close of business on the Wednesday of the last week of the month:
    6.1. Report total wild stock individuals in refugia, total in quarantine, total number of individuals that were captive bred, and the number of mortalities in refugia.
    6.2. Other numbers and data not required by the monthly report still need to be collected and reported, just not for this particular monthly report.
7. Update documents
    7.1. Online documents
        7.1.1. HACCP plans and logs
        7.1.2. Field collections

7.1.3. EAA species records

7.1.4. Outside take records

7.1.5. Inventory

7.1.6. Daily Water Quality and Feed logs

7.2. Paper copies are to be scanned and stored on the local drive or transferred to the correct filing excel sheet system in place for EAA contract. Keep backups of all documents on a separate computer or drive.

8. Check A/C unit
   8.1. Clean/replace the air filter
   8.2. Clear debris. Remove the filter and clean with a water hose if necessary.
9. Clean walls
   9.1. Wear the correct PPE. Fill a bucket with a bleach solution and use a mop to cleanse and rinse the walls to prevent molding. Focus attention to the doors and ceiling near windows. Avoid areas around refugia containers to prevent drips or spills into containers.

Yearly Activities

1. Keep good records of monthly collections into the Federal and TPWD collections permit log. Submission is per calendar year.
2. Keep good records of monthly files to utilize and compile for SMARC station annual reports; submission is for full fiscal year.

COLLECTION AND INITIAL QUARANTINE

Texas troglobitic water slaters (TTWS) are collected primarily through drift netting (Fig.1b) and lure collection (Fig. 1a) at Spring Lake, San Marcos, Texas and Comal Springs, New Braunfels, Texas. Drift nets are placed over known spring sources, for example, the large permanent net at Spring Lake Diversion Spring.

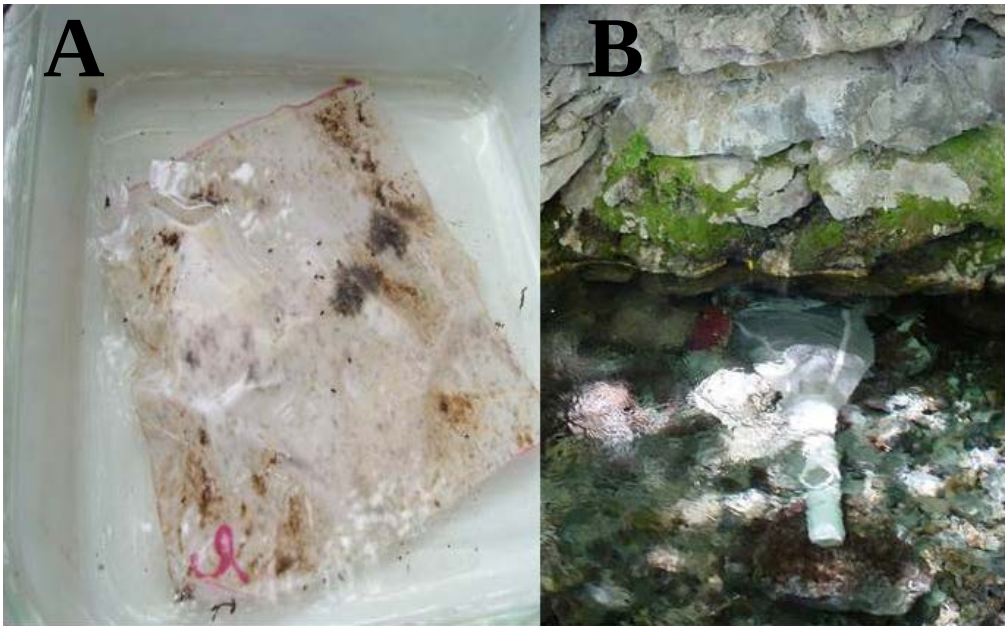


Figure 1 Unfolded cotton cloth lure (A). Drift net set at a spring source (B).

In the field, contents from the drift net cup are placed in a large cooler and transported to the station. Occasionally, TTWS are found on lures targeting other invertebrate species in the Comal Springs system. These should be placed in their own holding container that is then placed inside a transport cooler filled with cool water to keep temperature stable.

In the lab, carefully sift through the contents of the cooler and look for TTWS. They are often found clinging onto debris or other dead species thus can be tricky to find if not looking carefully. Move to holding container (i.e., a well or petri dish with debris for them to cling when in transport to the invertebrate room) using a piece of material to transfer. Use either a leaf, wood, piece of cloth or fine mesh to get the individual to crawl or cling to and then place this debris into holding container. When transferring to quarantine container allow the organisms to crawl off the debris and move elsewhere into the container. Be careful not to add more organic debris to the quarantine container, just the specimens. Soft forceps or sucking them up in a pipette are not recommended for TTWS as they are very fragile and often curl up and die after being transferred by either of these methods. It is common to use small plastic pipettes to prevent trauma or death, but also makes it more difficult to catch and prevent damage to the body itself, many times it is easy to get impatient with this method, transfer too quickly and cause damage. Other culturists have used a soft paint brush to gently collect and transfer TTWS. Do not scrape the TTWS off any surface, or depress the pipette too quickly or with too much force, as this can cause injury or death.

Research into distinguishing between *Lireolus* species is still tentative and there is no

non-lethal method in order to distinguish *L. smithii* from the others. At SMARC, all species of *Lirceolus* found in the field are collected as "TTWS" for the time being until a new method of distinguishing species is made. When the phrase TTWS is used in this SOP, it is applied to all species of *Lirceolus* in husbandry.

Water quality in receiving container should be similar to that in quarantine container. Acclimate the newly collected species to the water temperature as necessary before introducing into their designated quarantine container within the SMARC invertebrate room. A rule of thumb would be changing the water temperature 1 °C every half-hour; however, no studies have been done parameterizing acclimation speeds for these invertebrates, more research is warranted. Keep juveniles and adults apart due to potential cannibalism in TTWS; cannibalism, although unlikely (Hutchins et al. 2016), has been neither confirmed nor disproven this is done out of caution.

Organisms are kept in containers marked "Quarantine" for 30 days before joining containers of Refugia population. During this time we observe for other potential ANS species that might have come in with the collection. Observe the general health of the organisms and watch for large die-offs that might indicate a disease. If none of these occur then they can be moved to the Refugia population at the end of the quarantine period.

CULTURE SYSTEMS

Easy-Upwelling Systems

The Easy-Upwelling system mimics the hydrology of an upwelling with water flowing vertically rather than horizontally. Thus far, they have been run completely on Edwards Aquifer well water, but could easily be modified to accept re-circulating water.

Easy-Upwelling culture containers are constructed from plastic tubs/totes in 9-quart and 15-quart sizes (Figure 2). Water enters the system via a large hole cut into the container that is fit with adaptors and connected to PCV piping. For a 9 qt. size container, a single ½ in PVC pipe is used to facilitate water flow throughout the entire bottom of the container; a series of small holes ($1/16^{th}$ inch) are drilled along the pipe which is covered with fine mesh to prevent organisms from getting into the pipe. In the 15 qt. container, a square manifold of PVC pipe around the perimeter of the container is used. The small holes are drilled on the inner facing surfaces of the pipe and again are covered with fine mesh. For both types of up-welling manifolds, the piping is on or near the bottom of the container. Water flows out of the container via a horizontal standpipe capped with 300-400 µm mesh, held by hot glue, to prevent escape of

specimens and smaller life cycle stages. Containers should be assembled using PVC glue and/or aquarium safe silicone, allowed to cure-dry, and water run through them for a period of time to flush out any potentially harmful residual chemicals before organisms or habitat are added (this is also a good time to check and fix any leaks).

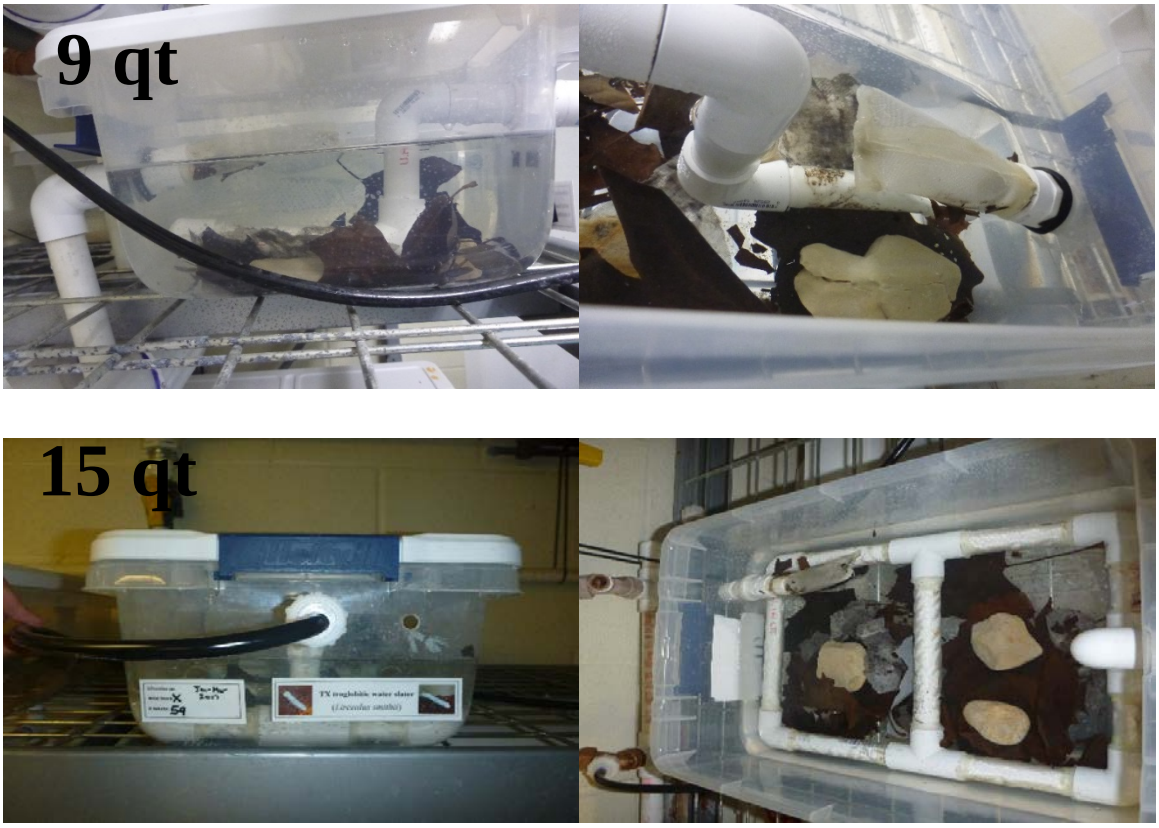


Figure 2 Views of 9 qt (upper row) and 15 qt (lower row) containers.

After containers are made, habitat items can be assembled in the container. It is best to put the container in its place on the rack system and get the water lines hooked up (but not filling) before adding rocks and leaves, then fill with water. This prevents items from shifting while the container is being moved. In addition, organisms should only be placed in the container after it is securely on its shelf, habitat is in place, and water has filled the container.

Leaves are layered on the floor of container and both new and conditioned poly cotton cloth. Small, lightweight rocks can be placed on top of these layers if leaves are not conditioned and float rather than sink on the floor. TTWS does not swim well and cannot utilize much vertical space within the container. Flow rate should be at a minimum as to not dislodge TTWS off the habitat items and cause undue stress by the TTWS being caught in current eddies and not being able to maneuver to cover/habitat to cling to.

- Limestone rocks (and all rocks) should be cleaned and fully dried before using in containers to prevent the potential transfer of other species.
- The leaves must come from a terrestrial source (not an aquatic source) so that accidental introduction of non-target aquatic organisms is minimized. These leaves should be collected from trees around the river system that the organisms are coming from and can include, but are not limited to: Anaqua, Sycamore, Pecan, or Black Walnut. Leaves should be quarantined for at least 2 weeks (following corresponding HACCPs). Leaves should be dried in the drying oven and then can be stored in a dry, sealed container until they need to be used. Leaves may be used by the organisms as cover or as a potential source of food. When conducting inventory save out some of the leaves from a container to use when placing habitat back into that container.
- Cotton cloth is used in the field to attract a variety of invertebrates for monitoring efforts. It has also been found to be a useful food source in culture for other invertebrates but we do not know what role(s) it may play for TTWS. Conditioned cloth, that is cloth that has been colonized by biofilm, can be added to the container. Additionally, new cotton cloth can also be added for cover and so that it can start the process of colonization by biofilm.

See section on "Production" for further instruction of use of flow-thru tubes with brooding females.

Suggested Maximum Capacity per Container (*these numbers have not been experimentally tested, just a basic guideline, these may be updated as more life history and husbandry information is learned).*

Container	TTWS
Small (9 qt)	50
Medium (15 qt)	150
Large (34 qt)	300
Flow-thru tube (1 in)	15

WATER QUALITY

Water quality values (see table below) should be checked weekly, unless problems arise and then should be checked more frequently. These values give here are a rule-of-thumb based on observational and anecdotal evidence, and are not necessarily backed by scientific analysis or experimentation. If water quality deviates from normal see "Container Issues and Resolutions" section for potential trouble-shooting or seek help from other biologists as to ways to correct the situation and be sure to inform your supervisor of changes and potential problems.

Metric	"Safe Range"
Temperature	18-24°C
Dissolved Oxygen	6-8 mg/L
pH	7-8.2
Total dissolved gas	≤ 100%
Ammonia	< 0.05 mg/L

Temperature should be monitored daily. Temperatures at or above 24°C will cause stress and possible death to TTWS and chilled well water should be added in addition to pure well water as sources for these systems in the event temperatures rise close to or above 24°C. Typically in late summer these actions will need to be implemented for all arthropod species. It's important to have a backup recirculation system with a heater/chiller and a pump that can recirculate water from the sumps in the event flow-through water quantity or quality is insufficient for Edwards Aquifer invertebrates. Observations by staff suggest that TTWS do better at the cooler end of the water temperature range.

FEEDING PROCEDURES

Flaked Feed: Small amounts of red fish flake may be beneficial to TTWS for additional protein, however we do not know for sure if the TTWS are eating this or what their diet mainly consists of in the wild. A very small amount can be added once a week. The flake is ground into a fine powder and mixed with water in a cup before injecting into the refugia containers. Stick pipette tip down into the bottom of the container to inject the food slurry. This ensures the food does not float on top and become covered in fungus. Adding too much flake to the containers can cause unhealthy fungus buildup and anoxia.

Within a brooding chamber, caution is to be exercised when removing the upper portion of the chamber before adding in flake feed. This prevents loss of gravid female or offspring if they are clinging onto the mesh within that adapter piece instead of within the tube.

Container Size	Pipette Amount
9 qt	0.25 ml
15 qt	0.5 ml
34 qt	1 ml
1 inch X 4 inch tube	0.25 ml

Leaves: Leaves added into the container during habitat construction may be a food source for TTWS, but this is not known for sure.

Cloth: Cotton cloth colonized by biofilm may be a food source, but again this is not known for sure. A few strips of cloth can be added to the system for the purpose of cover and potential food.

PRODUCTION

When inventorying TWWS from refugia containers, each isopod should be carefully observed for brooding. TTWS females hold their eggs and young in a brood pouch under the body (Figure 3). The appearance from the dorsal side will have a protrusion on each side of the middle area of the organism. If gravid females are located, they should be isolated in brooding chambers (Fig. 3) until they release their young. Brooding chambers provide both a refuge for the young, as well as an easy method for their collection. Brooding chambers can have mesh on the inflow and outflow around 55-75µm size. Contents within the chamber include leaves and stiff nylon mesh in the center for something the gravid females can hold on to and move vertically. Offspring should be transferred to a separate culture container after they are release from the brood pouch and collected from the brooding chambers. These are typically 9 qt. containers labeled as F1 generation. A plastic pipette works well to move the young and basters work well for adults. Put them into a beaker and then move them to the receiving container.

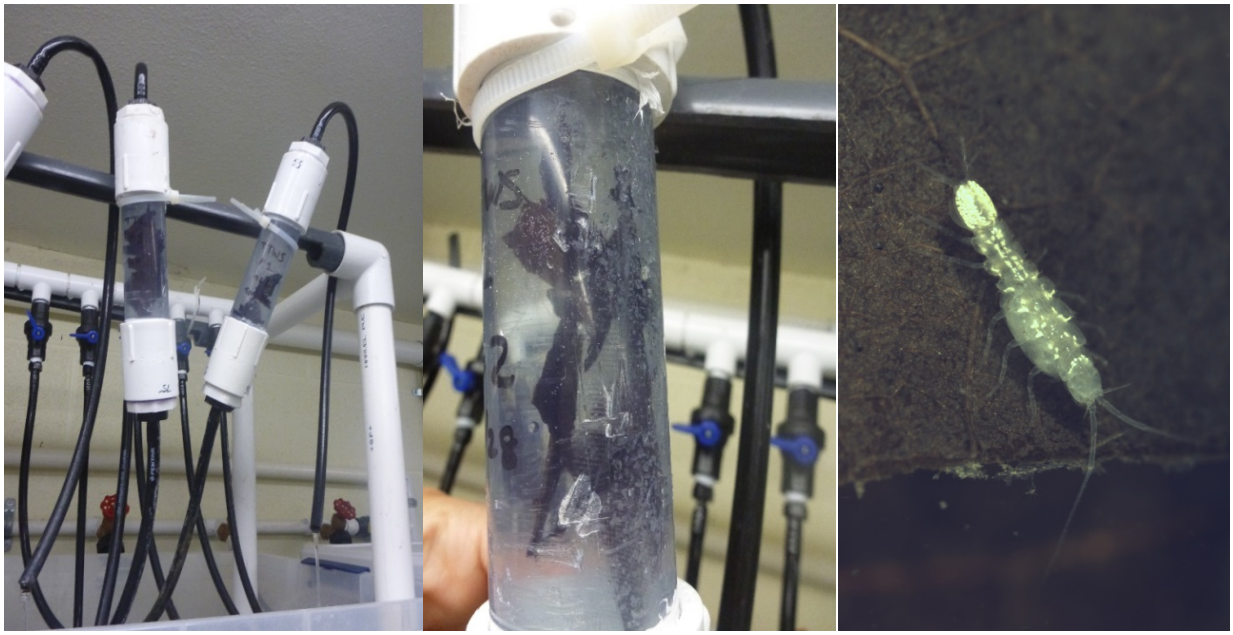


Figure 3 Clear 1 inch PVC brooding chambers and close-up of contents inside (left), gravid female brooding young (right).

CLEANING SYSTEMS

Culture Systems

Containers with TWWS generally are deep cleaned whenever the container must be disturbed (e.g., bi-monthly inventory or removing young). This is cleaning that goes beyond the routine daily care and cleaning of up-welling manifolds, outflow standpipes, and checking for fungus. Since the debris on the bottom may contain offspring or fragile brooding females, it must be carefully examined by pouring into a sieve before discarding. Adult TTWS can also cling to underside of small leaf pieces and stems and need to be checked thoroughly.

In order to remove $CaCO_3$ deposits without toxic chemicals, rinse empty containers with well water in the sink and scrape or wipe the containers and/or spray bars using brushes and sponges. If $CaCO_3$ deposits cannot be removed or are particularly bad, a replacement container or parts may be required. Any valves or tubing needs to be checked for $CaCO_3$ deposits and functionality and should be replaced if damage or obstructions are found. *Do not acid-wash*: most chemicals on station are not safe for aquatic invertebrates.

- Do not use bleach
- Do not use acid (e.g., HCl)

- Do not use Virkon

If a container or components have particularly stubborn, $CaCO_3$ deposits they can be soaked in a vinegar and water solution to loosen up the deposits. The containers should be triple rinsed and allowed to completely air dry before using again for invertebrates.

Brooding Chambers

Chambers are also a fragile environment and should not be disturbed needlessly. Cleaning is only to be done once a month or less often based on how clogged the mesh on the outflow or inflow adapters are. Have clean adapters ready to be swapped out for cleaning to prevent water sitting in the tube too long.

VIRKON® BIOSECURITY

A blue disinfection mat is located at each entrance to the invertebrate room. These mats help reduce the spread of nuisance species both into and out of the invertebrate room. Both mats should be refilled weekly with 1% Virkon® Aquatic solution. Each mat holds approximately 2.65 gallons of liquid. Record date Virkon® solution changed on data sheets located in invertebrate room notebook. If mats become dry during the week, add water as needed.

CONTAINER ISSUES AND RESOLUTIONS

1. If the floor is wet, there is probably a slow drip leak or water gushing somewhere. Find the source and adjust to cease.
    a. A nozzle may need to be tightened on either the inflow spray bar or upwelling connector or from the water inline on the back wall.
    b. Check outside the greenhouse door to see if a Texas wild rice refugia outflow pipe has been dislodged or moved away from the drainage system on the floor. The invertebrate room is not level and water from outside the room can travel under the greenhouse door inside the invertebrate room. Sand bags are in place outside the greenhouse door as a precaution, but are not a fail-safe.
    c. Check with other technicians and supervisors to see if any large alterations in water usage have occurred: such as starting or draining a pond, well location

switchover, immense well water pressure change, power was down and came back on, water turned off intentionally, break in the water line, etc. Any minor adjustments or changes in water usage at SMARC have the possibility to affect the invert rooms well water pressure. The pressure regulator valve on the water line into the invert room is designed to reduce high pressure issues.

2. Water levels in containers
    a. If the water line is above the outflow bar or stand pipe:
        i. Check for clogging and use a toothbrush or a pipette to remove the debris. Debris larger than a quarter can be placed under a rock. Do not siphon the container for debris as various life stages could be in this debris and accidentally discarded. If you see a pocket of fungused or anoxic debris you can remove it with a 3 ml pipette, but be sure to check the material on a petri dish for organisms before it is disposed of.
        ii. Flow into the container may also be too strong and turn the nozzle down and adjust the flow to a slower rate and see if that reduces the water line back down to the correct level.
    b. If container is overflowing of water outside the lid:
        i. Check the outflow bar or stand pipe for same conditions as 2a. Check to see if a hose has come loose from proper insertion point.
    c. If there are slow drips:
        i. Make sure the thread seal tape is not damaged or nonexistent and replace with new tape. Also, check the PVC fittings are flush to the PVC pipe on the outside the container may need to be glued to prevent drips from occurring. PVC glue or aquatic safe silicone can be used. However, this cannot be done with organisms in the container, as the glue requires time to cure and be water leached to prevent the organisms from coming into contact with harmful residues.
3. Change in water quality
    a. Increased/ Decreased Temperature:
        i. If temperature has decreased under 18 °C levers outside the invertebrate room in the greenhouse need to be adjusted to allow more non-chilled well water to stabilize the temperature. Check the pressure before and after making adjustments, the pressure should be between 10-13 psi, over 13 psi

is too high. First, have a hammer or rubber mallet to gently raise well water lever (yellow) and gently lower the chilled well water lever (blue) on. No water source lever will be on at full capacity; it is a mix of both levers to gently adjust the temperature. Changes can be tested in the sink with a temperature gun relatively soon after alterations. Drastic changes from hot to cold can stun CSDB and the possibility of death, so be careful and use gentle taps to adjust the amount of each water source.

ii. If temperature has increased to over 24 °C, levers outside the invertebrate room in the greenhouse need to be adjusted to allow chilled well water to stabilize the temperature. . Check the pressure before and after making adjustments, the pressure should be between 10-13 psi, over 13 psi is too high. First, have a hammer or rubber mallet to gently lower/turn off well water lever (yellow) and gently raise the chilled well water lever (blue) on. No water source lever will be on at full capacity; it is a mix of both levers to gently adjust the temperature. Changes can be tested in the sink with a temperature gun relatively soon after alterations. Drastic changes from hot to cold can stun CSDB and the possibility of death. So be careful and use gentle taps to adjust the amount of each water source.

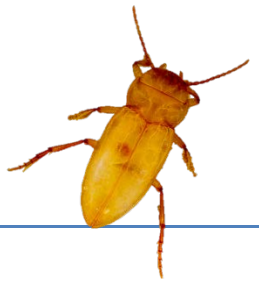
iii. If these changes do not cause a great enough effect to change the water temperature to within the safe range the recirculation system connected to the heater/chiller unit and the sumps may need to be initiated.

b. Increased Dissolved Oxygen:

i. Refer to the Supersaturation SOP on the local drive to address this immediate issue. Bubbles will be visible in all containers and a change in pressure will occur. Refer to the supersaturation diagrams above the sink in the invertebrate room to know which valves to turn off and/or on. Have the heater/chiller and pump ready to recirculate water from the sump if the supersaturation event is prolonged over 8 hrs. There is not much else you can do to prevent the bubbles from appearing. Use a toothbrush to disturb the water to release some bubbles to the surface. Lids on containers can be left ajar to allow gas exchange.

Standard Operation Procedures for the Edwards Aquifer diving beetle, *Haideoporus texanus*

Updated 2017



Daily Activities

1. Log water temperature and pressure
   1.1. Find datasheet on a clipboard above the desk and take the thermometer to the sink.
   1.2. Run the water for about 20 sec and then fill any beaker greater than 400mL and let it overflow.
   1.3. While water is overflowing and still running, take a temp reading and record the value on the datasheet.
   1.4. Pressure gauge will be on the same nozzle in the sink. Make sure water is on full before taking a reading, if pressure reading deviates from normal check the pressure gauge outside the invert room in the greenhouse. Change in pressure can be the first indication that there may be problems with flow in the containers.
2. Log feedings
   2.1. Find datasheet on a clipboard above the desk and fill in the appropriate boxes for the type of feed and which species were fed. Use the abbreviations listed in the legend.
3. Check for outflow in all flow through tubes. If flow is either too high or too low first refer back to the water pressure reading to see if it is abnormal before adjusting any valves. See also the Container Issues and Resolutions section for further trouble-shooting. With horizontal flow through tubes, check that the flow is not so high it looks turbulent. Flow should a steady stream coming out of the outflow, but not so high the diving beetles are unable to swim without strain or food suctions to the outflow mesh of the adapter.
4. Check the outflow tubing in all containers and remove debris from the mesh within the adapter with designated toothbrush for the species.
5. If mortalities are seen preserve individual in 95% EtOH in small glass vial with appropriate labeling on Rite-In-The-Rain paper slip inserted into vial.
6. Check for flooding, water puddles, and/or drips.
7. Clean floors, desks, equipment, and tools.

8. Work spaces must be neat and tidy. Make sure walkways are clear. Dry containers and desk with a rag. Put away tools in proper place and make sure the light is turned off at the end of the day.

Weekly Activities

1. Wear the correct PPE. Replace disinfectant (e.g. Virkon®) in mats by the door. Mix chemicals outside of the invertebrate room (see Virkon® Biosecurity section for details) and carefully fill the mats making sure not to overfill. Virkon® is highly toxic to invertebrates so be sure not to mix chemicals inside room and that it does not spill out into the room or come into contact with containers.
2. Check for $CaCO_3$ deposits interfering with valve functionality and/or water flow. Obstructions should be removed or valves replaced as needed.
3. Check all water quality parameters using the YSI water quality meter. Use the same method as Daily Activities #1 but with a beaker large enough to fully immerse the probes. Record on the data sheet on same clipboard as the daily log.
4. Check chlorination system.

Monthly Activities

5. Record all inventories on data sheets, keep track of daily metrics datasheet, and transfer both to appropriate files using the folders on the local drive.
    5.1. Keep a copy of these records on another computer. This prevents loss of data in case of a system failure and serves as a backup to paper documents.
6. The Deputy Director requires a monthly total of each species completed by close of business on the Wednesday of the last week of the month:
    6.1. Report total wild stock individuals in refugia, total in quarantine, total number of individuals that were captive bred, and the number of mortalities in refugia.
    6.2. Other numbers and data not required by the monthly report still need to be collected and reported, just not for this particular monthly report.
7. Update documents
    7.1. Online documents
        7.1.1. HACCP plans and logs
        7.1.2. Field collections

7.1.3. EAA species records

7.1.4. Outside take records

7.1.5. Inventory

7.1.6. Daily Water Quality and Feed logs

7.2. Paper copies are to be scanned and stored on the local drive or transferred to the correct filing excel sheet system in place for EAA contract. Keep backups of all documents on a separate computer or drive.

8. Check A/C unit
   8.1. Clean/replace the air filter
   8.2. Clear debris. Remove the filter and clean with a water hose if necessary.
9. Clean walls
   9.1. Wear the correct PPE. Fill a bucket with a bleach solution and use a mop to cleanse and rinse the walls to prevent molding. Focus attention to the doors and ceiling near windows. Avoid areas around refugia containers to prevent drips or spills into containers.

Yearly Activities

1. Keep good records of monthly collections into the Federal and TPWD collections permit log. Submission is per calendar year.
2. Keep good records of monthly files to utilize and compile for SMARC station annual reports; submission is for full fiscal year.

COLLECTION AND INITIAL QUARANTINE

Follow HACCP plan. Edwards Aquifer diving beetles (EADB) are collected via an artesian well opening into a drift net at Texas State University campus, San Marcos, Texas. After species identification is verified, move individuals to target container (e.g., plastic jar with lid containing stiff larger mesh pore nylon mesh). It is better to use small plastic pipettes or soft forceps when collecting in the field. In the field, contents from the drift net cup are placed in a large cooler filled with cool water to keep temperature stable and transported to station.

Figure 1. Drift net at the artesian well at TXST University campus

In the lab, carefully sift through the contents of the cooler and look for EADB. They are often found clinging onto debris or hiding on the bottom under mesh or debris. Using soft forceps carefully move to holding container (e.g., a small cup, ~ 100 ml, with debris for them to cling onto when in transport to the invertebrate room). Alternatively, you can move the piece of debris they are clinging on to the holding container. If they are moving quickly, a turkey baster can be utilized to carefully transfer them from the cooler to the cup. Wait until the EADB moves on its own, then use a pipette to extract it from its surroundings. Do not scrape the EADB off any surface, or depress the turkey baster too quickly or with too much force, as this can cause injury or death.

Water quality in receiving container should be similar to that in quarantine flow through tube. Acclimate the newly collected specimen to the water temperature as necessary before introducing into their designated quarantine flow through tube within the SMARC invertebrate room. A rule of thumb would be changing the water temperature 1 °C every half-hour; however, no studies have been done parameterizing acclimation speeds for these invertebrates, more research is warranted.

Organisms are kept in tubes marked “Quarantine” for 30 days before joining tubes to the Refugia population. During this time we observe for other potential ANS species that might have come in with the collection. Observe the general health of the organisms and watch for large die-offs that might indicate a disease. If none of these occur then they can be moved to the Refugia population at the end of the quarantine period.

CULTURE SYSTEMS

Horizontal Flow through Tube Systems

Culture containers currently favored at the SMARC for EADB are Horizontal Flow through Tube Systems. Tubes measure 1 inch diameter by 4 inch length (Figure 2). In general, no more than 2-3 beetles should be placed in each tube. Water flows into the tube via tubing attached to male-adapter nipple connections. A flow restrictor may be needed to regulate flow depending on the water supply valve used. At both ends of the tube (water in-flow and out-flow) 75-150 µm mesh should be stretched across the opening and secured to prevent escape. After construction tubes should be flushed with water for a period of time to flush out any potentially harmful residual chemicals before organisms or habitat items are added (this is also a good time to check and fix any leaks).

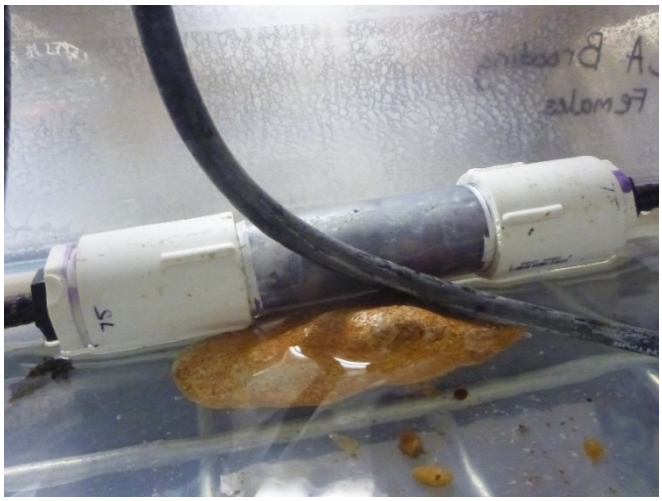


Figure 2. An example of a horizontal flow-through tube; this one is filled with contents for Comal Springs riffle beetles, not EADB.

After tubes are made, habitat items can be assembled within the center. It is best to put the tube in its place on the rack system and get the water lines hooked up (but not filling) before adding cloth, mesh, and leaves, then fill with water. This prevents items from shifting while the container is being moved. In addition, organisms should only be placed in the container after it is securely on its shelf, habitat is in place, and water has filled the container.

A few leaves are layered on the horizontal bottom of tube and both a small piece of new and conditioned poly cotton cloth. Small, lightweight pebbles can be placed on top of these

layers if leaves are not conditioned and float rather than sink. The stiff mesh is placed vertically across the diameter of the tube to provide refuge or something to cling to. EADB does swim well, but also sporadically and can easily get beached or caught in a crack and get stuck.

- Pebbles (and all rocks) should be cleaned and fully dried before using in containers to prevent the potential transfer of other species.
- The leaves must come from a terrestrial source (not an aquatic source) so that accidental introduction of non-target aquatic organisms is minimized. These leaves should be collected from trees around the river system that the organisms are coming from and can include, but are not limited to: Anaqua, Sycamore, Pecan, or Black Walnut. Leaves should be quarantined for at least 2 weeks (following corresponding HACCPs). Leaves should be dried in the drying oven and then can be stored in a dry, sealed container until they need to be used. Leaves may be used by the organisms as cover or as a potential source of food. When conducting inventory save out some of the leaves from a container to use when placing habitat back into that flow-through tube.
- Cotton cloth is used in the field to attract a variety of invertebrates for monitoring efforts. It has also been found to be a useful food source in culture for other invertebrates but we do not know what role(s) it may play for EADB. Conditioned cloth, that is cloth that has been colonized by biofilm, can be added to the tube. Additionally, new cotton cloth can also be added for cover and so that it can start the process of colonization by biofilm.

Suggested Maximum Capacity per Container (*these numbers have not been experimentally tested, just a basic guideline, these may be updated as more life history and husbandry information is learned).*

Container	EADB
1 inch X 4 inch tube	3
2 inch X 4 inch tube	6

WATER QUALITY

Water quality values (see table below) should be checked weekly, unless problems arise

and then should be checked more frequently. These values give here are a rule-of-thumb based on observational and anecdotal evidence, and are not necessarily backed by scientific analysis or experimentation. If water quality deviates from normal see "Container Issues and Resolutions" section for potential trouble-shooting or seek help from other biologists as to ways to correct the situation and be sure to inform your supervisor of changes and potential problems.

Metric	"Safe Range"
Temperature	18-24°C
Dissolved Oxygen	6-8 mg/L
pH	7-8.2
Total dissolved gas	≤ 100%
Ammonia	< 0.05 mg/L

Temperature should be monitored daily. Temperatures at or above 24°C will cause stress and possible death to EADB and chilled well water should be added in addition to pure well water as sources for these systems in the event temperatures rise close to or above 24°C. Typically in late summer these actions will need to be implemented for all arthropod species. It's important to have a backup recirculation system with a heater/chiller and a pump that can recirculate water from the sumps in the event flow-through water quantity or quality is insufficient for Edwards Aquifer invertebrates. Observations by staff suggest that EADB do better at the cooler end of the water temperature range.

FEEDING PROCEDURES

Flaked Feed: Diving beetle species in general are typically carnivorous, but a diet research shows that EADB are scavengers who scrape materials from substrates. Small amounts of fish flake may be beneficial to diving beetles for additional protein. A small amount can be added once a week; about a pinch. The flake is ground into a fine powder and mixed with water in a cup before injecting into the flow through tubes. This ensures the food does not float on top and become covered in fungus or get plastered to the mesh outflow once the water source is turned back on. EADB also have issues balancing in the water column (per obs.) and attempting to reach food at the surface could cause EADB to float as well. Adding too much flake to the containers can cause unhealthy fungus buildup and anoxia.

Container Size	Pipette Amount
1 inch X 4 inch tube	< 0.25 ml
2 inch X 4 inch tube	0.25 ml

Leaves: Leaves from terrestrial sources should be dried. Leaves can be added directly to the tube or container without conditioning or they can be soaked in well water for a week or more, decanting and replacing water several times. Walnut, pecan, anacua, elm, and sycamore leaves are usable types.

Cotton cloth: Biofilm on cotton cloth has also been found to be a useful food source in culture in other beetle species at SMARC and could potentially be used for EADB, but no research has been evaluated. Cotton cloth should always be used in combination with leaves and both replenished with new sources within a 2 month period or replaced when the cloth piece becomes 90% black.

PRODUCTION

Production in the EADB has not been recorded at SMARC. The life history of EADB is unknown thus it is difficult to hypothesize on how to best perform production culture techniques.

CLEANING SYSTEMS

Culture Systems

Flow through tubes with EADB are deep cleaned whenever the tube must be disturbed (e.g., bi-monthly inventory). This is cleaning that goes beyond the routine daily care and cleaning of adapter mesh, outflow tubing, and checking for fungus. Since the debris on the bottom may contain offspring, it must be carefully examined by pouring into a sieve before discarding the debris down the drain. EADB don't typically cling on, but check pieces of materials inside the tube upon disturbance thoroughly.

In order to remove $CaCO_3$ deposits without toxic chemicals, rinse empty containers with

well water in the sink and scrape or wipe the containers and/or spray bars using brushes and sponges. If $CaCO_3$ deposits cannot be removed or are particularly bad, a replacement container or parts may be required. Any valves or tubing needs to be checked for $CaCO_3$ deposits and functionality and should be replaced if damage or obstructions are found. *Do not acid-wash*: most chemicals on station are not safe for aquatic invertebrates.

- Do not use bleach
- Do not use acid (e.g., HCl)
- Do not use Virkon

If a container or components have particularly stubborn $CaCO_3$ deposits they can be soaked in a vinegar and water solution to loosen up the deposits. The containers should be triple rinsed and allowed to completely air dry before using again for invertebrates.

VIRKON® BIOSECURITY

A blue disinfection mat is located at each entrance to the invertebrate room. These mats help reduce the spread of nuisance species both into and out of the invertebrate room. Both mats should be refilled weekly with 1% Virkon® Aquatic solution. Each mat holds approximately 2.65 gallons of liquid. Record date Virkon® solution changed on data sheets located in invertebrate room notebook. If mats become dry during the week, add water as needed.

CONTAINER ISSUES AND RESOLUTIONS

1. If the floor is wet, there is probably a slow drip leak or water gushing somewhere. Find the source and adjust to cease.
    a. A nozzle may need to be tightened on either the inflow spray bar or upwelling connector or from the water inline on the back wall.
    b. Check outside the greenhouse door to see if a Texas wild rice refugia outflow pipe has been dislodged or moved away from the drainage system on the floor. The invertebrate room is not level and water from outside the room can travel under the greenhouse door inside the invertebrate room. Sand bags are in place outside the greenhouse door as a precaution, but are not a fail-safe.

c. Check with other technicians and supervisors to see if any large alterations in water usage have occurred: such as starting or draining a pond, well location switchover, immense well water pressure change, power was down and came back on, water turned off intentionally, break in the water line, etc. Any minor adjustments or changes in water usage at SMARC have the possibility to affect the invert rooms well water pressure. The pressure regulator valve on the water line into the invert room is designed to reduce high pressure issues.

2. Water levels in containers
    a. If the water line is above the outflow bar or stand pipe:
        i. Check for clogging and use a toothbrush or a pipette to remove the debris. Debris larger than a quarter can be placed under a rock. Do not siphon the container for debris as various life stages could be in this debris and accidentally discarded. If you see a pocket of fungused or anoxic debris you can remove it with a 3 ml pipette, but be sure to check the material on a petri dish for organisms before it is disposed of.
        ii. Flow into the container may also be too strong and turn the nozzle down and adjust the flow to a slower rate and see if that reduces the water line back down to the correct level.
    b. If container is overflowing water:
        i. Check the outflow bar or stand pipe for same conditions as 2a. Check to see if a hose has come loose from proper insertion point.
    c. If there are slow drips:
        i. Make sure the thread seal tape is not damaged or nonexistent and replace with new tape. Also, check the PVC fittings are flush to the PVC pipe on the outside the container may need to be glued to prevent drips from occurring. PVC glue or aquatic safe silicone can be used. However, this cannot be done with organisms in the container, as the glue requires time to cure and be water leached to prevent the organisms from coming into contact with harmful residues.
3. Change in water quality
    a. Increased/ Decreased Temperature:
        i. If temperature has decreased under 18 °C levers outside the invertebrate room in the greenhouse need to be adjusted to allow more non-chilled well

water to stabilize the temperature. Check the pressure before and after making adjustments, the pressure should be between 10-13 psi, over 13 psi is too high. First, have a hammer or rubber mallet to gently raise well water lever (yellow) and gently lower the chilled well water lever (blue) on. No water source lever will be on at full capacity; it is a mix of both levers to gently adjust the temperature. Changes can be tested in the sink with a temperature gun relatively soon after alterations. Drastic changes from hot to cold can stun CSDB and the possibility of death, so be careful and use gentle taps to adjust the amount of each water source.

ii. If temperature has increased to over 24 °C, levers outside the invertebrate room in the greenhouse need to be adjusted to allow chilled well water to stabilize the temperature. . Check the pressure before and after making adjustments, the pressure should be between 10-13 psi, over 13 psi is too high. First, have a hammer or rubber mallet to gently lower/turn off well water lever (yellow) and gently raise the chilled well water lever (blue) on. No water source lever will be on at full capacity; it is a mix of both levers to gently adjust the temperature. Changes can be tested in the sink with a temperature gun relatively soon after alterations. Drastic changes from hot to cold can stun CSDB and the possibility of death. So be careful and use gentle taps to adjust the amount of each water source.

iii. If these changes do not cause a great enough effect to change the water temperature to within the safe range the recirculation system connected to the heater/chiller unit and the sumps may need to be initiated.

b. Increased Dissolved Oxygen:

i. Refer to the Supersaturation SOP on the local drive to address this immediate issue. Bubbles will be visible in all containers and a change in pressure will occur. Refer to the supersaturation diagrams above the sink in the invertebrate room to know which valves to turn off and/or on. Have the heater/chiller and pump ready to recirculate water from the sump if the supersaturation event is prolonged over 8 hrs. There is not much else you can do to prevent the bubbles from appearing. Use a toothbrush to disturb the water to release some bubbles to the surface. Lids on containers can be left ajar to allow gas exchange.

CAPTIVE PROPAGATION MANUAL FOR *EURYCEA* SP.

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PREFACE

As large declines in amphibian populations are documented across the globe (Houlahan et al 2000), with dramatic declines within protected areas in North America in particular (Halliday 2005), concerns have been raised about the efficacy of conservation by habitat protection alone. An alternative and particularly proactive conservation measure for imperiled amphibian species has been the establishment of captive assurance colonies, or refugia, by zoological parks (zoos), wildlife organizations, universities, and federal agencies. These colonies provide an insurance policy for species at risk of extinction in the wild, help to preserve genetic diversity, and simultaneously allow opportunities for researchers to gather valuable information regarding biology and conservation needs. For example, captive breeding of several federally listed salamander species at the San Marcos Aquatic Resources Center (SMARC) has led to the development of effective breeding techniques (Cantu *et al*. 2016), establishment of rearing protocols (Fenolio *et al*. 2014), and determination of valuable life history information.

The following document was developed to serve as a guide to the establishment and culture of captive assurance colonies, refugia, for the federally listed endangered Texas blind Salamander (*Eurycea rathbuni*), the federally listed threatened San Marcos Salamander (*Eurycea nana*), and the petitioned Comal Springs Salamander (*Eurycea* sp.). Additionally, this document may provide valuable reproductive, developmental, and basic life history information relevant to other federally listed obligate aquatic salamander species. When information is limited on the species of concern, guidance derived from closely related species is applied when appropriate.

Introduction

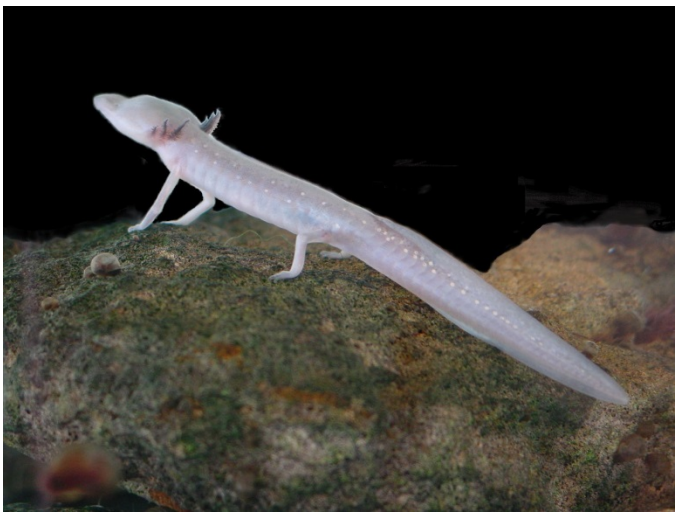
The Edwards Plateau region of central Texas is home to a variety of highly endemic, spring- and cave-dwelling salamander species of the genus *Eurycea* (Chippindale *et al*. 2000), including three of which are federally listed as endangered and four federally listed as threatened (USFWS: Environmental Conservation Online System. Endangered species search: Texas http://ecos.fws.gov/tess_public/reports/species-listed-by-state-report?state=TX&status=listed). These wild populations of *Eurycea* have extremely limited distributions, which are restricted to caves and/or the vicinity of spring outflows, and are highly dependent on clean groundwater from the Edwards Aquifer (Chippindale *et al*. 2000; Chippindale and Price 2005). Further, these habitats are stenothermal: water temperatures, under normal conditions, remain relatively constant. Captive assurance colonies for Texas blind Salamander (*Eurycea rathbuni*), San Marcos Salamander (*Eurycea nana*) and Comal Springs salamander (*Eurycea* sp.) are currently held by the United States Fish and Wildlife Service (USFWS) at SMARC in San Marcos, Texas.

The survival of all of these imperiled *Eurycea* species is highly dependent on the water level and quality of the Edwards Aquifer (Chippindale and Price 2005). Yet, these species face multiple threats to their continued existence in the wild. Major threats include the alteration of natural spring flow regimes, drought, and the effects of urbanization. A severe drought from 2011-2015 in Texas prompted the governor to issue an emergency disaster proclamation. This drought served to highlight the threats and fragility of natural habitat for aquatic species, spurring on the need/utility of refugia populations in captivity to ensure the survival of the species. Additionally, the central Texas region has consistently been among the fastest growing areas in the nation. The San Antonio-New Braunfels metropolitan area (of approximately 2.4

million people and growing) draws 90% of its water from the Edwards Aquifer, furthering concern of the stability and sustainability of the aquifer habitat.

Texas blind salamander (*Eurycea* [formerly *Typhlomolge*] *rathbuni*)

Eurycea rathbuni (Stejneger 1896) is a state (Texas) and federally listed endangered troglobitic (cave dwelling) plethodontid (lungless) salamander endemic exclusively to the Edwards plateau region in Hays County, Texas, USA (Petranka 1998). This species was among the very first to be listed as threatened or endangered in the United States under the 1966 Endangered Species Preservation Act. Given their subterranean habitat within the Edwards Aquifer, the full extent of their geographic range, as well as accurate population estimates, remain unknown. They have relatively large bodies for this group of salamanders (family Plethodontidae): adults are about 4 inches measured from the nose to tip of the tail. The Texas blind salamander is adapted for living in darkness; it lacks eyes, though a pigmented epithelial layer is present where eyes occur in related species. It has scarce skin pigment, is generally white in color, and has red external gills used for gas exchange with water.



The Texas blind salamander is an active predator. It hunts food using specialized receptors on its head to sense water pressure waves created by prey in the still underground waters where it lives. Small snails, shrimp, and other aquatic invertebrates make up its diet.

Reproduction appears to occur year-round based on collection records from the wild, but this has not been confirmed (Longley 1978).

San Marcos Salamander (*Eurycea nana*)

Eurycea nana (Bishop 1941) is a state (Texas) and federally listed threatened plethodontid salamander endemic to the headwaters of the San Marcos River in Hays County, Texas (Chippindale *et al*. 1998). The habitat of *E. nana* includes Spring Lake, which is fed by over 200 spring openings emanating from San Marcos Springs, and continuing 150 meters downstream of Spring Lake Dam (Nelson 1993). Past population estimates for this species indicate that approximately 116 to 129 salamanders per square meter occur in vegetation within this habitat (Tupa and Davis 1976; Nelson 1993).

Moss and algae provide hiding places for the small-bodied salamanders and habitat for small organisms that serve as their food. The dark color on the back of the San Marcos Salamander almost perfectly matches the dark reddish-brown color of the algae. These salamanders do not occur where the bottom is muddy or bare.

Comal Springs Salamander [Texas Salamander (Eurycea sp.)] — The Comal Springs salamander is only known to occur in the Comal Springs system in Landa Park and Landa Lake in Comal County, Texas. Life history information is limited for this species. However, it is physically similar to the San Marcos salamander and both species display similar

habitat, feeding habits, and food preferences. In addition, SMARC staff have observed both species employing similar mating dances/behaviors and social grouping in captivity.

Taxonomic classification of the Comal Springs salamander remains unclear. This species was originally described by Bishop and Wright (1937) from a spring in Bexar County, Texas.

However, molecular and morphological data suggests that this species as potentially representing a unique taxon (Chippindale et al. 1998, 2000). Currently no formal species description has been published. The USFWS recognizes this species as "*Eurycea* species 8." Given the current threats of habitat modification and loss to the Comal Springs ecosystem, this species is currently under review and being considered for federal listing (USFWS 2009).

Collection of Wild Stock/Brood Stock Organisms

Salamanders are collected from the wild in the San Marcos and Comal River systems via a variety of methods (nets, traps in cave and wells, and hand collection with dip nets) depending to the species and location targeted. Texas blind salamanders are collected by traps from caves and wells that connect to the aquifer and also from drift nets. San Marcos salamanders are found in Spring Lake on the bottom in vegetation, under large rocks, and buried in loose rocks near various spring upwellings. These salamanders are collected by hand using small dip nets and SCUBA or collected by a drift net attached to Diversion Spring. They can also be found and

collected just below Spring Lake dam. Comal Springs salamanders are found near spring orifices and upwellings, in similar habitats as San Marcos salamanders, in the Comal River system with targets in the spring runs and around Spring Island in Landa Lake. In general, collection is stressful to the organisms; care should be taken to reduce stress and damage to the salamanders when possible. In order to reduce body damage, minimize the time salamanders are exposed to air, prevent excess handling, and choose the time of year for collection to reduce thermal stress when these animals will be out of water or in transport.

Drift nets

Drift nets are a passive way of collecting organisms from spring openings. Nets are usually made from 200-350 µm mesh and are custom fit to the type of opening on which they are installed. In general, nets are conical shaped with a removable cod end made out of hard PVC. Mesh may be placed in the cod-end collection cup as refuge for captured organisms. Nets should be checked every few days for organisms, but more frequently, if required (e.g. due to low survival, high water volume, or any other reason).

Figure 1 Diversion Spring drift net.

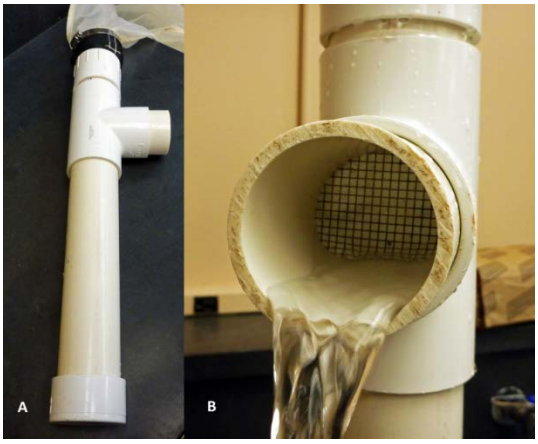


Figure 2 Example of cod-end collection cup for drift nets. Design may need to be modified depending on spring characteristics.

Figure 3 Bottle trap design for collecting salamanders.

Trapping

Minnow traps or bottle traps (Figs. 1 and 2) can be very effective in sampling deep caves and wells. Traps are baited with organic material (i.e. pistachio nuts, potato peels, catfish pellets) to lure invertebrate organisms that, in turn, attract salamanders. However, the bait itself may attract salamanders. More study on bait preference is encouraged. Traps should be checked at least twice a week and more often if time permits. Rotten bait should be removed and replaced as needed. To encourage conservation not deplete the wild population, we only take one of every three Texas blind salamanders (either physically or visually) captured for incorporation into the refugia.

Figure 4 Minnow trap being lowered into well to collect salamanders.

Hand collection

Collectors using masks and snorkels or SCUBA can search for the salamanders under rocks and in vegetation near spring orifices. Once found, salamanders can be carefully collected with a small dip-net using one hand to gently flush the salamander into the net. Individual salamanders should be transported to the surface immediately.

Salamander swabs

Salamanders should be swabbed for disease monitoring before being transported to the facility. Swabbed each collected salamanders in duplicate along its body and limbs using sterile cotton tipped swabs then place swabs individually into labeled, 1.5mL vials and store on ice in the field. Once back at the station, store swabs in a freezer until they are sent to the USFWS Fish Health Unit for testing. The Fish Health Unit will test swabs for two types of chytrid fungus. These tests are based on DNA. Although it is best to keep samples cool and stored in the freezer, they do not have to be kept ultra-cool (like RNA samples) and can be shipped, with an ice pack, using overnight, regular delivery to Fish Health Unit. Always verify preferred procedures with the Fish Health Unit before shipping.

Transporting salamanders

Salamanders should be transported to the station in coolers with water from their collection location if possible. It generally is difficult to get water from caves or wells. Thus, prior to collecting Texas blind salamanders from caves or wells, small coolers should be filled with well water from the station. Coolers should be monitored for temperature, especially during prolonged collection events, and water changes carried out as needed. Loose mesh or PVC shavings/curls can be placed in coolers as refuge for the salamanders. Heavy substrate, such as rocks, should not be placed in coolers for refuge because they can shift and crush salamanders

during transport. During long transportation times, when water changes are not possible, bubbles may need to be added if many individuals are in a cooler. Caution should be exercised with bubblers as salamanders are prone to getting gas bubbles trapped under their skin due to their physiology in highly saturated water. Once at their destination salamanders should be slowly acclimated to their quarantine water quality, paying attention to temperature changes.

Quarantine

All wild caught salamanders must go through a 30-day period in quarantine. Daily observations of behavior and health (brief visually inspections for maladies) should be made of each salamander aquarium in the quarantine system. As part of quarantine, all salamanders of each species must have returned test results of their swabs from the Fish Health Unit prior to incorporation into the refugium system. The swab samples will be sent to Dexter Fish Health Unit to screen for *Batrachochytrium dendrobatidis* (Bd, commonly referred to as chytrid) and *Batrachochytrium salamandrivorans* (Bsal) prior to specimen incorporation into the general refugia population. Chytrid (Bd) fungus has caused mortalities in some amphibian species; however, some species show little to no symptoms from the fungus. Previous tests of wild caught salamanders at SMARC (both Texas Blind and San Marcos) have tested positive for Bd with varying rates by location and year caught, but all sites have had salamanders that tested positive. Clinically, the salamanders appear normal and do not have any lesions or signs of disease. Positive testing for Bsal will be treated more cautiously as it has not been documented in this area before; these salamanders would remain in quarantine until further study and recommendations from FWS Fish Health.

Culture

Best Practices for Bio Security

- Wash or disinfect hands before or between working on separate tanks or refugium systems. Even better to wear disposable gloves covering hands when they are in contact with water and to change when moving to another system.
- Do not casually place hands into tank water while feeding or checking system equipment. Diseases and pathogens can easily be carried between tanks on hands covered in water.
- Make sure to disinfect with bleach or Virkon, and then thoroughly rinse, any equipment before initial use on a tank system.
- Preferably, each system would have its own equipment (nets, brushes, probes, buckets, etc.), but if not, be sure to follow proper sterilization protocols to prevent the spread of pathogens.
- Do not move salamanders from one aquarium to another without appropriate authorization and/or purpose.

Culture systems

Here we describe a general set up for salamander culture systems used at the SMARC; other designs may be used. The main concern for these salamanders is to maintain optimum temperature and avoid supersaturation of gases in water. Temperature is maintained in the tank systems by a recirculation system that passes the tank water through a heater/chiller unit. Gas saturation in the water is decreased as water passes through a degassing valve that causes negative pressure in the plumbing (designed based on Herman 1995) that helps release gases in solution. Each tank is plumbed with an overflow drain pipe to maintain a water level of approximately half of the tank volume. Chilled well water (non-degassed) is added to each

container holding salamanders at a low rate such that water turn over in the system occurs once every 24-48 hours. Thus, each tank with salamanders should have two sources of water at all times (e.g. well water input and recirculating water flow). The number of salamanders per container varies, but we have found that it should not exceed about one adult per liter for extended periods (also depends upon size of salamander). Habitat for salamanders to climb on or take cover should be placed in each container, including clean limestone rocks (from a terrestrial source to avoid accidental introduction of non-target aquatic organisms, and should be cleaned and disinfected before using), PVC halves, PVC spiral shavings, black or clear acrylic houses ("hotels"), and/or plastic vegetation. Plastic vegetation is a good option that can easily be taken out and disinfected and does not have to be tended to like natural plants. Generally, *Amblystegium* species that has been salt treated can be added for cover to help clean the water, and to provide substrate for egg-laying. Do not place so much habitat that it obscures being able to see mortalities or hinder cleaning of tanks.

Always make sure that water levels are no more than about half to two-thirds height of containers to prevent escape. Use the biggest mesh size for overflow pipes as appropriate for size of salamander and its food. Make sure that mesh covers for overflow pipes are very closely fitted and zip-tied tightly as salamanders can use this as an avenue of escape. Additionally, make sure that water does not splash walls or separating screens of the containers housing salamanders; this allows salamanders to climb the wet area on the walls of the tank and provides an escape route for the salamanders.

Aquarium certified silicone sealant is commonly used to seal gaps between screen dividers and tank walls. Seal inspections should be done regularly. Worms may find small gaps between the silicone and the tank wall and make them larger. Should a gap be found where

worms or salamanders are hiding under the silicone the seal should be repaired. Once the tank system is clean, remove the old silicone with a scraper, clean the tank surfaces (where the silicone will be applied) with alcohol, and reapply the silicone along the gap as a uniform bead. Slide your finger (wearing a surgical glove) along the bead to compress the seal. Allow silicone to completely dry before refilling with water. Flush tank with water and check water quality parameters before adding salamanders back to the system.

Water Quality

Maintaining water quality is essential for captive survival and effective propagation of all three of the salamander species. Weekly testing of water quality should be conducted on all systems, with more frequent sampling as needed. Water quality should be more frequently and closely monitored after any type of disturbance to the system, such as a main station well going down, a system repair, or when setting up a system after acid washing. Water quality should also be measured if two or more mortalities are discovered in a single tank system within 24 hours; this includes checking ammonia levels. Data should be logged so care takers can get a general sense of what water quality should be and recognize when parameters are out of range and action is required. Below are ranges for various parameters:

Parameter	**Safe range**
Temperature	18-23 °C
Dissolved oxygen	4-8 mg/L
Total dissolved gas	≤ 100%
Ammonia	≤ 0.05 mg/L
pH	7-8.5

Temperature

Texas blind, San Marcos, and Comal Springs salamanders are typically found in and around springs, which vary little in temperature year-round (spring water from the Edwards Aquifer averages 21-22 °C year round (Tupa and Davis 1976, Najvar 2001). For this reason, temperatures in the refugia are targeted at 21-22 °C year-round with help from an inline heater/chiller unit. Primary system temperature controller should be set to 21 or 22°C with a differential of ±1°C. Set secondary system overheat and overcool protection devices 3-5°C above and below, respectively, target temperatures on controllers. Studies have shown San Marcos salamanders will begin to die off if temperatures increase above 31°C (Berkhouse and Fries 1995), but experience at the SMARC has been that San Marcos and Texas Blind salamanders experience die-offs at temperatures of 26°C. Troubleshooting temperature problems may include:

- Making sure temperature controls are working and are properly set
- Checking how the pump is operating
- Clearing water lines (pipes) for calcium buildup or debris
- Check on power sources (i.e. circuit breakers)

Ammonia should be checked as part of the weekly water quality monitoring. Ammonia test strips (Hach Company, Product #: 2755325) may be used to determine levels in tank systems. It is common for ammonia, nitrite, and/or nitrate to be undetectable in salamander tanks because of the water turnover rate in each tank. However, if ammonia levels are above 0.25 mg/L (0.25 mg/L is typically the second smallest increment of measure on ammonia test strips) then the tank system should be flushed with chilled well water (i.e. temporarily increase the rate of chilled well water input until ammonia levels are undetectable). See Crow et al. 2017

for further details on toxicity of ammonia, nitrite, and nitrates for a closely related salamander species, the Barton Springs salamander (*Eurycea sosorum*).

Total dissolved gas also should also be measured weekly in a sink with flowing well water and in one representative container with salamanders per culture system to compare non-degassed well water with degassed water in refugia tanks. When a well is repaired after a well failure or is replaced, the total dissolved gas in the well water can drastically increase above 100%. For this reason, the total dissolved gas should be maintained between 80 to 90 % as a buffer. Salamanders that are exposed to supersaturated waters can develop gas bubbles under their skin and are seen floating in containers (refer to Supersaturation Events and Health Maintenance sections for more details).

Supersaturation Events

If the station well water supply pipe becomes only partially filled with water, which can happen when the station well pump goes down, after the pump comes back on and water fills the line, the increased pressure in the line forces the air into solution in the water coming through the pipes causing gas supersaturation to spike (101-145% total dissolved gas) in the supply water. This can harm salamanders by causing air bubbles to become trapped under their skin and leave them floating in tanks. Some salamanders can recover from this if given structure to hold themselves down in the water column and allowing the trapped gas to naturally pass out of their system over a few days in less saturated water. In case of a potential supersaturation event or if you see excessive bubbles in the tanks, check incoming well water gas saturation. It is supersaturated if above 100% and water often appears whitish due to many tiny gas bubbles being trapped in the water. In all individual containers with salamanders, reduce well water flow until it barely drips. Ensure that all containers have a much higher exchange rate of recirculating

conditioned (degassed and heated or chilled) water than well water. When well gas saturation returns to 100% or below (often takes several days to a week), readjust all lines back to full open position.

Some containers have only well water (e.g., hospitals), if possible, move salamanders to a safer location with conditioned water or come up with a contingency plan based on the estimated length of supersaturation event. You may be able to adjust water flow until well water just begins to trickle into containers. Freeze several Nalgene containers with well water in case they are needed to cool temperatures in containers. When well gas saturation returns to 100% or below (often takes several days to a week), readjust all faucets back to full open position.

Food Production and Maintenance

Worms come from a commercial source and should be acclimated upon arrival from shipping conditions to destination container conditions. Make sure the destination containers have been properly cleaned and disinfected prior to arrival. Place worms in isolation in their containers (on flow through water) for a week to observe for any anomalies with the order and remove worms that die due to shipping (usually 20-30% mortality). Mark date of worms' arrival. Keep an eye on worm levels to know when to order more so as not to completely run out before more are ordered. After a container of worms is empty of useable worms it should be thoroughly cleaned of any remaining snails and leeches and disinfected before restocking with new worms. Worms should be fed flaked feed twice weekly to maintain them and to gut load them for better nutrition for the salamanders they will be eaten by.

Small worms, for feeding smaller salamanders, are made by slicing larger worms into small pieces (either with a blade or a quick pulse with immersion blender). The newly-sliced

worms take a few days to be active enough to be used for feeding salamanders. Make up a large batch and place in a separate container. They should not be mixed with larger worms.

Amphipods can be collected from the bottom of ponds set aside for food cultivation on station. Although outdoor collection has been successful, indoor culture can be set up to supplement pond collections and to potentially reduce nuisance species mixed in with amphipods.

The following describes the collection of amphipods and the use of an amphipod separator (Cantu et al. 2009). Collect amphipods from a designated detritus-filled cultivation pond using a long-handled small mesh net. Drag the net through the sediment and/or grab pieces of vegetation and shake them off into the net. Rinse the net and its contents at the surface of the pond to allow the finer material to pass through the mesh. Place the contents of the net into a separation bucket containing clean water. Next, place a crowder (blue bottle top) into the bucket so that it sits tightly above the contents at the bottom. Then add flowing well water above this crowder at a moderate rate. Metabolic activity will create an anaerobic environment under the crowder. This will cause the amphipods to swim out of the hole at the top of the crowder, and its unique funnel shape will prevent the amphipods from returning to the detritus layer. The amphipods will then be forced by flow into a collection bucket and can be siphoned or netted from this bucket and fed out directly or placed into their own culture system. Allow amphipods to separate for 24 hours or longer. While in their own culture system or in the collection bucket gut-load amphipods with nutrient rich flake food to better enhance their nutritional value to the salamanders.

Figure 5 Picture of a amphipod separation system. Muck from the bottom of a pond is placed into a bucket fitted with a drain pipe mounted on the side. A cover made from the top of a 19l water jug is placed over the muck. Fresh water is pumped into the bucket over the cover. Amphipods and other organisms fleeing anoxic conditions under the cover swim up and are flushed out the drain pipe into the second bucket where they can be collected.

Daily Husbandry

Two "walk-throughs" at a minimum should be conducted daily (during weekend only once daily is acceptable) to ensure tanks systems are functioning properly. The first walk-through should be conducted in the morning and the second, one hour before closing (to allow enough time to correct system malfunctions). Though, when working in the area of the organisms, vigilance and watching of tanks should be done, always observing and being aware of the systems and the behaviors of the organisms in them.

1. Check water temperatures to be sure they are in the range given. Temperature is one of the most critical elements of these salamanders well-being, checking temperatures cannot be stressed enough.
2. Check to make sure water sources are flowing and functioning in each system/tank.

3. Check to water levels in tanks to make sure they are not either overflowing or running dry (starting to run dry). If either is occurring locate the source of the problem and take appropriate measures. Common causes are clogged outflow screens or leaks in lines.
4. Observe the general health and behavior of the salamanders in the system. If any are showing signs of disease remove them to isolation/ quarantine system and resolve the situation with the appropriate treatment.
5. Check for dead salamanders. If found, remove and place in appropriate sized glass vial, add label with date and specific tank number, and add 10% Neutral Buffered Formalin. Be sure to record death in appropriate logs.
6. Look for salamander eggs and note with date, time, number of eggs seen, and tank number. Eggs should be removed to a smaller tank to avoid them from being eaten by adult salamanders, amphipods, or worms. A baster can be used to gently suck up the eggs in water, be sure to cover the end of the baster so eggs do not fall out. For further instruction see Egg Care section.
7. Once a week all habitat enrichment items should be moved/picked-up to check for salamander mortalities that might have gotten trapped under the rocks or plants. During this time visually check salamanders for off-coloring, abnormal swimming, and other abnormal behavior or maladies.
8. Routinely check flow restrictors and drippers for clogs and regular cleaning.

Mortalities

Dead salamanders should be removed immediately from tank systems to reduce the spread of pathogens and preserved. Sometimes the tails continue to move even after the heart has stopped

beating. If you are unsure if a salamander is fully dead euthanize with a buffered solution of MS-222 at a dosage of at least 2 mg/L.

Feeding Procedures

A note on feeding**:** At SMARC we currently feed our salamanders Monday, Wednesdays, and Fridays, unless special cases warrant more often. We have found this is enough to keep our salamanders in good health and at above average weight from those found in the wild. Excess food should not sit in tanks as it can add to fouling. If you continually find excess food in your tanks, you might need to decrease the amount of food you are giving at a time. If there is a sudden jump in excess food, observe salamanders to see if they are actually eating and potentially consult a veterinarian on the change in eating behavior. See section on Food Production and Maintenance for details on food rearing and preparation.

Worms can be given to all juvenile and adult salamanders. Smaller worms that have been chopped up with an immersion blender should be fed to larval (after yolk sac has been absorbed) and small juvenile salamanders. The amounts of worms to feed will vary but as a general guideline estimate 5 worms per salamander. Worms should be fed once a week, unless there are not enough amphipods and then can be increased. Leftover worms are not encouraged in tanks as they can burrow holes in caulking around tank dividers. Dead worms can become trapped under habitat enrichment items causing fouling in tanks. Also, while worms are scavengers, they can scavenge on compromised salamanders.

Amphipods make up a larger portion of salamander diet in the wild, so when possible these should be a larger portion of the diet fed in captivity. If amphipods are plentiful feed twice a week. A good estimate is to feed 2 amphipods per salamander. After you have gathered the amount of amphipods needed for that day's feed put them in an isolation bucket/container and

treat with 1.5% salt solution in static water for 1 hour. This is to kill any hydra, flat worms, or introduced snails that might be mixed into the food. Allow well water to run through the bucket for 10 min to rinse off any salt from the food. Use a baster to feed the amphipods to salamanders. Do not feed amphipods to compromised salamanders in hospital tanks as amphipods are aggressive scavengers and have been seen to eat necrotic tissues off of live salamanders that are not in good condition.

Artemia can be fed to larval and juvenile salamanders three times weekly (M, W, F). Adult salamanders can also eat artemia and are a good choice of food item for compromised salamanders. Remove aeration from the brine shrimp culture separatory funnel. Wait 5-10 minutes for brine shrimp to settle, hold the funnel over a small plastic beaker, open the stopcock for about a second (try to get mostly live brine shrimp), and distribute evenly using a baster or pipette. Be careful that you do not add a lot of cyst cases - these can block the overflow screen. You may find that you need to temporarily stop or reduce water flow into tanks when feeding artemia so that the salamanders have time to hunt and eat them before they are flushed out of the container. Be sure to remove any uneaten artemia that settles to the bottom of the tank as it can quickly fungus and cause water quality problems.

Figure 6 This is an example overfeeding of salamanders. This amount of worms should never be left for long periods in a tank.

Tank Cleaning

Aquariums housing adult salamanders ideally should be cleaned (i.e. siphoned) once each week to remove fouling organisms and detritus buildup. It is preferred that each refugium system have its own equipment, including siphoning tubes, nets, and scrub pads. If not, be sure to disinfect all tools before using them on different refugium systems. Use a siphon to draw out any waste, debris, and uneaten food from each aquarium. Care should be taken to avoid pulling salamanders into the siphon tube as this can cause unnecessary stress and/or injuries, and as a precaution siphon into a bucket to capture any accidentally siphoned salamanders. Use a scrubbing pad to remove fouling organisms like algae and deposits of calcium from the walls, piping, and furnishings of each aquarium. Debris items and calcium build-up can clog stand pipe screens, so rotate screens regularly to prevent aquarium from overflowing. Additionally, make sure not to splash water on the walls or separating screens of aquariums housing salamanders as this could provide an escape route for the salamanders, which can climb up any moist surface. It is also beneficial to rotate habitat enrichment objects (rocks, PVC, fake plants) in and out of the system, which allows them to be dried out, cleaned, and sterilized periodically.

Hydra that comes in through the well water or with food can, at times, start to grow in tanks. These should be removed as soon as possible as they can eat the food meant for the salamanders (hydra mainly feed off artemia). Either remove the structure the hydra are growing on or siphon out the hydra. If a tank is completely over-run the salamanders may need to be moved and that tank drained and cleaned.

Inventory

A hand count of refugium population should be conducted periodically to reconcile mathematical population counts based on seen mortalities with an actual count of organisms. This can also help determine if salamanders are possibly escaping without being noticed. Physical inventory should be done at least twice a year, up to quarterly or more if counts are consistently off. Increased hand count inventories should be balanced with the amount of stress caused to the animals during this activity, recognizing when too much handling stress out-weighs counts. Visual counts should be conducted at least monthly. Care takers should always be cognizant and aware of salamanders keeping an eye on numbers as best they can.

The method for counting population size depends on the tank setup and the number of salamanders per aquarium. When the salamanders are in individual or small aquaria, counts can possibly be performed visually rather than using any type of handling to avoid disturbing the animals, as long as all animals can be counted and seen. Otherwise, habitat structures and salamanders should be removed until an accurate count is determined. In the larger holding tanks, the salamander population may be too numerous to count visually due to constant movement. To count the population in these larger tanks, start by netting out individuals and transferring them into a bucket. Count each salamander as they are netted. It may be beneficial

to use a hand tally counter while performing this task to help keep track of the numbers of salamanders. After a certain point you may be able to visually count the remaining salamanders in the tank. When possible, two people should do inventory to verify count numbers and to reduce handling time out of tank. Carefully, pour the contents of the bucket back into the aquarium once all the salamanders have been counted. Unfortunately, this causes a great deal of stress to the animals and care should be taken to minimize handling. During these inventories, also take extra time to inspect the tanks for needed repairs such as new pipe-stand screens, new tubing for water lines, peeling sealant, etc.

A detailed record of all population counts and daily mortalities should be kept in a data base or log book. Numbers from the previous inventory minus the mortalities should correspond to the results of the latest count. If numbers do not match, thought should be put into why there is a difference and what can be done to prevent this discrepancy in the future. Be sure to note the discrepancy of numbers in the data sheets and logs. Inventories are also a good time to separate male and female salamander to prevent any unwanted breeding (see Identifying Broodstock section for more on how to sex salamanders). Take this opportunity to closely inspect salamanders for any signs of maladies; if any are found they should be relocated to hospital tanks

Reproduction

Gender determination

Currently the most reliable way to determine gender for smaller bodied salamanders (San Marcos and Comal Springs) is by the method of candling (Gillette and Peterson 2001). For larger bodied Texas blind salamanders candling is not always effective to visibly see testes;

though on younger Texas blind candling can work, but balance against if the animal is mature enough for sexual organs to be seen. Sever (1985) demonstrates a method of cloacal identification of sexes that could be useful. Otherwise, you might have to wait until a female becomes visibly gravid to accurately identify as a female, at that time they can be marked as female for future identification.

To candle a fiberoptic light is used to shine through the dorsal side of an adult salamander that has been very gently "flattened" into the bottom of a small, clean, clear plastic bag. Transfer a salamander into a wet zip-lock bag (wetted with well-water) and carefully remove as much air from the bag as possible. Verify the salamander is not a male by searching for eggs on the ventral side of the salamander. If eggs are present, they will be seen beneath the skin of the salamander. If no eggs are present, gently stretch apart the plastic bag near the pelvic area of the salamander while holding up the bag to a light source. If two (rarely just one) thin vertical parallel lines are seen through the skin, the salamander is a male. No lines would indicate a non-gravid female.

Figure 7 A pair of salamanders undergoing candling. A) Male with paired black testes circled in red. B) Female with mature cream colored ovum visible just beneath her skin.

Triggering reproduction

We are currently refining reproduction protocols for all three species of salamanders with experiments designed to test several techniques developed over the years of salamander care. While there is not a current, documented, reliable method to trigger reproduction for these species, a method that has shown some success in the past (with a closely related species, the Barton Springs salamander) is separation of the sexes followed by pair grouping (Cantu et al. 2016).

Briefly: Males and females are separated for at least one month. The separation will occur in the same system where salamanders do not have physical access to each other, but adjacent so hormones can be detected/transmitted in the water by the opposite sex. Randomly selected pairs are then stocked into individual tanks that contain a rock to allow for mating dances. After a two week period to allow for mating, males are removed from the tanks to allow for the females to deposit eggs undisturbed. Aquatic moss (*Ambystigium* sp.) can be put into

aquarium for females to deposit eggs on. Group breeding can also be utilized, same as pairwise, but more than just a single m/f pair in a tank, optimum numbers have yet to be tested.

Texas blind salamander reproduction

Given the Texas blind salamander's restriction to subterranean environments, knowledge of their reproductive cycles is limited thus, breeding patterns for this species have yet to be established. However, incidental observations conducted year-round (Longley 1978) suggests a continuous breeding season.

The SMARC is one of the few institutions that has managed to successfully captively breed *E. rathbuni*, producing 71 clutches of eggs and three generations of *E. rathbuni* between 2008 and 2016. Yet, despite recurrence of oviposition events reported by the SMARC, egg deposition remains relatively rare and not predictable (Figure 1). The clutch size laid by the SMARC population of Texas blind salamanders (wild and captive bred) was 23.6 ± 1.95 SE with an average hatch rate of 23%.

Figure 8 Adult Texas blind salamander curled into a C-shape and depositing eggs (eggs show by arrows approximately 2 mm in size) in holding tank at the SMARC.

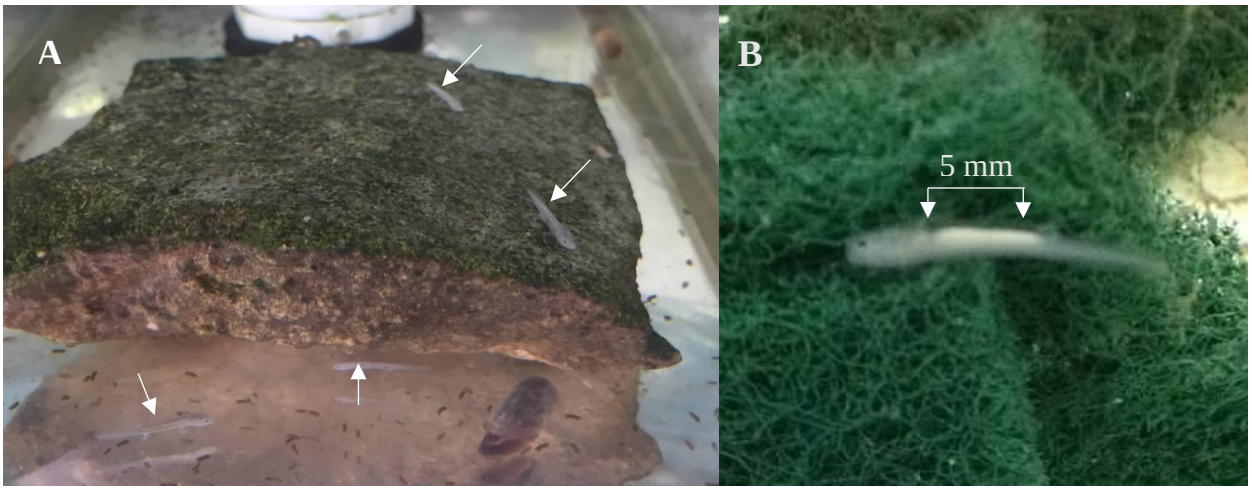


Figure 9 Recently hatched E. rathbuni larvae (A and B). Yolk sac shown (B) in a seven day old larvae.

Analysis of the reproduction records from the captive *E. rathbuni* population at the SMARC suggests a tendency for breeding to increase during winter and spring followed by a reduction during summer months (see Table 2). Further inquiry of ongoing captive reproduction and additional examination of historical data from wild caught individuals that include confounding factors, such as spring flows, rain events, and drought events may yield a more definitive understanding of the presence or lack of a seasonal breeding cycle in *E. rathbuni*.

TABLE 2. Summary of seasonal mean, standard deviation and range of 49 oviposition events between 2008 to 2014.

Season	N	mean	SD	range
Winter	18	2.6	3	0 - 9.0
Spring	16	2.3	1.6	1.0 - 5.0
Summer	6	0.9	0.7	0 - 2.0
Autumn	9	1.3	1.1	0 - 3.0

San Marcos salamander reproduction

From 1997-2011 San Marcos salamanders at SMARC had 221 ovipositions, average clutch size was 32.9 ± 1.3 SE, (range 1-95), of these the average hatching percentage was 22.8% ±2.0 SE range (0-100).

Egg care

Eggs can be moved by gently suctioning them up into a baster after all eggs have been deposited (full oviposition can take 48 hours). If necessary eggs can be removed a few hours after deposition if they are in danger of predation (by other salamanders or more likely by amphipods and worms) or other deleterious circumstances like equipment failure. Eggs are transferred cautiously by an appropriate sized baster to small holding container first (i.e. wide mouth beaker) and then into 5-gallon tank set up for egg rearing. When moving the baster out of

the water place gloved finger over the end to keep eggs from falling out. Eggs should remain in water at all times. Place egg on nylon mesh or matting in the rearing tank at evenly spaced intervals. Make sure there is enough water flow in the tank, but not so much that causes eggs to swirl around. Eggs should be checked daily for fungus and if fungused removed immediately—they will not survive and only serve as an agent to spread fungus to other eggs. The first cell cleavage should begin within the first 24 hours after oviposition and neurulation (formation of neural plate and neural folds) typically occurs within the first 48 hours after deposition. Distinct gill folds should be visible around day 17 and limb buds can be seen around 34 days. Larvae generally hatch out around 30 days depending on water temperature.

Newly hatched larvae can feed off the remainder of their yolk for 10-14 days post hatch. However, start adding artemia in small amounts 10 days after the first egg hatched. Increase amount of food added as more larvae develop and grow. Larvae should not be in a density greater than 20 hatched

Juvenile transfer

Once the hatchlings are about 15 mm in total length, they can be transferred to grow-out aquaria. This could be a 20-gallon aquarium of with no more than 20 of these small juveniles or separate out into fewer numbers in smaller tanks. Continue to monitor and adjust densities as the organisms grow. A baster does not work well because the salamanders are too active and swim out of the baster, possibly onto the floor. Use a small net and a baster to coax the young into the net. Put them into a beaker and then move them to the receiving aquarium. Salamanders should be housed with similarly sized individuals to avoid cannibalism.

Health Maintenance

Current knowledge and understanding of diseases in salamanders is very limited. Furthermore, diagnosis and treatment of maladies in salamanders is often insufficient. Currently the best tool

to maintain health in salamanders in refugia is prevention. However, as noted below, there are several options if salamanders begin to show signs of illness. Always consult a veterinarian or the Fish Health Unit for accurate diagnosis and suggested treatment.

Isolation of sick salamanders

Sometimes salamanders will show signs of possible sickness or abnormal behavior, such as not feeding, swimming in circles and spirals, will develop curved bodies, or turn very pale. These salamanders should be isolated from all other salamanders in small individual hospital tanks with flow through water. Provide a small object for cover such as a mesh cylinder and continue regular feeding. Many times, salamanders can recover on their own when moved to a less stressful environment without harassment and competition from other salamanders.

San Marcos salamander egg rupture

Gravid female San Marcos salamanders have been found to have their eggs rupture from their abdominal wall or cloacal region. They also can become very swollen and bloated with eggs and fluid in their abdominal cavity. Swollen/bloated females should be moved to hospital tanks and monitored; they might recover and end up reabsorbing their eggs. However, after a point if the salamander is not improving or if eggs have ruptured from the body, the decision should be made to euthanize the animal to end needless pain and suffering. Exact cause is not known, but we are currently working with veterinarians to try to determine a cause and work out appropriate treatment.

Microsporidia

During November 2013, the SMARC began working with Texas A&M University Veterinarian Medical and Biomedical Sciences Team (VMBST) to investigate possible causes of unexplained Barton Spring salamander mortalities. These salamanders presented lesions and abnormal

colored tissue loss on their skin. Since then the same symptoms have also been seen in San Marcos salamanders. The investigation identified a microsporidia parasite in both species. A treatment of Voraconazole was tried, but results were inconclusive as to its effectiveness in treatment. At this time there is still no treatment for microsporidia in salamanders. Currently the only known way to diagnose microsporidia is a histopathology test. If salamanders show symptoms they should be removed to a hospital tank. It is important to quickly remove dead salamanders from tanks as upon death the body sheds the parasite that could then infect other salamanders. The extent of the disease among wild and captive salamanders and ability of salamanders to recover are unknown. Preliminary tests revealed that zooplankton and native ramshorn snails (*Helisoma anceps*) were the only food infected with microsporidia.

Supersaturation/Gas bubble disease

At times gas becomes tapped under the skin of salamanders and the can start to float or hover in the water. If you see this happening immediately check the total dissolved gas levels in your system. Some salamanders can recover from this if given structure to hold themselves down in the water column and allowing the trapped gas to naturally pass out of their system over a few days in less gas saturated water. Sometimes the trapped gas can cause ruptures of organs that are fatal to the salamanders.

Fungus Saprolegnia spp.

Salamanders can develop fungus around their gills, face, and appendages. A note here: that based on evidence we, as biologists, have called this fungus, but this has not been clinically diagnosed by a trained veterinarian at this time. If caught early we have been successful at stopping the spread of the fungus and death by giving salt treatments to the salamander. Effected salamanders should be moved to hospital tanks and given a 2% salt treatment immediately. Salt

treatment should continue every other day until fungus has cleared. Be sure to vigilantly check the other salamanders in the tank the salamander came from for signs of the fungus that could indicate a system wide problem that needs to be addressed.

Acid Washing Tank Systems

Most of the systems need to be acid-washed annually to remove calcium carbonate deposits which affect efficiency of the temperature-conditioning equipment, valve functionality, flow, degassing, etc. Obviously, this cannot be done with the salamanders present.

Protocol for Acid Washing Refugia System with Muriatic Acid

Step 1 – Ensure all salamanders have been transferred to a holding tank or another system before adding acid to the system. Muriatic acid will kill any organisms left in the system, so double check all tanks and sumps for escapees.

Step 2 – Follow proper safe protocol and always wear appropriate protective gear while working with muriatic acid. Thick rubber gloves and aprons protect the skin from burns. Protective eyewear or face shield will guard the eyes from splashes. Always work in a well ventilated area as the acid reacts with calcium deposits and produces carbon dioxide gas. If needed use respiratory masks and gear. Consult your chemical safety officer to ensure all safety steps are being taken for your system, building, and set-up.

Step 3 – You can place smaller tank, pipes, hoses, and fixtures that also need to be acid washed into the larger base tank of a system. Fill the system with water and put a taller stand pipe in drain or plug drain. Turn on the recirculating system. Slowly and evenly pour

muriatic acid across water in the system. The general acid concentration we use is 5ml (HCl) per liter of water. A higher concentration of acid might be needed for systems that are calcified or that have not been acid washed for an extended period of time. Allow the acid to run throughout system for at least 1 to 3 days. Make sure to label the tanks being washed with signs that alert visitors and fellow employees about the presence of acid in the tanks.

Step 4 – Check the pH of the tank to see if the water has become more basic since the initial addition of acid. Calcium carbonate does somewhat neutralize the acid. Once the pH is at 4 or above the water could be safely drained into drainage system where it will be diluted with other water. If this pH and residual acid is still of concern the water may need to be neutralized further before draining. Again, consult your chemical safety officer for appropriate neutralization agents for your systems and drainage. Before the water is drained, while still wearing protective gear, the sides of the tank can be scrubbed to remove any residual calcium deposits that did not dissolve with the treatment.

Step 5 –Use a freshwater hose to flush the entire system (while recirculation system is still on to get acid out of lines) for several hours to remove any acid residue. At the end of the flush period, remove the hose and reassemble the system. Fill the system with freshwater and allow to sit for an additional day, drain and repeat as necessary to fully remove all acid from the system. Remember while an acid wash most likely kills any pathogens in the system, it is not considered or used as disinfectant. If you are concerned about disinfecting a system use another method after the acid has been flushed from the system.

Step 6 – Acid washing can be harsh on the seals of the system and on various components of the heater-chiller units. Be sure to check all seals and silicone on screens for damage and make appropriate repairs. Check to make sure the heater-chiller unit is running properly.

Step 7 – Before moving salamanders back into the system, it is essential to run a water quality tests. If conditions are ideal, transfer the salamanders back into the system. Re-label the tanks and record all activities and data into a log book for future reference.

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CAPTIVE PROPAGATION MANUAL FOR THE

COMAL SPRINGS RIFFLE BEETLE (*HETERELMIS COMALENSIS*)

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Contents

Introduction

In 1997, the U.S. Fish and Wildlife Service (USFWS) listed the Comal Springs riffle beetle (CSRB) as endangered (USFWS, 1997). CSRB have a fairly limited spatial distribution, concentrated in areas immediately adjacent to spring orifices and up-wellings within the Comal Springs system and immediate headwaters of the San Marcos Springs system (Gibson et al., 2008; Cooke et al., 2015). The entire critical habitat for this species consists of surface and subsurface components of 1.4 km of Comal Springs and 0.3 km of Spring Lake (USFWS, 2013). This range includes the 15 m around each spring outlet and extends 110 m down into the aquifer (USFWS, 2013). As part of the Edwards Aquifer Habitat Conservation Plan off-site refugia of all endangered species of the Edwards Aquifer are to be maintained, including the Comal Springs riffle beetle, in the event of sudden demise or radical reduction of the wild population due to catastrophic events (e.g., spring flow severely reduced, desiccation of habitat, or anthropogenic contamination).

Ecology and Life History

Comal Springs riffle beetles are members of the family Elmidae (Brown, 1981) and belong to the genus *Heterelmis* (Sharp, 1882), a group of fully aquatic beetles with 20 described species (Manfred et al., 2016). At least four species of *Heterelmis* are found in the state of Texas: *Heterelmis comalensis, H. glabra* (Horn), *H. obesa* (Sharp), and H. *vulnerata* (LeConte) (Burke 1963; Brown 1972; Brown 1983; Bosse et al. 1988). Even though the Comal Springs riffle beetle inhabits the same river system as *H. vulnerata*, it appears to be more closely related to *H. glabra* (Bosse et al., 1988; Gonzales, 2008; Nice and Lucas, 2015). The Comal Springs riffle beetle (length: 1.7 – 2.1 mm; width: 0.8 – 0.91 mm) can be distinguished from *H. glabra* (length: 1.9 – 2.35 mm; width: 1.0 – 1.17 mm mm) by its smaller size, more slender shape, paler

coloration and truncated wings (Figure 1; Bosse et al., 1988). Of the four Texas species of *Heterelmis*, the Comal Springs riffle beetle has one of the smallest habitat distributions (Bowles et al., 2003; Gibson et al., 2008).

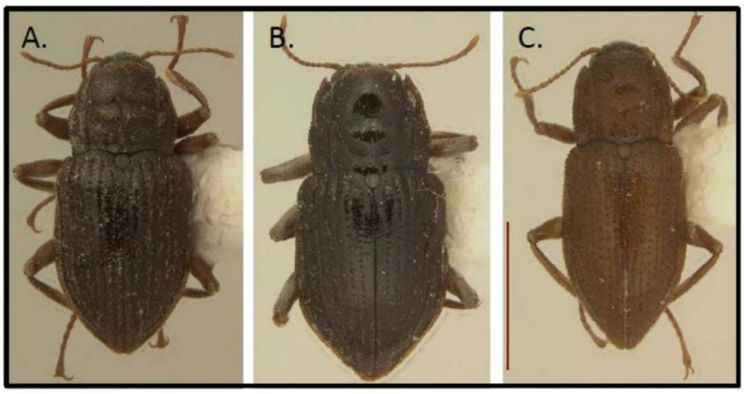

Figure 1. A side to side comparison of three species of Heterelmis riffle beetles. (A.) H. cf. glabra, (B.) H. vulnerata, and (C.) H. comalensis. As seen in Nowlin et al. 2014.

Riffle beetles in general typically inhabit shallow, clear areas of swift flowing streams where levels of dissolved oxygen (DO) are elevated (Brown, 1972). Elmid larvae have external gills, which allow them to absorb oxygen directly from the water (Graham, 1990). Adult beetles can respire underwater through a film of air that covers the spiracle of their trachea system (Brown, 1987; Resh et al., 2008). Within this thin sheet of air, known as the plastron, is a dense layer of fine specialized hydrophobic setae (hairs and scales) used for gas exchange (Hinton, 1976; Brown, 1987).

Riffle beetles are considered to be biofilm scrapers, slowly crawling over rocks and wood gathering up small feed items like bacteria, fungi, and diatoms (LeSage and Harper, 1976; Brown, 1987; Elliott, 2008). Stable isotope and gut content analyses show that CSRB are detritivores, feeding on wood, leaf, and root decaying microorganisms within spring sources of the Edwards Aquifer (BIO-WEST, Inc., 2015; Nowlin et al., 2017b)

This species seem to show no seasonality in mating, larval development, or emergence patterns and have overlapping, asynchronous generations in the wild and in captivity (Bowles et al., 2003; Cooke, 2012; BIO-WEST, Inc., 2017). Life history studies showed that CSRB deposit eggs singly or in clutches predominantly on leaves; eggs incubate for approximately 3 weeks before hatching; larval development fit a linear trend across time reaching their final (7^{th}) instar in 4 months; the final instar typically takes 4 months for pupation; and overall generation time was found to be 2 years (BIO-WEST, Inc., 2017). Currently, requirements for pupation of Comal Springs riffle beetle are still unknown. The combination of leaves and cotton cloth substrate for biofilm production was found to be important to the success for development and egg production (BIO-WEST, Inc., 2017).

Refugia Protocol

Methods described in this manual were designed and implemented by the staff of the USFWS at the San Marcos Aquatic Resources Center for CSRB refugia systems. It is the intent of this manual to share general procedures and guide future development of culture methodologies. This is considered a living-document and will be up-dated and refined are more information is learned about this species.

Culture Holding Containers

Culture containers currently favored at the SMARC for CSRB are Easy-Spring model systems. This is a spray bar type system (Figures 2 & 3) uses a single spray bar and one or two horizontal outflow standpipes. Designed and specially constructed by SMARC staff in order to better replicate the hydrology of Comal Springs, the Easy-Spring systems have thus far provided

success in survival and potential reproduction. These systems have been run completely on flow-through Edwards Aquifer well water, but could easily be modified to accept re-circulating water.

Easy-Spring culture containers are constructed from plastic tubs/totes (Figure 2). Easy-Spring containers have holes drilled in the sides to allow for adaptors/parts to be inserted for water inflow and outflow. Containers should be assembled using PVC glue and/or aquarium safe silicone, allowed to cure-dry, and water run through them for a period of time to flush out any potentially harmful residual chemicals before organisms or habitat are added (this is also a good time to check and fix leaks).

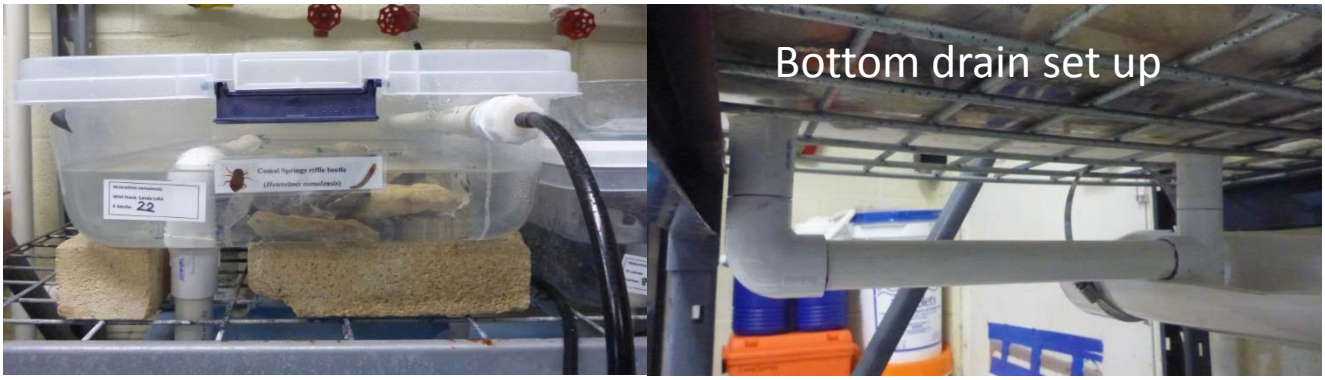

Figure 2 Outer construction and design of an easy spring container. Outflow design is one of many options and can be changed to fit your refugia preferences

Figure 3. Inner construction and design of an easy spring container. (Left) Spray bar extends to the container wall before trickling to the water line. (Right) Rocks are spaced far enough to allow water to flow between the stacks to prevent anoxia.

Holes in the front side near the top allow piping to provide water into the system via a spray bar (Figure 3). The spray bar has 1/16 inch (1.6 mm) holes drilled evenly spaced along a horizontal line. The spray bar is pointed so that the water sprays onto the side of the container, not down into the container or out over the surface. The intention of the inflow spray bar design is to simulate the hydrology of spring orifices at Comal Springs in which water moving down the side of the container will move in a laminar fashion through the culture medium into the outflow standpipe.

The outflow standpipe is constructed from 1in PVC pipe that is affixed to the bottom of the culture container and runs horizontally at the desired water level height. A series of 5/16 to 3/8 in diameter holes drilled into the outflow standpipe allowing water to passively exit the culture container. These holes are covered with 50 to 100 µm nylon mesh to prevent the escape of the beetles, larvae, and eggs. Lids are fitted to the top of containers to prevent other organisms from getting into the culture. Part of the lid can be removed and replaced with 700

µm nylon mesh to help control humidity while preventing entry of unwanted organisms. Culture containers should be kept dark to mimic the underground environment. This may be accomplished by partially covering containers with black plastic or shade cloth. Bottoms and sides of containers may require shading as well to prevent light from causing distress.

After containers are made, habitat items can be assembled in the container. It is best to put the container in its place on the rack system and get the water lines hooked up (but not filling) before adding rocks, leaves, and cloth, then fill with water. This prevents items from shifting while the container is being moved. The culture medium is made up of leaves, cloth lures, and smooth flat rocks piled up under to spray bar. This culture medium acts as both food and shelter for the beetles and their offspring.

- Limestone rocks (and all rocks) should be cleaned and fully dried before using in containers to prevent the potential transfer of other species.
- The leaves must come from a terrestrial source (not an aquatic source) so that accidental introduction of non-target aquatic organisms is minimized. These leaves should be collected from trees around the river system that the organisms inhabit and can include, but are not limited to: Anaqua, Sycamore, Pecan, or Black Walnut. Leaves should be quarantined for at least 2 weeks (following corresponding HACCPs). Leaves should be dried in the drying oven and then can be stored in a dry, sealed container until they need to be used. Leaves may be used by the organisms as cover or as a potential source of food. When conducting inventory, save some of the leaves from a container to use when placing habitat back into that container.

- Cotton cloth is used in the field to attract CSRB for monitoring efforts. It has also been found to be a useful food source in culture for CSRB. Conditioned cloth, that is cloth that has been colonized by biofilm, can be added to the container. You can save conditioned cloth from a container as you are conducting inventory and add back when re-assembling the habitat. Alternatively, condition cloth can be added by placing new strips in a container with flow-thru well water, but no organisms, for several weeks to allow biofilm to colonize. Additionally, new cotton cloth is added for cover and so that it can start the process of colonization by biofilm.

To begin habitat assembly (after container is in designated location) put a layer of smooth rocks with pebble legs on the bottom of the container. The pebble legs allow for water flow underneath the habitat, reducing anoxic spaces. Then begin layering leaves between limestone rocks. Leaves should not be stacked too densely in one spot as they can reduce the flow of water and create anoxic areas. Cotton cloth strips (both conditioned and un-conditioned) can be placed near, but not stacked with, the leaves as to avoid reduction of water flow and anoxia. Once habitat is constructed, turn on the water flow and allow the container to fill. Observe for any problems or adjustments that need to be made. Add in the organisms. Containers may also be completed and water running weeks before invertebrates are added so the further conditioning and colonization by biofilm can occur.

Water Quality

Due to the unique spring environment, the CSRB should be supplied specific water parameters in order to thrive in captivity. Temperature in the refugia should be kept near 23 °C

and monitored daily. Nowlin et al. (2014, 2017a) showed that the Comal Springs riffle beetle experienced agitation at 27 - 29°C and a lost ability to respond to stimuli at 44 - 46°C when exposed to these temperatures for a short period of time (3 min).

Water quality values (see table below) should be checked weekly, unless problems arise and then should be checked more frequently. These values given here are a rule-of-thumb based on observational and anecdotal evidence, and are not necessarily backed by scientific analysis or experimentation. More research into optimum water quality parameters is encouraged.

Metric	"Safe Range"
Temperature	18-24 °C
Dissolved Oxygen	6-8 mg/L
pH	7-8.2
Total dissolved gas	≤ 100%
Ammonia	< 0.05 mg/L

Refugia Field Collection

The current method of collection for CSRB is using cotton cloth lures. Until further genetic analysis is conducted collections from several locations at Comal Springs following finding by Lucas et al. (2016) is suggested in order to get a good representation of the species' genetic diversity.

Collection Locations

In 2013, the Service designated critical habitat of the Comal Springs riffle beetle to be approximately 169 acres (68 hectares) of the Comal and San Marcos Rivers impounded headwaters (USFWS, 2013). Within these aquatic habitats, CSRB is largely concentrated to spring openings and adjacent areas (Gibson et al., 2008, Cooke et al., 2015) (Figure 4). CSRB

can be found at a few spring openings at the headwaters of Spring Lake in Hays County near the Meadows Center for Water and the Environment (Gibson et al., 2008).

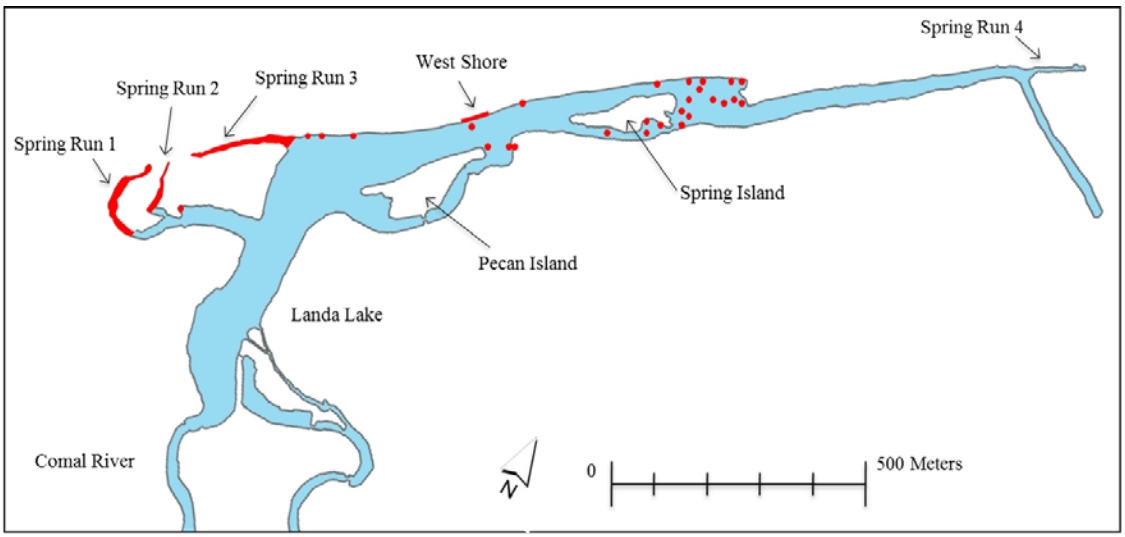

Figure 4. Distribution of Comal Springs riffle beetles in Landa Lake, Comal County, Texas.

Cotton Cloth Lures

A standardized cotton cloth lure protocol (EAHCP, 2016) and collection methods has been developed by several researchers following Huston et al. (2015). These lures can be made from a 225 cm^2 piece of cotton-60% / nylon-40% (eg. a bed sheet) folded into a 5×5×1cm rectangle, then placed in galvanized steel screen box cages with a mesh size of 1 cm^2 (Figure 5, Figure 6). The cages help to prevent anoxic conditions from occurring within the cloth lures as a result of compaction into the substrate and aid in standardizing the method. The lures should be placed inside spring sources where they will be colonized by local microorganisms, creating a biofilm which serves as a food attractant. Do not bury the lure(s) if the substrate is primarily silt, this will cause anoxic conditions over time. Sand grain size or larger is preferable. Lures should be covered with rocks to prevent dislodgment.

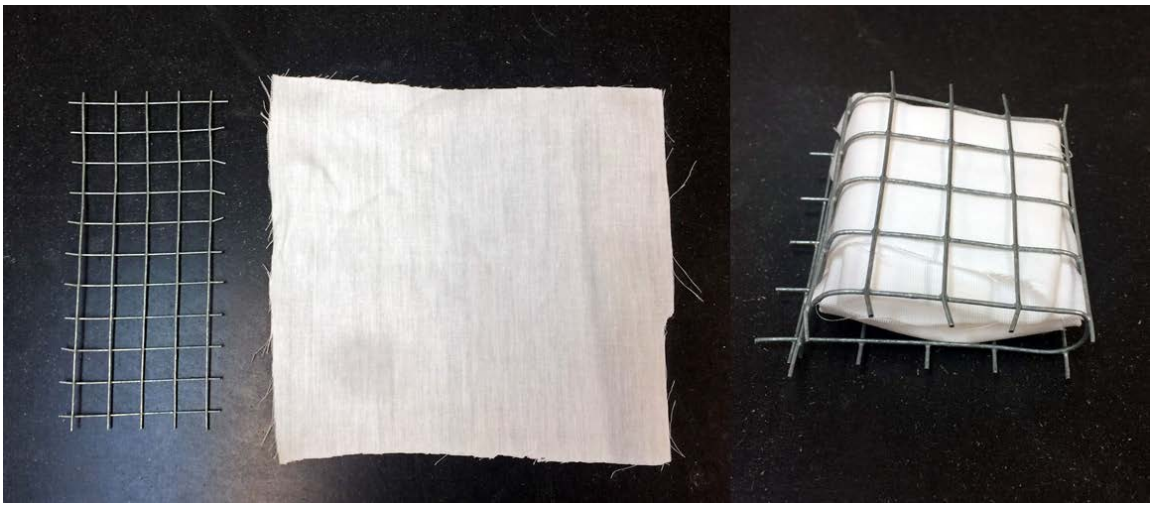

Figure 5. Materials and construction of poly cotton cloth lures.

Figure 6. Collection cup (700 ml) containing lures for riffle beetles and other invertebrates before proceeding to sorting the contents. Biofilm and beetles (black specks- one circled in red) on a lure brought in from conditioning in the field for approximately.

Upon collection of a retrieved lure, open the wire cage and unfold the lure (Fig. 1a) to count and separate species and life stages. Cloth should be placed into a small dish containing fresh spring water to sort. Use small plastic pipettes for larvae or soft forceps for adults when separating/counting. After species identification is verified, move individuals to a target

container (e.g. Polypropylene jar with lid ranging in sizes from 200 ml – 1 L). The holding containers are then moved to a transport container (small drink coolers filled with spring water to maintain temperature during transport).

After arrival back at the station, compare the water temperature inside the transport container that is holding the invertebrates from the field to the water temperature within the appropriate destination container; you may use the invertebrate room sink water temperature as a proxy if needed. Acclimate the water temperature as necessary before introducing the newly collected species into their designated quarantine container within the SMARC invertebrate room. A rule of thumb would be changing the water temperature 1 °C every half-hour; however, no studies have been done parameterizing acclimation speeds for these invertebrates, more research is warranted. All culture containers should be clearly marked with the name of the species and the location that they were collected from and the date period they were collected.

Organisms are kept in containers marked “Quarantine” for 30 days before joining containers of Refugia population. All culture containers should be clearly marked with the name of the species and the location that they were collected from and the date period they were collected. During this time we observe for other potential ANS species that might have come in with the collection. Observe the general health of the organisms and watch for large die-offs that might indicate a disease. If none of these occur, then they can be moved to the Refugia population at the end of the quarantine period. No bacteria or viruses are currently known to infect riffle beetles, but these beetles are prone to fungal infections (Benjamin, 1973; Brown, 1987). Infected beetles will sprout white cotton like tuffs of fungal spores from the joints in their exoskeleton (Figure 7). Protozoa (ciliates and rotifers) are commonly found attached near the

mouthparts of riffle beetles. These symbiotic protozoa likely feed on biofilms and debris produced by the beetles as they feed (Steffan, 1967; Brown, 1987; Fries, 2003; Figure 8).

Figure 7. Left is a fruiting body of an aquatic fungus protruding from the exoskeleton of a riffle beetle. Right is a fungal "cocoon" encapsulating a dead beetle. Whether these are the cause of the individuals' death is unknown.

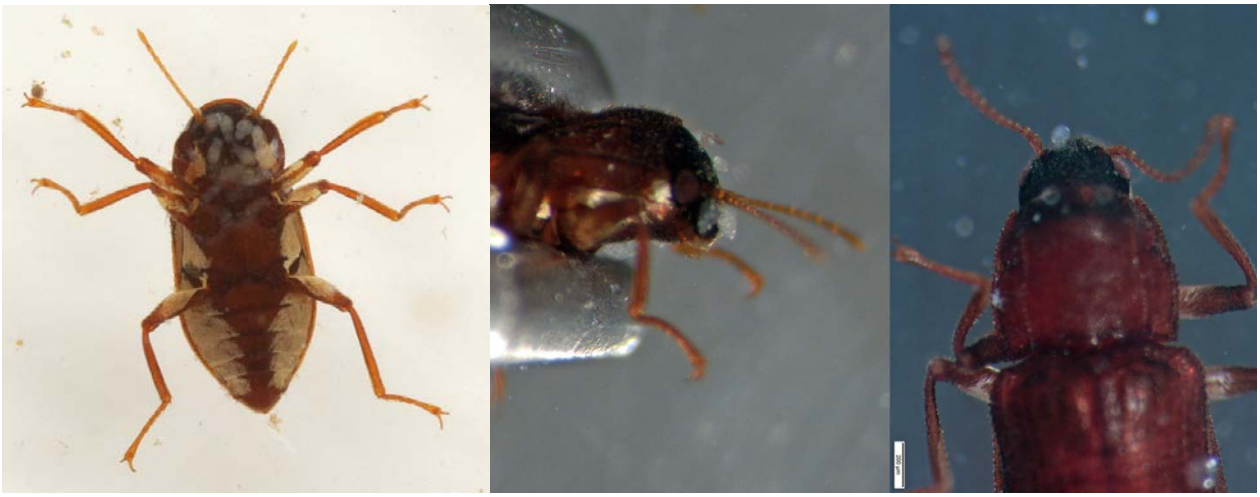

Figure 7. Comal Springs riffle beetle adults with protozoans attached to their heads.

Biosecurity

A disinfection foot mat (or some type of foot bath) containing a 10% Virkon solution should be placed at all the entrances to the refugia; these mats help reduce the spread of nuisance species both into and out of the invertebrate room.

For field collection equipment, all should be visually inspected and sterilized before and after leaving the refugia facility to prevent introductions of organism into new environments. Use caution with chemicals like Roccal, bleach and Virkon, commonly used for equipment sterilization to kill bacteria and viruses on station, these are highly toxic to invertebrate organisms. Leftover residue from poorly rinsed containers and soft mesh can leach into the water even after being dried by direct sunlight in the case of surfactants. Think about how the equipment will be used again and if these chemicals will get back into the invertebrate environment. Best practices would be to have a set of equipment for each system.

Transport water brought back from the field should be poured into a drain that leads to a proper waste water treatment system (e.g. chlorinator/sand filter). If forceps need to be sterilized, use isopropyl alcohol. All containers and storage equipment should be completely dried before sealing lids and closing storage containers.

Refugia Maintenance

Riffle beetles in general are sensitive to pollutants and degraded water quality. Ensuring a clean stable environment is essential for the captive propagation of the CSRB. The refugia should be housed in a dark, temperature-controlled room set between 20 - 23 °C (representative of the wild habitat of the Comal Springs riffle beetle). The refugia should receive Edwards Aquifer well water and the capability to recirculate water through a pump and heater/chiller as a

backup to maintain a constant environment in the event well water is not available or has insufficient water quality. A walkthrough of the entire refugia system should be performed at least two times daily: once in the morning and once again in the afternoon. During the walkthrough, checks should be performed on the water flow rate entering the system and in each individual tank.

Daily Activities

1. Log water temperature and pressure
   1.1. Find datasheet on a clipboard above the desk and take the thermometer to the sink.
   1.2. Run the water for about 20 sec and then fill any beaker greater than 400mL and let it overflow.
   1.3. While water is overflowing and still running, take a temp reading and record the value on the datasheet.
   1.4. Pressure gauge will be on the same nozzle in the sink. Make sure water is on full before taking a reading, if pressure reading deviates from normal check the pressure gauge outside the invert room in the greenhouse. Change in pressure can be the first indication that there may be problems with flow in the containers.
2. Check for inflow in all containers. If flow is either too high or too low first refer back to the water pressure reading to see if it is abnormal before adjusting any valves.
3. Check the outflow standpipes in all containers and remove debris with designated toothbrush for the container.
4. If mortalities are seen preserve individual in 95% EtOH in small glass vial with appropriate labeling on Rite-In-The-Rain paper slip inserted into vial.
5. Check for flooding, water puddles, and/or drips.
6. Clean floors, desks, equipment, and tools.
7. Work spaces must be neat and tidy. Make sure walkways are clear. Dry containers and desk with a rag. Put away tools in proper place and make sure the light is turned off at the end of the day.

Weekly Activities

1. Wear the correct PPE. Replace disinfectant (e.g. Virkon®) in mats by the door. Mix chemicals outside of the invertebrate room (see Virkon Biosecurity section for details) and carefully fill the mats making sure not to overfill. Virkon is highly toxic to invertebrates so be sure not to mix chemicals inside room and that it does not spill out into the room or come into contact with containers.
2. Check for $CaCO_3$ deposits interfering with valve functionality and/or water flow. Obstructions should be removed or valves replaced as needed.
3. Check all water quality parameters using the one of the water quality meters available. Use the same method as Daily Activities #1 but with a beaker large enough to fully immerse the probes. Record on the data sheet on same clipboard as the daily log. Check a random flow through container weekly for water quality parameters using test strips or meter if box is deep enough.
4. Check chlorination system.

Monthly Activities

1. Record all inventories on data sheets, keep track of Daily Log datasheet, and transfer both to appropriate files using the folders on the local drive. Make sure to scan the hard copies of daily and weekly logs (and all data sheets) and save these files.
    1.1. Keep a copy of these records on another computer. This prevents loss of data in case of a system failure and serves as a backup to paper documents.
2. The Deputy Director requires a monthly total of each species completed by close of business on the Wednesday of the last week of the month:
    2.1. Report total wild stock individuals in refugia, total in quarantine, total number of individuals that were captive bred, and the number of mortalities in refugia.
    2.2. Other numbers and data not required by the monthly report still need to be collected and reported, just not for this particular monthly report.
3. Update documents
    3.1. Online documents
        3.1.1. HACCP plans and logs

3.1.2. Field collections

3.1.3. EAA species records

3.1.4. Outside take records

3.1.5. Inventory

3.1.6. Daily Water Quality and Feed logs

3.2. Paper copies are to be scanned and stored on the local drive or transferred to the correct filing excel sheet system in place for EAA contract. Keep backups of all documents on a separate computer or drive.

4. Check A/C unit
   4.1. Clean/replace the air filter
   4.2. Clear debris. Remove the filter and clean with a water hose if necessary.
5. Clean walls
   5.1. Wear the correct PPE. Fill a bucket with a bleach solution and use a mop to cleanse and rinse the walls to prevent molding. Focus attention to the doors and ceiling near windows. Avoid areas around refugia containers to prevent drips or spills into containers.

Yearly Activities

1. Keep good records of monthly collections into the Federal and TPWD collections permit log. Submission is based on the calendar year for both, but with different start and endpoints. For example the Federal permit is due in December while the TPWD permit is due in June.
2. Keep good records of monthly files to utilize and compile for SMARC station annual reports; submission is for full fiscal year.
3. Same files are used for the EAA annual reports; submission is for the calendar year.

Inventory

An inventory of the refugia CSRB populations should be conducted routinely to determine the population size and track changes in reproduction or health. Inventory should be

performed every 1 or 2 months; too much disturbance could add undue stress on the organisms, too little disturbance does not allow for an accurate accounting of the species.

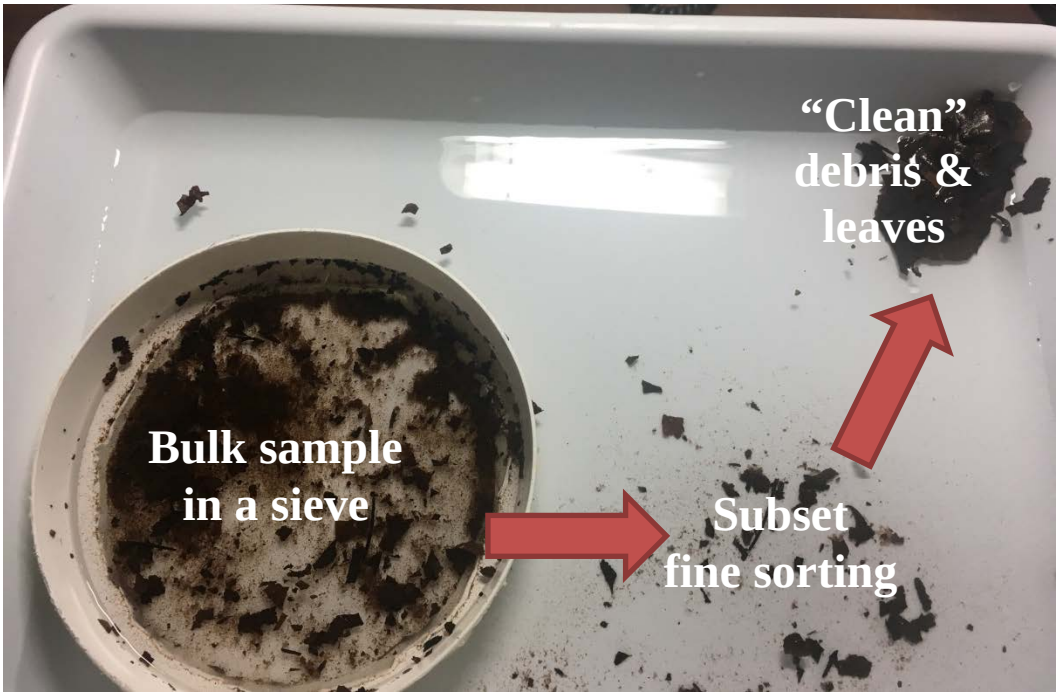

Figure 9. CSRB inventory set up in the first step of the total process: sorting through the sieve of debris, excrement, decayed leaf materials.

In order to accurately count the population, the entire contents of the container needs to be removed. Have a white tray partially filled with well-water ready on a desk to begin sorting and prevent desiccation (Figure 9). First carefully remove rocks, cloth lures, and large leaves from the container. You may either carefully inspect these items at this time for beetles, eggs, or larvae or place them aside in containers of fresh well water for inspection after the debris of the container is sorted through. When just water and the smallest debris are left inside the container, tilt the container to pour into a fine mesh net sieve (< 100 µm). Rinse out the container with fresh well water into the sieve to completely empty the container. Next, run fresh well water through the sieve to wash any fine silt from the leaf litter debris while retaining organisms. Sort through debris under a magnifying glass and remove any found CSRB, larvae, pupa, or eggs

using a pair of soft lightweight forceps. Collected CSRB life-stages can be placed into small (150 ml) appropriately labeled beakers filled with fresh well water. If any mortalities are present, remove the bodies and keep a count of the mortalities, preserve in 95% EtOH. If rocks, cloth, and leaves were not thoroughly inspected before debris, go through each of these items now, placing them in the tray with well-water to prevent desiccation.

After counting, any adult beetles can be returned to the refugia, while any eggs or larvae discovered during inventory periods are removed to a F1 (1^{st} generation) culture system. Pupae can be placed into pupae chambers or placed into a F1 culture system. Record all population counts, F1 transfers, and pertinent notes on to data sheets.

Cleaning Containers

Containers with CSRB generally are deep cleaned whenever the container must be disturbed (e.g., bi-monthly inventory or removing young). This is cleaning that goes beyond the routine daily care and cleaning of flow bars and outflow standpipes. In order to remove $CaCO_3$ deposits without toxic chemicals, rinse empty containers with well water in the sink and scrape or wipe the containers and/or spray bars using brushes and sponges. If $CaCO_3$ deposits cannot be removed or are particularly bad, a replacement container or parts may be required. Any valves or tubing needs to be checked for $CaCO_3$ deposits and functionality and should be replaced if damage or obstructions are found. *Do not acid-wash*: most chemicals on station are not safe for aquatic invertebrates.

- Do not use bleach
- Do not use acid (e.g., HCl)
- Do not use Virkon

If a container or components have particularly stubborn $CaCO_3$ deposits they can be soaked in a vinegar and water solution to loosen up the deposits. The containers should be triple rinsed and allowed to completely air dry before using again for invertebrates.

Nutrition

It is believed that CSRB feed off the biofilms that grow on decaying terrestrial plant-matter found around spring openings (Gibson et al., 2008; BIO-WEST, Inc., 2015, Nowlin et al., 2017b). In the wild, CSRB are often found on leaves and woody debris near spring sources (Gibson et al., 2008). Dried leaves and conditioned cloth have been shown to be effective nutritional sources under culture conditions (BIO-WEST, Inc., 2017).

For the refugia, leaves are picked from trees around the springs. Trees that produce useable leaves include walnut, pecan, anacua, elm, and sycamore. Leaves should be dried in the drying oven and then can be stored in a dry, sealed container until they need to be used. It generally takes weeks before enough biofilm accumulates on new leaves and lures (Huston et al., 2015). This means that newly constructed containers should be to be allowed to mature for a week or two before CSRB are added to the system. Alternatively, when conducting inventory save some the larger leaves from a container to use when placing habitat back into a container with new leaves to help start the biofilm colonization process. The same holds for cotton cloth that can be saved during inventory. Both should be replenished with every two months during inventory.

Refugia Propagation

Propagation methods for CSRB are still being tested and refined and are considered a work in progress.

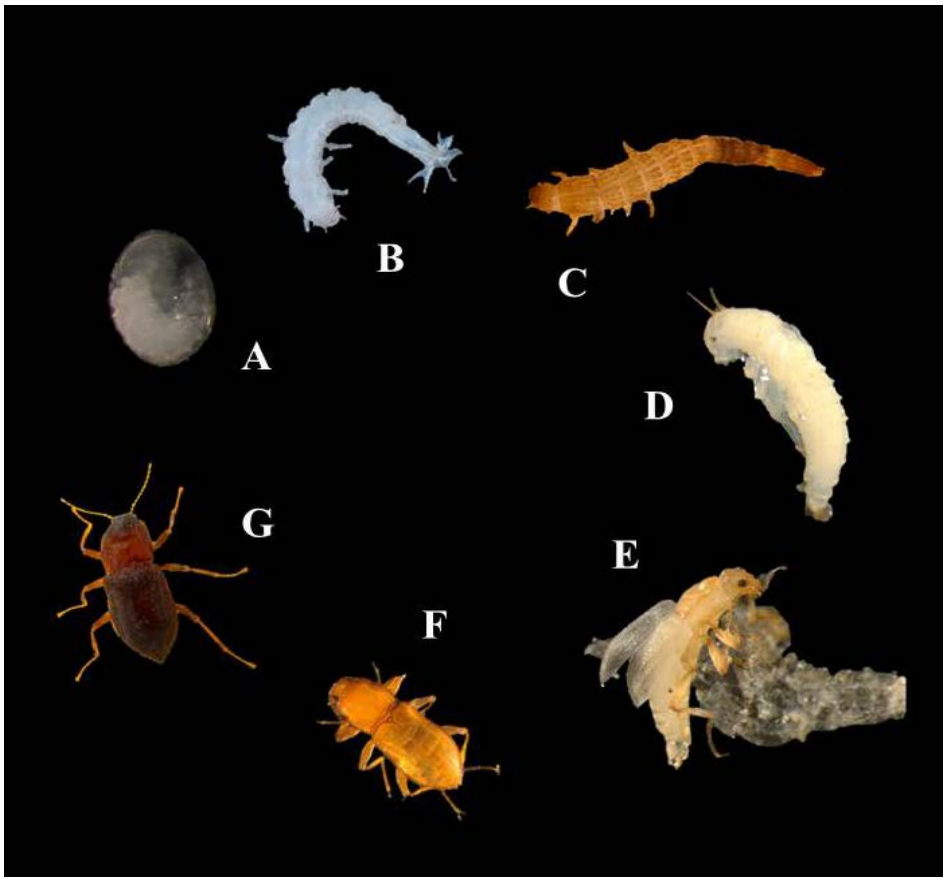

Figure 10. Development of the Comal Springs riffle beetle divided into smaller stages to distinguish between molts and size differences. Order clockwise: A) Egg B) early instar larvae C) late instar larvae D) pupa E) pupa emergence F) teneral adult G) adult. Pictures are not to scale.

Sex Determination

Research by BIO-WEST, Inc. (2017) comparing the length of the posterior most sternite (terminal sternite) shows differences between male and female CSRB. Male beetles have a terminal sternite measuring less than 0.26 mm (85% within range) in length, while the females have a terminal sternite length greater than 0.28 mm (95% within range) (Figure 11).

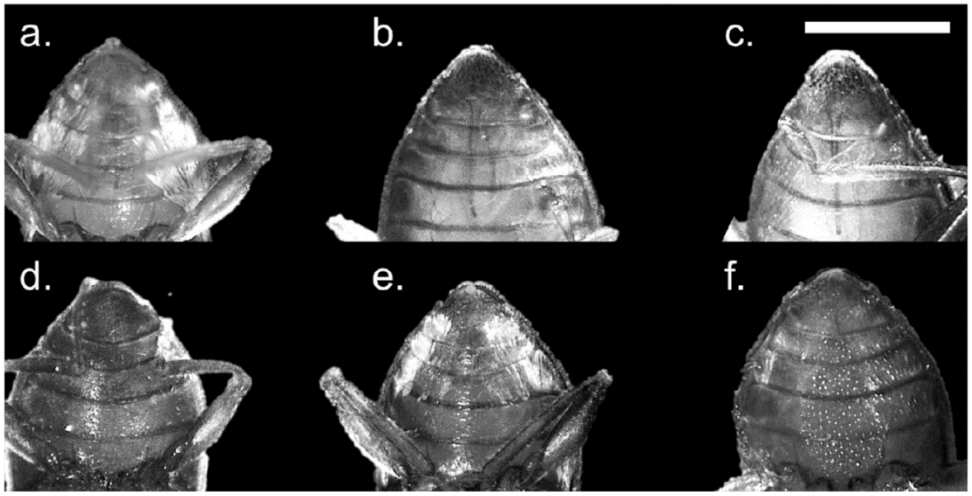

Figure 11. Photo micrographs of female (a-c) and male (d-f) abdomens. Females are recognizable by noticeably more elongate posterior most sternites. Scale bar is 500-µm. (BIO-WEST, Inc., 2017.

Egg Production and Collection

Due to the constant nature of its spring habitat, CSRB exhibit no seasonal reproductive cycle with larvae and adults found at the spring openings year-round (Bowles et al., 2003; Gibson et al., 2008). Eggs have not been positively identified in the wild, and refugia propagation is the easiest and most certain method to obtain them. Based on research in refugia, the female beetle retains a clutch of eggs in her abdomen and lays relatively large eggs (300 µm × 200 µm) in clutches of 3 ± 2.5 eggs every week depositing eggs primarily on leaves in their containers (BIO-WEST, Inc., 2017) (Figures 12 & 13). Riffle beetle eggs hatch-out in ~25 days and newly hatched larvae begin to feed on biofilm.

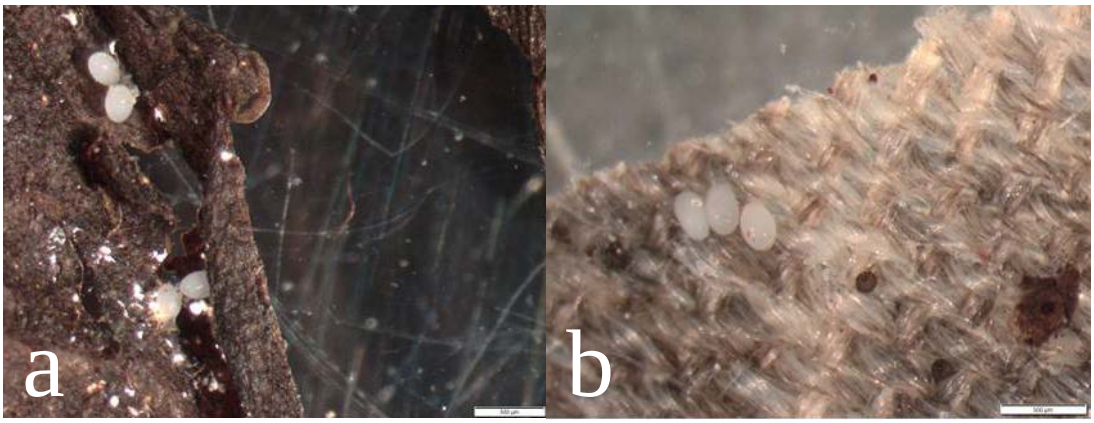

Figure 12. Egg deposition on various materials. a) sycamore leaf; b) poly-cotton cloth.

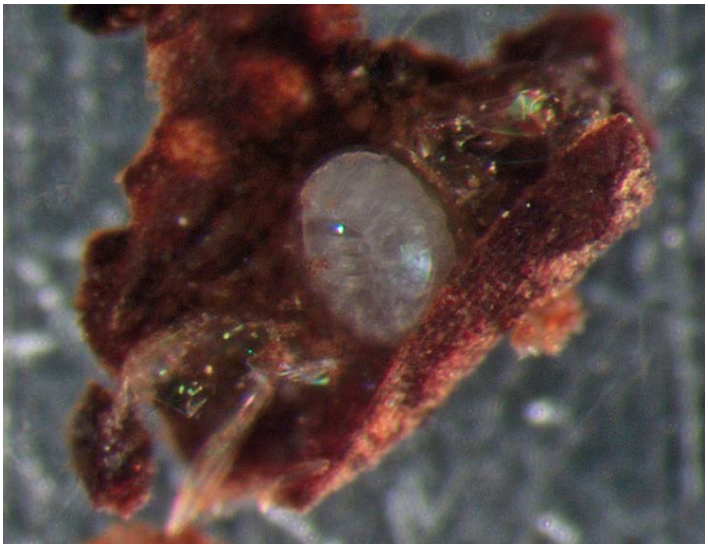

Figure 13. A Comal Springs riffle beetle egg attached to a piece of leaf. The developing larvae can be seen inside.

Larval Grow-out and Pupation

Comal Springs riffle beetle are highly productive with adult beetles producing 10 or more larvae per adult per month (Huston and Gibson, 2015). CSRB are thought to have 7 instar stages before pupation (Cooke 2012, BIO-WEST, Inc., 2017). First instar larvae of the CSRB have a head capsule measuring ca. 0.013mm across, while the final instar's head capsule is ca. 0.45mm

across, roughly a 40 fold increase in growth (Cooke, 2012; BIO-WEST, Inc., 2017). Larvae follow a linear growth cycle with 50% mortality observed at each stage until the final instar where survival increased to 90%. During this final stage pupation take around four months. Unlike other species of elmids, the CSRB can pupate under the water line (Huston and Gibson, 2015). Larval CSRB do not construct pupal chambers nor do they anchor themselves to substrate (Huston and Gibson, 2015). Currently, the requirements for pupation of Comal Springs riffle beetle are still unknown. Pupae collected during inventory should be isolated and transferred to a pupation chamber or F1 tank. Pupation chambers can be made from small flow-through filters or PVC chambers modified with 200 µm mesh covering each end. These chambers are connected to a well water line that supplies the chambers with fresh water that upwell through the tube (Figure 14).

Figure 14. Pupation chamber with pupa.

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CAPTIVE PROPAGATION MANUAL FOR PECK'S CAVE AMPHIPOD

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Introduction

The Peck's Cave amphipod (Figure 1) was listed as endangered due to its limited range and sensitive habitat requirements (USFWS, 1997). This species is also included on the IUCN Red List. This crustacean species, of the order Amphipoda, is restricted to two subterranean spring systems in central Texas (Comal and Heuco Springs) that are threatened by increasing use of the Edwards Aquifer and/or other human activities that impact water resources within the Edwards Aquifer (Barr, 1993; Gibson et al. 2008; USFWS, 1995). The entire critical habitat for this species consists of surface and subsurface components of these two spring systems and surrounding wells (USFWS, 2013). This range includes the 15 m around each spring outlet and extends 110 m down into the aquifer (USFWS, 2013).

Figure 1 A white morph Peck's cave amphipod from Hueco Springs on the left and an orange morph Peck's cave amphipod from Comal springs on the right.

The main threat to the amphipods' survival is the loss of suitable habitat from reductions in spring flow leading to a drop in water quantity and quality, primarily due to human extraction (USFWS, 1997). These springs are connected to the underground reservoir used by the City of San Antonio, a major metropolitan area whose wells can discharge up to half a million acre-feet per year (U.S. Geological Survey, San Antonio, 1996). During drought years, this high level of pumping can affect spring flows putting these sensitive species at risk. Daily flows in Comal

Springs average between 200-300 cubic feet per second (cfs) in a typical rain year. During a drought, reduction of this surface habitat begins to occur at approximately 120 cfs, as a daily average, at the main spring runs. Flows below 120 cfs are unsuitable for spring associated organisms (EARIP, 2012). During a severe drought in 1956, Comal Springs stopped flowing for a period of 144 days. Landa Lake dried up. The Peck's Cave amphipod was not yet described at the time (Holsinger, 1967). However, presumably amphipods were present in the Comal Spring system prior to the 1956 drought, and thus were able to persist through the event. It remains uncertain how drought events affect the amphipods population structure, or if they have the ability to rapidly recover from a large drought events.

Human activity can threaten the long term survival of the PCA in other ways, such as groundwater contamination from urban runoff, agricultural chemicals, and leaking oil or sewage pipelines. Major rainstorms, usually considered beneficial for spring associated organisms, can also increase contaminate concentrations, as runoff brings pesticides, fertilizer, and other chemicals from the surrounding urban and agricultural lands. Some chemical pollutants have been shown to affect the sex ratios of amphipods (Gross et al., 2001).

The threats facing the PCA encouraged the Edwards Aquifer Authority (EAA) to contract the USFWS to construct a refugia for the amphipod at San Marcos Aquatic Resources Center and the Uvalde National Fish Hatchery. The primary goal of these refugia is to safeguard populations of these amphipods in captivity, representing the genetic structure of those found in the wild. In the event of a catastrophic event that threatens the extirpation of the species or population from the wild, the refugia could reintroduce a population of genetically fit individuals once conditions in the wild improve. Genetically fit individuals, in this context, are organisms that, collectively, retain as much of the wild gene pool as possible.

The Edwards Aquifer Recovery Implementation Program (EARIP) recommended off-site refugia for all endangered species of the Edwards Aquifer to be maintained (EARIP 2011). This included protection for the PCA in the event of sudden demise or radical reduction of the wild populations due to catastrophic events (e.g., severely reduced spring flow, habitat desiccation, or anthropogenic contamination). The San Marcos Aquatic Resources Center (SMARC) and the Uvalde National Fish Hatchery (UNFH), both operated by the United States Fish and Wildlife Service (USFWS), have been awarded the opportunity to establish and maintain captive refuge populations of Edwards Aquifer (EA) species of concern. Since 2003, USFWS- SMARC has achieved moderate success in holding Peck's cave amphipods (*Stygobromus pecki*) in captivity and producing progeny. The lack of available knowledge of these amphipods, especially information about their life history and environmental requirements, impedes SMARC and UNFH from effectively maintaining captive populations. SMARC is working with researchers on population genetics of this species (and close relatives) to further understand isolation in the wild and successful establishment of refugium populations.

Goals for the controlled propagation of listed species support recovery related research, maintaining refugia populations, providing organisms for reintroduction or augmentation of existing populations, and conserving species or populations at risk of imminent extinction or extirpation (USFWS/NOAA 2000). Scientific research has commenced to address the lack of knowledge and to increase the effectiveness of captive survivability and propagation. This should be conducted in a manner that will, preserve the genetic and ecological distinctiveness of the listed species while minimizing risks to existing wild populations. It is uncertain whether amphipods born in captivity should be stocked into the wild. However, captive propagation is a goal of the refugia, with the expectation that genetic management should allow captive-bred

populations to reflect the genetic diversity of wild populations. Captive propagation should seek to minimize the amount of genetic drift (changes in gene frequencies) experienced by subsequent generations. Management of the genetic structure of a captive population is imperative as genes frequencies under captive conditions can be detrimental to long term survival in the wild (Robert, 2009). A target of 500 adult amphipods, to insure that heterozygosity (genetic variation), should be maintained throughout the life span of the refugia (Franklin, 1980; Soulé, 1980).

Biology

Taxonomy

In Comal Springs there are six species of amphipods divided among four genera comprising four families (Gibson et al. 2008). The largest group of these amphipods is the genus *Stygobromus*, which has about 140 species found primarily in North America with a few species known from Eastern Europe (Holsinger, 1994). The PCA was first collected by Peck in 1964 and later named *Stygonectes pecki* (Holsinger, 1967) in his honor. *Stygonectes* was later synonymized with *Stygobromus* (Holsinger, 1978), the later precedent.

Anatomy and Morphology

All *Stygobromus* amphipods lack the presence of eyes, a characteristic associated with subterranean aquifer habitats. Despite the PCA's lack of sight, recent findings have shown the species possesses some light sensitivity (Nowlin et al. 2016). PCAs are relatively large, growing to more than 1 cm in length. These stygobiontic fauna often occur mainly around spring openings, large upwellings, and occasionally in deeper wells (Barr, 1993; Gibson et al. 2008). This species is not normally found in the shallower seeps where *S. russelli* is more predominate (Holsinger, 1967). Like other troglomorphic species, PCAs have long antenna to search for food and navigate through the dark aquifer (Holsinger, 1967).

Figure 2 A Peck's cave amphipod found on a log placed in an upwelling at Landa Lake.

The Comal Springs populations of PCAs have an orange hue, likely resulting from food available in phreatic habitats (Figure 1, right panel; Figure 2). In contrast, little to no pigment is seen in PCAs from Heuco Springs (Figure 1, left panel). This is more typical for troglomorphic organisms restricted to deeper groundwater habitats (Christiansen, 1962).

Genetics

Genetically, PCAs are more closely related to *Stygobromus dejectus* and *S. longipes*, both of which occur in adjacent Kendall County, Texas, than *S. fagellatus* from the nearby San Marcos Springs in Hays County or *S. russelli* which occurs sympatric with Peck's cave amphipod (Ethridge et al. 2013; Lucas et al. 2016). Despite their limited range, PCAs have higher levels of genetic variation in mtDNA then would be expected (Ethridge et al. 2013; Lucas et al. 2016). This suggests that the Peck's cave amphipod has relatively lower migration rates between populations (Lucas et al. 2016). This might be due to the uniquely compartmentalized nature of their spring habitats.

Feeding

Individual Peck's cave amphipods also feed on woody debris, but PCAs are opportunists, and have been observed consuming other small invertebrates, including conspecifics. Radio isotope analyses indicate that the PCA has broader feeding strategies than other spring-associated invertebrates, which feed mainly on terrestrial woody debris (Bio-West, 2015).

Life History

Stygobromus amphipods of the Edwards Aquifer region are still largely understudied, and much about their life history is still unknown. Based on the findings of Crawford and Tarter (1979), subterranean amphipods, like the PCA, typically take about a year to mature, a slower rate of development and reproduction than epigean species. It is unclear if PCAs breed continuously throughout the year or if they have specific mating seasons. The sex ratios of amphipod offspring fluctuate (Crawford and Tarter 1979). This may be the result of the mechanism of sex determination in amphipods. Amphipod sex-determining alleles are distributed across several different chromosomes. Some amphipod pairings lead to exclusively male or female offspring (Sutcliffe 1992). Sex determination in this species may also be affected by environmental and other factors, such as chemical pollutants (Gross et al. 2001) and microsporidian infections (Bulnheim and Vávra 1968).

Bollache and Cezilly (2004a) suggested that amphipods may only be able to mate after the female cuticle is flexible enough to release eggs, after molting. Males typically guard females through amplexus during the short timing of female receptivity. Amplexus can be energetically costly (McLister, 2003) and prevents the male from being able to forage for food. Amplexus is often avoided unless the male has sufficient stored lipids and glycogen to last the duration of a female's molt (Plaistow et al. 2003). Additional nourishment may be necessary to offset the cost of amplexus and reduce cannibalism in captive populations.

Research so far is insufficient to conclusively predict hatching success in this species. However, initial work at SMARC suggests that PCAs likely produce multiple broods of about ten young per reproductive event (Fries et al. 2004). USFWS is currently working with BIO-WEST, Inc. to research the early stages of brooding release and neonate development. Fries et al. (2004) reported that PCAs hatch when reaching about 2 mm in length, but the number of molts that are undergone before becoming sexually mature remains unknown. Observations of SMARC's F_1 generations have shown PCAs grow to about 9 mm (length) in 14 months.

Refugia

Collecting for Refugia

Peck's cave amphipods are an occasional bycatch on cotton lures targeting other invertebrate species in the Comal Springs system. See cotton cloth standard operations procedure for more information. Hand collection of PCA with small, fine-meshed aquarium nets is a successful method. Collection is made by locating an upwelling and scooping a net full of gravel. The net contents are then carefully sifted through for PCAs. To prevent the PCAs from being crushed in the net, the gravel can be placed in a shallow white tray filled with enough water to allow the PCAs to swim. The PCA can be transferred using a turkey baster to a small jar or container with a screw-on lid. Large-mesh nylon netting is added to provide structure during transport. After species identification is verified, the jars are moved to small coolers filled with spring water to buffer temperature changes during transport.

Figure 3 Cloth lure and wooden dowel before biofilm has developed.

Collection Locations

Peck's cave amphipod can be found in Hueco Springs, Landa Lake (Figure 4), and Panther Canyon Well in Comal County (Barr, 1993; Gibson et al. 2008). The Hueco Springs area predominantly private property; access to this area is problematic. Collection efforts through 2017 have concentrated mainly on Landa Lake, however, future collections will expand to incorporate Panther Canyon.

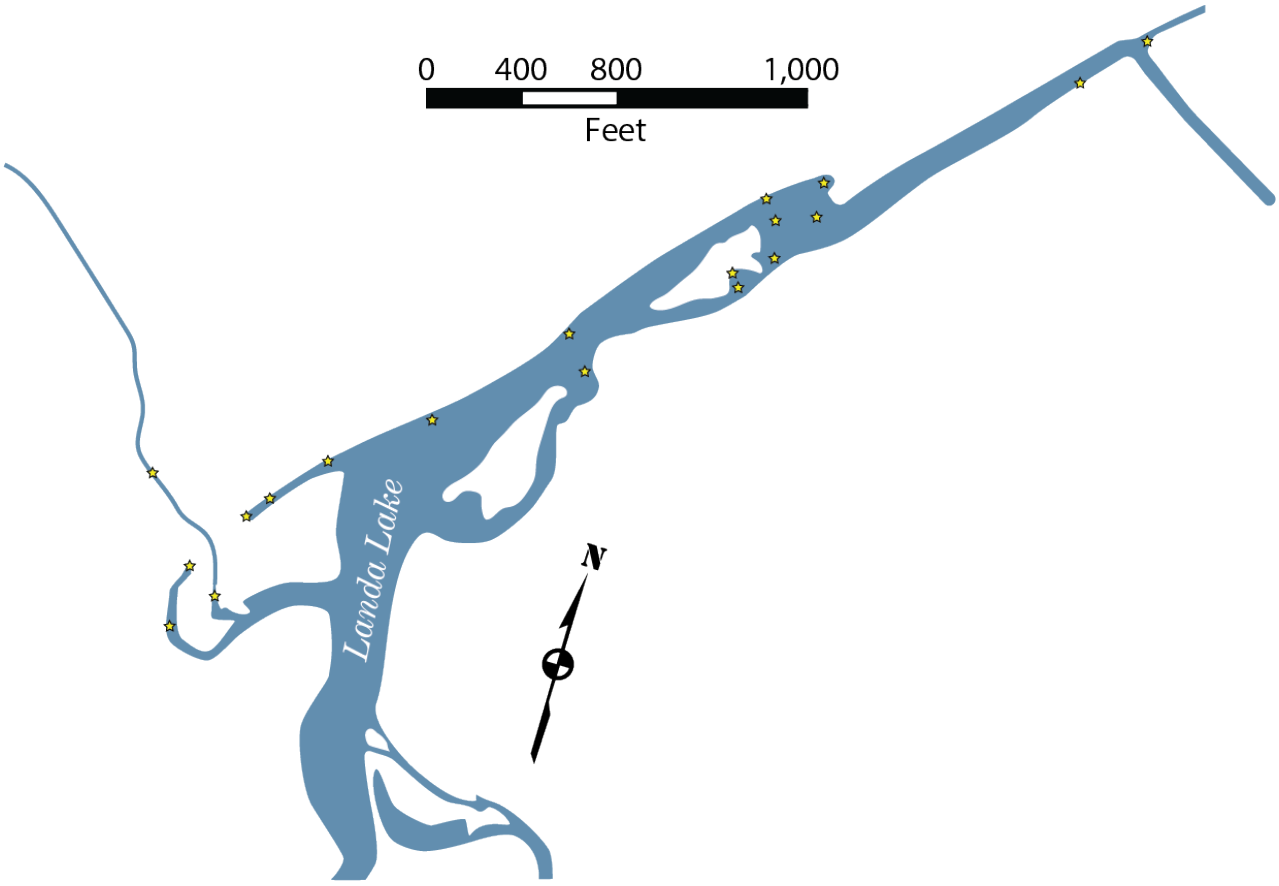

Figure 4 Map of Landa Lake slowing the collection sites for Peck's cave amphipods

Quarantine Procedures

At the station, compare the water temperature inside the transport container to the temperature within the appropriate destination container. Adjust the water temperature as necessary before the newly collected species are introduced into their designated quarantine container. Experience has shown that adjusting water temperature 1 °C every half-hour is sufficient; however, no studies have examined acclimation rates for these invertebrates; more research is warranted.

Once the temperature in the transport jar has equalized with the destination container, the PCAs can be carefully poured into a labeled quarantine container. Prior to incorporation into the refugia system, all PCAs must undergo a 30 day quarantine period. The quarantine system is

setup in the same way as the refugia. During this time we observe for any other species that might have come in with the collection. Observe the general health of the organisms and watch for large die-offs that might indicate a disease. If none of these occur, then the PCAs can be moved to the refugia population at the end of the quarantine period.

Culture Systems

Easy-Spring Culture Container

The Easy-Spring upwelling system mimics the hydrology of a natural upwelling, with water flowing vertically rather than horizontally (Figure 5). Easy-Spring culture containers are constructed from plastic tubs/totes in 8 quart, 15 quart and 34 quart sizes. Water enters the system via a hole cut into the container that is fitted with adaptors and connected to PCV piping. For a 9 quart size container, a single 1/2 inch PVC pipe is used to facilitate water flow throughout the entire bottom of the container; a series of small holes (1/16 inch) are drilled along the pipe which is covered with fine mesh to prevent organisms from entering the pipe. In the 15 and 34 quart containers, a square manifold of PVC pipe around the perimeter of the container is used. The small holes are drilled on both the inner and outer facing surfaces of the pipe and again are covered with fine mesh. For both types of flow bars, the piping is on or near the bottom of the container. Water flows out of the container via a horizontal standpipe capped with 300-400 µm mesh, held by hot glue, to prevent escape of specimens and smaller life cycle stages. Containers should be assembled using PVC glue and/or aquarium safe silicone, allowed to cure-dry. Run water through them for at least 24 hours to flush out any potentially harmful residual chemicals before organisms or habitat are added (this is also a good time to check and repair any leaks).

After containers are made, habitat items can be assembled in the container. Place the container on the rack system and hook up the water lines before the leaves, mesh and, rocks are

added. Place leaves on the floor of the container and with large nylon mesh for cover. Small, lightweight rocks can be placed on top of these layers if leaves are not conditioned and float rather than sink on the floor. PCAs do not swim well and cannot utilize much vertical space within the container. Set flow rate at a minimum to prevent dislodgement of PCA from the habitat items. Place PCAs in the container only after it is securely on its shelf, habitat is in place, and water has filled the container. Though PCA lack eyes, light can cause stress. To mimic the underground environment, the systems should be covered (e.g., with black plastic, shade cloth, or weed control cloth.

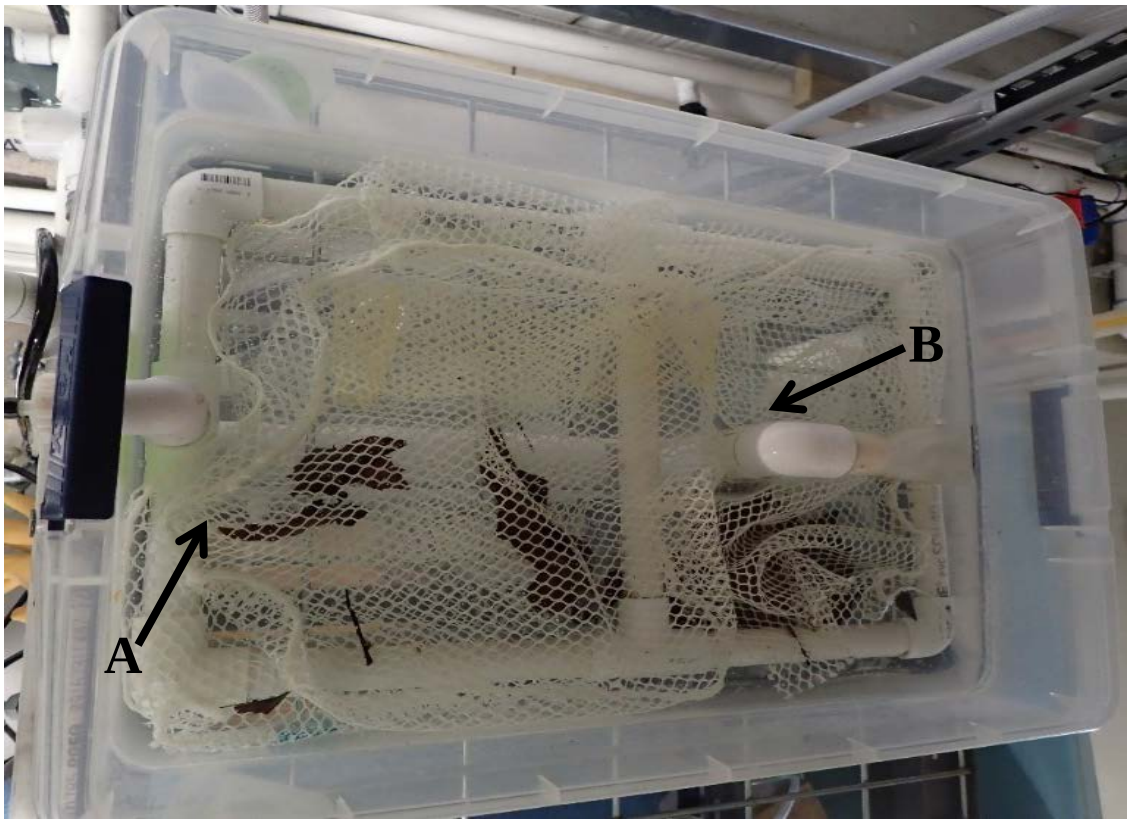

Figure 5 An easy-spring culture container with: A. a square upwelling spray bar and B. stand pipe. Nylon mesh netting provides shelter in the easy-spring culture container.

Flow-through Chambers

The flow-through chambers (Figure 6) are designed to mimic the hydrology of the spring orifices. Water enters the system through a barb adaptor at the bottom with a flow rate of about

two liters per minute. Place fine micron mesh between the adaptors to prevent escapement. The "viewing chamber" is constructed from clear PVC that is threaded at both ends. The viewing chamber contains nylon mesh netting and leaves to provide food and shelter (Figure 4). The water flows out at the top through a barb adaptor and hose and is collected in a sump basin. The design of Brooding chambers is similar to that of flow-through chambers, but with two "viewing chambers." The downstream chamber is separated from the upstream chamber by a 1 mm mesh screen (Figure 7), and each end of the brooding chambers is capped with 50 µm mesh to prevent the young from escaping. Place a gravid female in the bottom, or upstream chamber along with leaves and nylon mesh. Once the female amphipod releases her young, they are carried through the 1 mm mesh screen into the top, downstream chamber. Transfer the young amphipods to a different container for grow out.

Figure 6 A Peck's cave amphipod taking shelter among nylon mesh netting in a flow-through chamber

Figure 7 (A) Flow-through separation chambers with clear threaded PVC and (B) mesh screen for the separation of the young. Picture curtesy of Bio-West.

Water Quality

Traditional Systems

The Peck's cave amphipod is adapted to the unique environmental conditions of the Edwards Aquifer. Temperature is an important variable in this particular aquatic environment as it is generally constant throughout the year, thus the species is not eurythermal. Temperatures in Comal Springs average 23°C and temperatures in Hueco Springs average 21°C (Fries et al. 2004). Temperatures in the refugia should be kept between 18-24°C, with the ideal setting between 22-23°C. Long-term temperatures over 24.0°C are not recommended. The temperature in the refugia should be checked daily. Water quality is tested weekly for DO (safe between 6-8 mg/L), pH (safe between 7-8.2), total dissolved gas (safe at or below 100%) and ammonia (safe at less than 0.05 mg/L).

Upwelling Grid Systems

At the San Marcos station these systems run on pure well water, which comes in at a constant 22-23°C. Water quality is not taken routinely in these systems, but temperature and conductivity are recorded every 15 minutes from well sources. In Uvalde, the well water is warmer. Systems are in place to keep the water below 24°C. Temperatures at or above 24 °C will cause stress and possible death to arthropods. Chilled well water should be mixed with pure well water as sources for these systems in the event well water temperatures are not cool enough. Typically in late summer, these actions will need to be implemented for all arthropod species.

Feeding – Related Procedures

The PCA is an omnivorous opportunist feeder, attracted to the biofilm that grows on decaying terrestrial plant-matter and other small invertebrates around such food sources (Biowest, 2015). In captivity, offer dried leaves (Figure 8) and fish flakes as food sources for amphipods. Pick fresh green leaves from trees around the amphipods' habitat. Trees that produce suitable leaves include walnut, pecan, anacua, elm, and sycamore. Bring these leaves back to the refugia facility and place them into a drying-oven at 150°C. Leave them to dry for about 48 hours. The dried leaves can be added directly to refugia. It generally takes a week before sufficient biofilm accumulates on new leaves and lures for the amphipods (Gibson et al. 2008). Add leaves during semi-monthly inventory or upon inspection.

Small amounts of fish flaked feed may be beneficial to amphipods for additional protein. Grind the flakes into a fine powder before use. Mix the finely ground powder with dechlorinated water to form a slurry so that it does not float on top of the water and become covered in fungus. Use a pipette to add the flake and water solution under the netting and leaves. Adding too much flaked food to the containers can cause unhealthy fungus buildup and increase the biological oxygen demand. Add the flake slurry mixture to PCA containers at most two times a week.

Figure 8 Freshly dried leaves can be stored in an air-tight container until they are needed.

Propagation

PCA mating begins with amplexis, which often begins before the female molts. The eggs are thought to be released after the female molts (Bollache and Czilly, 2004). Female amphipods hold their eggs and young in a brood pouch under the body (Figure 9) while they are developing and released fully formed young resembling miniature adults. A female PCA can hold approximately ten eggs during each breeding cycle (Fries et al. 2004). It is still unknown how often the PCA breeds in the wild. In captivity, PCA are thought to breed once every three months (Gibson obs.). Young PCA grow from 2 mm to 9 mm in 14 months and will then produce offspring during the following year.

When counting PCA from refugia containers, carefully observe each amphipod for brooding. If you discover gravid females, isolate them in brooding chambers until they release their young. Small PCAs are often cannibalized by adults, and the brooding chambers provide both a refuge for the young, as well as an easy method for their collection. Transfer offspring to a separate culture container after collection.

Figure 9 A female Peck's Cave amphipod with young in the brood pouch under her body

Inventory

Periodically (at least every two months) count (inventory) the refugia population to determine its population size. These inventory periods provide an opportunity to view the organisms closely, and collect information about the size and health of the refugia population. Set up temporary holding containers, one for each life stage, partially filled with well water. Allow the water is to drain out of the stand pipe as much as possible. Carefully remove the netting and rocks and check for adults and young. Rinse the netting in well water several times and make sure all PCA are removed. Place leaves in a separate cup to be examined later. Pour the remaining water and debris into a 75 µm or smaller mesh sieve. Take care when rinsing and moving the container, as PCAs may be under or on the upwelling bars. Rinse the container

several times into the sieve to be sure that no organisms are clinging to the container. When water is not being poured through the sieve, the sieve should be placed in enough water to ensure that is the mesh is submerged, but does not go over the top of the sides, so that amphipods remain wet. Once the container is clear of amphipods, rinse it well, wipe it down, and scrub to remove any calcium build-up, if necessary.

Place each leaf in a white tray partially filled with well water. Put each PCA life stage into a different receiving container. The PCA may be transferred using a turkey baster for adults and a wide tipped pipette for the young. Carefully examine the debris in the sieve is for all life stages (Figure 10). Minimize handling as this may cause damage to the amphipods exoskeleton, loss of limbs, and increased vulnerability to disease or even death. Keep a tally to ensure a proper count. If any gravid females are discovered during inventory periods, transfer these to a brooding chamber until they release their young.

Once all of the structures have been thoroughly examined, some of the leaves with less degradation should be returned to the system, in addition to new leaves. Rinse the netting to remove any buildup of detritus. Then, replace it into the system. Refill the system with well water and replace the adults. Place offspring (F1 generation), if any are found, in a separate enclosure. Document all population counts, transfers, and disease issues in the data sheets.

Figure 10 During inventory debris from the refugia is removed to white tray and sifted through for amphipods. (Inset) Peck's Cave amphipods stand out against the dark color of the debris.

Cleaning Systems

Clean the PCA container whenever it must be disturbed (e.g., semi-monthly inventory or when removing young). This cleaning should be more thorough than the routine daily care and cleaning of flow bars, outflow standpipes, and when checking for fungus on dowels. Since the debris on the bottom may contain offspring, it must be carefully examined. Pouring the debris into a sieve and examine it carefully before discarding it. PCAs can cling to the underside of small leaves, nylon mesh, out-flow cover mesh, or rocks and need to be checked before cleaning or discarding these pieces.

In order to remove CaCO2 deposits without toxic chemicals, empty containers and rinse them with well water in the sink, then clean with sponges or brushes. If CaCO2 deposits cannot

be removed or are particularly stubborn, a replacement container or parts may be required. Check all valves and tubing for CaCO2 deposits and functionality. Replace these if damage or obstructions are found. Acid washing must not be done, as most chemicals on station are not safe for aquatic invertebrates, this includes bleach, hydrochloric acid, and Virkon®.

- DO NOT USE BLEACH, HYDROCHLORIC (MURIATIC) ACID, or VIRKON®

If a container or its components have particularly stubborn CaCO2 deposits they can be soaked in a vinegar and water solution to loosen up the deposits. The containers should be triple rinsed and allowed to completely air dry before using again for invertebrates.

Biosecurity

Proper biosecurity procedures are vital to insure the long-term health and maintenance of the refugia and facility as a whole. As the chemicals typically used for disinfection are not safe for invertebrates, separate equipment must be used for the Comal River systems and the San Marcos River systems, in the field as well as on station. Completely dry equipment from the field before storing. Dispose of transport water brought back from the field into a drain that leads to the chlorine and sand filtration system. All water leaving the amphipod refugia should also pass through this treatment system. Clearly mark all culture containers with the name of the species and the location from which they were collected.

To prevent organisms from entering or escaping the refugia on shoes, a foot-bath containing a 1% Virkon® Aquatic solution is placed at all the entrances to the refugia. These mats should be refilled at least once weekly and recorded on the data sheets.

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In Reply Refer To:
FWS/R2/FR-SFHU/847

Memorandum: May 12, 2017

To: Lindsay Campbell, San Marcos ARC
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the wild salamanders (SNARRC Case Number 17-20)

On February 28, 2017, thirty three salamander skin swabs were received from San Marcos ARC. These swabs came from wild salamanders that were captured and moved into quarantine at San Marcos ARC. Thirty one samples were from Comal Springs salamanders (*Eurycea* spp or *Eurycea neotenes*) and two swabs were from Texas Blind Salamanders (*Eurycea rathburni*). Samples were submitted in duplicate to be tested for *Batrachochytrium dendrobatidis (*Bd*)* and *Batrachochytrium salamandrivorans* (Bsal). Swabs were tested using PCR. Duplicate samples were labeled "A" and "B". Only the A samples were tested. All "B" samples will be stored in our -80 freezer in case of the need for any further testing. Unless otherwise requested, these samples will be tossed at the end of the fiscal year.

Results:
All thirty three salamander samples (100%) were negative for Bsal. Several samples developed inhibition during the PCR; therefore, we could not conclude they were negative. Of the remaining samples 8/33 (24.2%) were positive for Bd, 21/33 (63.3%) were negative and 4/33 (12.1%) could not be determined. An individual breakdown of the results is attached on page 2.

Final Diagnosis: *Batrachochytrium dendrobatidis (Bd)*

The finding of Bd in these animals is not surprising as it has been previously detected in this species. Historically, these species have not shown any clinical signs related to the fungus. However, any future movements of these animals should be done with care, as group housing has likely resulted in many of the previously negative animals to become positive. If they are moved back into the wild, they could transmit the pathogen to new waters. Therefore, no movement of these animals is recommended unless the receiving body of water has already been determined to be Bd positive.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 17-20 for any follow-up correspondence.

Vial #	Date	Collection Location	Species	TL (mm)	Lesions present (Y/N)	Facility SMARC or UNFH	Notes/ Observations	Date Swabs sent to Fish health	Test results	Date moved out of QT	Data Collector	Swab collector
1	2/21/2017	Spring Island	**ESPP**	28	N	UNFH		2/27/2017	I		KS	LM
2	2/21/2017	SR 3 A	**ESPP**	38	N	UNFH		2/27/2017	-		RB	RB
3	2/21/2017	SR 3 A	**ESPP**	51	N	UNFH	Very thin	2/27/2017	-		RB	RB
4	2/21/2017	SR 3 A	**ESPP**	57	N	UNFH	l gas bubble	2/27/2017	-		RB	RB
5	2/21/2017	SR 3 A	**ESPP**	70	N	UNFH		2/27/2017	+		RB	RB
6	2/21/2017	SR 3 A	**ESPP**	49	N	UNFH		2/27/2017	-		RB	RB
7	2/21/2017	SR 3 A	**ESPP**	41	N	UNFH	al tail ampu	2/27/2017	+		RB	RB
8	2/21/2017	SR 3 A	**ESPP**	50	N	UNFH		2/27/2017	-		RB	RB
9	2/21/2017	SR 3 A	**ESPP**	55	N	UNFH		2/27/2017	-		RB	RB
10	2/22/2017	SR 3 A	**ESPP**	48	N	SMARC		2/27/2017	I		JC	JC
11	2/22/2017	SR 3 A	**ESPP**	36	N	SMARC		2/27/2017	-		JC	JC
12	2/22/2017	SR 3 A	**ESPP**	33	N	SMARC	Resolved by	2/27/2017	-		JC	JC
13	2/22/2017	SR 3 A	**ESPP**	24	N	SMARC		2/27/2017	-		JC	JC
14	2/22/2017	SR 3 A	**ESPP**	24	N	SMARC	d tail (HOSP	2/27/2017	I		JC	JC
15	2/22/2017	SR 3 A	**ESPP**	56	N	SMARC		2/27/2017	I		JC	JC
16	2/22/2017	SR 3 B	**ESPP**	54	N	SMARC	Female (GF	2/27/2017	+		JC	JC
17	2/22/2017	SR 3 B	**ESPP**	35	N	SMARC		2/27/2017	-		JC	JC
18	2/22/2017	SR 3 B	**ESPP**	35	N	SMARC		2/27/2017	-		JC	JC
19	2/22/2017	SR 3 B	**ESPP**	54	N	SMARC		2/27/2017	-		JC	JC
20	2/22/2017	SR 3 B	**ESPP**	37	N	SMARC		2/27/2017	+		JC	JC
21	2/22/2017	SR 3 B	**ESPP**	46	N	SMARC	OT resolved	2/27/2017	-		JC	JC
22	2/22/2017	SR 3 B	**ESPP**	43	N	SMARC		2/27/2017	-		JC	JC
23	2/22/2017	SR 3 B	**ESPP**	30	N	SMARC		2/27/2017	-		JC	JC
24	2/22/2017	SR 3 B	**ESPP**	51	N	SMARC		2/27/2017	-		JC	JC
25	2/22/2017	SR 3 B	**ESPP**	43	N	SMARC		2/27/2017	-		JC	JC
26	2/22/2017	SR 3 B	**ESPP**	58	N	SMARC		2/27/2017	+		JC	JC
27	2/22/2017	SR 3 B	**ESPP**	43	N	SMARC		2/27/2017	-		JC	JC
28	2/22/2017	SR 3 B	**ESPP**	46	N	SMARC		2/27/2017	-		JC	JC
29	2/22/2017	SR 3 B	**ESPP**	43	N	SMARC		2/27/2017	-		JC	JC
30	2/22/2017	SR 3 B	**ESPP**	44	N	SMARC		2/27/2017	-		JC	JC
31	2/22/2017	SR 3 B	**ESPP**	-	N	SMARC		2/27/2017	+		JC	JC
32	1/13/2017	ttlesnake Ca	**ER**	-	N	SMARC		2/27/2017	+		LC	LC
33	2/10/2017	imer's Fissu	**ER**	100	N	SMARC		2/27/2017	+		KA	LC

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
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In Reply Refer To:
FWS/R2/FR-SFHU/851

Memorandum: May 30, 2017

To: Lindsay Campbell, San Marcos ARC
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the wild salamanders (SNARRC Case Number 17-39)

On April 13, 2017, 164 salamander skin swabs were received from San Marcos ARC. These swabs came from wild salamanders that were captured and moved into quarantine at San Marcos ARC. 162 samples were San Marcos salamanders (*Eurycea nana*) and two swabs were from Texas Blind Salamanders (*Eurycea rathburni*). Samples were submitted in duplicate to be tested for *Batrachochytrium dendrobatidis (*Bd*)* and *Batrachochytrium salamandrivorans* (Bsal). Swabs were tested using PCR. Duplicate samples were labeled "A" and "B". Only the A samples were tested. All "B" samples will be stored in our -80 freezer in case of the need for any further testing. Unless otherwise requested, these samples will be tossed at the end of the fiscal year.

Results:
All 164 salamander samples (100%) were negative for Bsal. One sample developed inhibition during the PCR; therefore, we could not conclude its status. Of the remaining samples 120/163 (73.6%) were positive for Bd, 43/163 (26.3%) were negative. An individual breakdown of the results is attached.

Final Diagnosis: *Batrachochytrium dendrobatidis (Bd)*

The finding of Bd in these animals is not surprising as it has been previously detected in this species. Historically, these species have not shown any clinical signs related to the fungus. However, any future movements of these animals should be done with care, as group housing has likely resulted in many of the previously negative animals to become positive. If they are moved back into the wild, they could transmit the pathogen to new waters. Therefore, no movement of these animals is recommended unless the receiving body of water has already been determined to be Bd positive.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 17-39 for any follow-up correspondence.

Vial #	Date	Collection Location	Species	TL (mm)	Lesions present (Y/N)	Facility SMARC or UNFH	Notes/ Observations	Date Swabs sent to Fish health	Test results (Bd)
34	3/14/2017	TSU Well	**ER**	42	N	SMARC	Looks good, food in gut	4/12/2017	-
35	3/20/2017	Spring Lake (SMR) - Catfish Hotel	**EN**	50	N	UNFH		4/12/2017	+
36	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	44	N	UNFH		4/12/2017	+
37	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	42	N	UNFH	Missing tail	4/12/2017	+
38	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	52	N	UNFH		4/12/2017	+
39	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	48	N	UNFH		4/12/2017	-
40	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	50	N	UNFH		4/12/2017	+
41	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	52	N	UNFH		4/12/2017	-
42	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	34	N	UNFH		4/12/2017	-
43	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	41	N	UNFH		4/12/2017	-
44	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	55	N	UNFH		4/12/2017	+
45	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	58	N	UNFH		4/12/2017	-
46	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	26	N	UNFH		4/12/2017	-
47	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	46	N	UNFH		4/12/2017	+
48	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	40	N	UNFH		4/12/2017	-
49	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	40	N	UNFH		4/12/2017	+
50	3/20/2017	Spring Lake (SMR) - Deep Crevass	**EN**	51	N	UNFH		4/12/2017	+
51	3/20/2017	Spring Lake (SMR) - Deep Crevass	**EN**	34	N	UNFH		4/12/2017	+
52	3/20/2017	Spring Lake (SMR) - Deep Crevass	**EN**	56	N	UNFH		4/12/2017	-
53	3/20/2017	Spring Lake (SMR) - Deep Crevass	**EN**	42	N	UNFH		4/12/2017	+
54	3/20/2017	Spring Lake (SMR) - Deep Crevass	**EN**	57	N	UNFH		4/12/2017	-
55	3/20/2017	Spring Lake (SMR) - Deep Crevass	**EN**	25	N	UNFH		4/12/2017	-
56	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	56	N	UNFH		4/12/2017	+
57	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	42	N	UNFH		4/12/2017	+
58	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	49	N	UNFH		4/12/2017	+
59	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	46	N	UNFH		4/12/2017	+
60	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	38	N	UNFH		4/12/2017	-
61	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	55	N	UNFH		4/12/2017	-
62	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	47	N	UNFH		4/12/2017	+
63	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	56	N	UNFH		4/12/2017	+
64	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	58	N	UNFH		4/12/2017	+
65	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	46	N	UNFH		4/12/2017	-
66	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	41	N	UNFH		4/12/2017	+
67	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	60	N	UNFH		4/12/2017	+
68	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	39	N	UNFH		4/12/2017	-
69	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	55	N	UNFH		4/12/2017	+
70	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	44	N	UNFH		4/12/2017	+
71	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	50	N	UNFH		4/12/2017	+
72	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	44	N	UNFH		4/12/2017	+
73	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	53	N	UNFH		4/12/2017	-
74	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	41	N	UNFH		4/12/2017	+
75	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	49	N	UNFH		4/12/2017	+
76	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	50	N	UNFH		4/12/2017	+
77	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	42	N	UNFH		4/12/2017	+
78	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	41	N	UNFH		4/12/2017	+
79	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	64	N	UNFH		4/12/2017	+
80	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	43	N	UNFH		4/12/2017	+
81	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	32	N	UNFH		4/12/2017	-
82	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	47	N	UNFH		4/12/2017	+
83	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	43	N	UNFH	Missing tip of tail	4/12/2017	+
84	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	44	N	UNFH		4/12/2017	-
85	3/20/2017	Spring Lake (SMR) - Catfish Hotel	**EN**	25	N	UNFH	Missing tail	4/12/2017	+
86	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	42	N	UNFH		4/12/2017	+
87	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	40	N	UNFH		4/12/2017	+

88	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	52	N	UNFH		4/12/2017	+
89	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	58	N	UNFH	Missing end of tail	4/12/2017	-
90	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	46	N	UNFH		4/12/2017	+
91	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	48	N	UNFH		4/12/2017	+
92	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	50	N	UNFH		4/12/2017	+
93	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	65	N	UNFH		4/12/2017	+
94	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	42	N	UNFH		4/12/2017	+
95	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	58	N	UNFH	Gravid Female	4/12/2017	+
96	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	51	N	UNFH		4/12/2017	+
97	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	40	N	UNFH		4/12/2017	+
98	3/20/2017	Spring Lake (SMR) - Diversion	**EN**	46	N	UNFH		4/12/2017	inhibited
99	3/20/2017	Spring Lake (SMR) - Deep Crevass	**EN**	41	N	UNFH		4/12/2017	+
100	3/20/2017	Spring Lake (SMR) - Deep Crevass	**EN**	65	N	UNFH		4/12/2017	-
101	3/20/2017	Spring Lake (SMR) - Deep Crevass	**EN**	40	N	UNFH		4/12/2017	-
102	3/20/2017	Spring Lake (SMR) - Deep Crevass	**EN**	31	N	UNFH	Missing end of tail	4/12/2017	-
103	3/20/2017	Spring Lake (SMR) - Deep Crevass	**EN**	49	N	UNFH		4/12/2017	+
104	3/20/2017	Spring Lake (SMR) - Deep Crevass	**EN**	45	N	UNFH		4/12/2017	+
105	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	57	N	UNFH		4/12/2017	+
106	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	61	N	UNFH		4/12/2017	+
107	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	45	N	UNFH		4/12/2017	+
108	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	50	N	UNFH		4/12/2017	+
109	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	55	N	UNFH		4/12/2017	+
110	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	53	N	UNFH		4/12/2017	+
111	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	48	N	UNFH		4/12/2017	+
112	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	50	N	UNFH		4/12/2017	-
113	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	47	N	UNFH		4/12/2017	+
114	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	52	N	UNFH		4/12/2017	+
115	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	36	N	UNFH		4/12/2017	-
116	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	32	N	UNFH	Missing tail	4/12/2017	-
117	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	53	N	UNFH		4/12/2017	+
118	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	43	N	UNFH		4/12/2017	-
119	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	53	N	UNFH		4/12/2017	-
120	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	48	N	UNFH		4/12/2017	+
121	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	42	N	UNFH		4/12/2017	+
122	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	46	N	UNFH		4/12/2017	+
123	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	29	N	UNFH		4/12/2017	+
124	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	51	N	UNFH	Gravid female	4/12/2017	+
125	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	40	N	UNFH		4/12/2017	+
126	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	39	N	UNFH		4/12/2017	+
127	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	49	N	UNFH		4/12/2017	+
128	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	51	N	UNFH		4/12/2017	+
129	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	31	N	UNFH	Tail amputated, already regrowing	4/12/2017	+
130	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	54	N	UNFH		4/12/2017	+
131	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	45	N	UNFH		4/12/2017	+
132	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	53	N	UNFH		4/12/2017	-
133	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	48	N	UNFH		4/12/2017	+
134	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	50	N	UNFH		4/12/2017	+
135	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	51	N	UNFH		4/12/2017	+
136	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	55	N	SMARC		4/12/2017	+
137	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	47	N	SMARC		4/12/2017	+
138	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	50	N	SMARC		4/12/2017	+
139	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	37	N	SMARC		4/12/2017	+
140	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	30	N	SMARC		4/12/2017	-
141	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	40	N	SMARC		4/12/2017	+
142	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	52	N	SMARC		4/12/2017	+
143	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	55	N	SMARC	Gravid female	4/12/2017	-
144	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	50	N	SMARC		4/12/2017	+

145	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	57	N	SMARC		4/12/2017	+
146	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	49	N	SMARC		4/12/2017	+
147	3/20/2017	Spring Lake (SMR) - Hotel	**EN**	57	N	SMARC		4/12/2017	+
148	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	55	N	SMARC		4/12/2017	+
149	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	42	N	SMARC		4/12/2017	-
150	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	49	N	SMARC		4/12/2017	+
151	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	46	N	SMARC		4/12/2017	+
152	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	56	N	SMARC		4/12/2017	+
153	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	47	N	SMARC		4/12/2017	+
154	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	51	N	SMARC		4/12/2017	+
155	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	49	N	SMARC		4/12/2017	+
156	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	43	N	SMARC		4/12/2017	+
157	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	50	N	SMARC		4/12/2017	+
158	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	44	N	SMARC		4/12/2017	+
159	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	55	N	SMARC		4/12/2017	+
160	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	54	N	SMARC		4/12/2017	+
161	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	46	N	SMARC		4/12/2017	+
162	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	45	N	SMARC		4/12/2017	+
163	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	44	N	SMARC		4/12/2017	-
164	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	45	N	SMARC		4/12/2017	+
165	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	52	N	SMARC	Somewhat gravid	4/12/2017	+
166	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	52	N	SMARC	Fatty (not gravid)	4/12/2017	+
167	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	57	N	SMARC		4/12/2017	+
168	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	35	N	SMARC		4/12/2017	-
169	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	31	N	SMARC		4/12/2017	-
170	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	43	N	SMARC		4/12/2017	+
171	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	39	N	SMARC		4/12/2017	+
172	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	51	N	SMARC		4/12/2017	+
173	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	56	N	SMARC	Somewhat gravid	4/12/2017	+
174	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	50	N	SMARC		4/12/2017	+
175	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	44	N	SMARC		4/12/2017	+
176	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	43	N	SMARC		4/12/2017	-
177	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	51	N	SMARC		4/12/2017	+
178	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	45	N	SMARC		4/12/2017	+
179	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	48	N	SMARC	Gravid (Full)	4/12/2017	+
180	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	53	N	SMARC		4/12/2017	+
181	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	32	N	SMARC		4/12/2017	+
182	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	60	N	SMARC	Broken tail, very pale	4/12/2017	+
183	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	49	N	SMARC		4/12/2017	+
184	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	45	N	SMARC		4/12/2017	-
185	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	35	N	SMARC		4/12/2017	+
186	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	25	N	SMARC		4/12/2017	-
187	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	43	N	SMARC		4/12/2017	+
188	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	47	N	SMARC	Gravid (Full) + amputated tail	4/12/2017	+
189	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	52	N	SMARC		4/12/2017	+
190	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	54	N	SMARC	Gravid (Full)	4/12/2017	-
191	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	54	N	SMARC		4/12/2017	-
192	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	61	N	SMARC		4/12/2017	+
193	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	44	N	SMARC		4/12/2017	+
194	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	49	N	SMARC		4/12/2017	-
195	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	34	N	SMARC		4/12/2017	-
196	3/21/2017	SMR - behind Saltgrass, upstream of bridge, downstream of old dam	**EN**	34	N	SMARC		4/12/2017	-
197	4/10/2017	Rattlesnake Well	**ER**	78	N	SMARC	Some blood on nose; very blue color with very fluffy pink gills	4/12/2017	-
198									
199									
200									

UNITED STATES DEPARTMENT of the INTERIOR

U.S. Fish and Wildlife Service

National Wild Fish Health Survey

This report is not evidence of future disease status.

Species	Sample Location		Collection Date	Collector
DX17-50	COMAL RIVER	TX	6/01/17	LINDA MOON

Latitude 29.7106 **Longitude** 98.1275

Fountain darter

60 (total)

Pathogen	Screening Method	NS*	NSP	NS+	Confirmation Method	NC**	NCP	NC+	Assay Result
IHNV	EPC-15	12	0	0	IHNV-M160	0	0	0	--
IPNV	CHSE-15	12	0	0	IPNV-PrD1	0	0	0	--
LMBV	FHM-25	12	0	11	LMBV-288F	2	0	2	+C
OMV	CHSE-15	12	0	0	OMV-F10	0	0	0	--
VHSV	EPC-15	12	0	0	VHSV-AJ84	0	0	0	--

Species	Sample Location	Collection Date	Collector

Two collection locations recorded for this case at N 29.7106, W-98.1275 and N 29.7106, W -98.1175.

LMBV= Largemouth Bass Virus
IHNV= Infectious Haematopoietic Necrosis
IPNV= Infectious Pancreatic Necrosis
VHSV= Viral Haemorrhagic Septicaemia
OMV= Oncorhynchus Masou Virus
CCV= Channel Catfish Virus

-- = Pathogen Not Detected
P = Pending Initial Screening
+P = Detected by Screening Method, Confirmation Pending
+C = Detected by Screening and Confirmation Method
--C = Not Detected by Confirmation Method

Rsal= Renibacterium salmoninarum
Yruc= Yersinia ruckeri
Asal= Aeromonas salmonicida

Mcer= Myxobolus cerebralis
Bach= Bothriocephalus acheilognathi

NS = Total number of pools Screened
NSP = Number of pools Pending initial Screening
NS+ = # of Pools Pathogen detected by Sceening Method
NC = Number of Pools Confirmed
NCP = Number of pools Confirmation Pending
NC+ = # of Pools Pathogen Detected by Confirmation Method

Diagnostician Address	Diagnostician
Southwestern Native Aquatic Resources & Recovery Ctr. - Southwestern Fish Health Unit P.O. Box 219 Dexter, NM 88230	Jason Woodland

CHN No. 17-50 revised 08/17/2016

Date: 6-6-17 Date Collected: 6-1-17

Facility: San Marcos Aquatic Resources Center

Name: Ken Ostrand

Address: 500 East McCarty Lane; San Marcos, Texas 78666

Fish species: Fountain Darter (60 fish)

Location: Comal River, TX

	fish #1	fish #2	fish #3	fish #4	fish #5	fish #6	fish #7	fish #8	fish #9	fish #10
Weight (mg)	370	219	88	100	188	197	249	212	270	142
Length (mm)	36	29	22	25	29	29	30	29	30	26

Haplorchis cysts **Body**

caudal (combine both sides)		2	6	3	2	2	3	4	3	6	3
head	L	2	2	0	0	0	2	0	0	2	0
	R	0	1	0	0	0	0	0	0	1	0

Centrocestus cysts **Gill Findings**

Mature (left gills only)	L	0,1,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,3,2	0,0,0,0	0,1,0,0	0,2,0,0,	0,0,0,0
Immature (left gills only)	L	1,7,4,1	1,5,4,8	1,1,2,0	0,0,2,0	0,0,0,0	6,5,6,1	1,1,2,0	1,3,3,1	5,8,11,2	2,2,2,0
Monogenea	L	0,0,0,0	0,0,0,0	0,0,0,0	1,0,0,0	0,0,0,1	0,0,0,0	0,0,0,0	0,0,0,0	0,0,1,0	0,0,0,0
Myxobolus sp.	L	1,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0
Other	L										

UNITED STATES DEPARTMENT of the INTERIOR

U.S. Fish and Wildlife Service

National Wild Fish Health Survey

This report is not evidence of future disease status.

Species	Sample Location		Collection Date	Collector
DX17-51	SAN MARCOS RIVER	TX	6/01/17	LINDA MOON

Latitude 29.8940 **Longitude** 97.9302

Fountain darter

60 (total)

Pathogen	Screening Method	NS*	NSP	NS+	Confirmation Method	NC**	NCP	NC+	Assay Result
IHNV	EPC-15	12	0	0	IHNV-M160	0	0	0	--
IPNV	CHSE-15	12	0	0	IPNV-PrD1	0	0	0	--
LMBV	FHM-25	12	0	5	LMBV-288F	5	0	0	--C
OMV	CHSE-15	12	0	0	OMV-F10	0	0	0	--
VHSV	EPC-15	12	0	0	VHSV-AJ84	0	0	0	--

Species	Sample Location	Collection Date	Collector

Two collection locations recorded for this case at N 29.8940, W-97.9302 and N 29.8901, W -97.9337.

LMBV= Largemouth Bass Virus
IHNV= Infectious Haematopoietic Necrosis
IPNV= Infectious Pancreatic Necrosis
VHSV= Viral Haemorrhagic Septicaemia
OMV= Oncorhynchus Masou Virus
CCV= Channel Catfish Virus

-- = Pathogen Not Detected
P = Pending Initial Screening
+P = Detected by Screening Method, Confirmation Pending
+C = Detected by Screening and Confirmation Method
--C = Not Detected by Confirmation Method

Rsal= Renibacterium salmoninarum
Yruc= Yersinia ruckeri
Asal= Aeromonas salmonicida

Mcer= Myxobolus cerebralis
Bach= Bothriocephalus acheilognathi

NS = Total number of pools Screened
NSP = Number of pools Pending initial Screening
NS+ = # of Pools Pathogen detected by Sceening Method
NC = Number of Pools Confirmed
NCP = Number of pools Confirmation Pending
NC+ = # of Pools Pathogen Detected by Confirmation Method

Diagnostician Address	Diagnostician
Southwestern Native Aquatic Resources & Recovery Ctr. - Southwestern Fish Health Unit P.O. Box 219 Dexter, NM 88230	Jason Woodland

CHN No. 17-51 revised 08/17/2016

Date: 6-6-17 Date Collected: 6-1-17

Facility: San Marcos Aquatic Resources Center

Name: Ken Ostrand

Address: 500 East McCarty Lane; San Marcos, Texas 78666

Fish species: Fountain Darter (60 fish)

Location: San Marcos River, TX

	fish #1	fish #2	fish #3	fish #4	fish #5	fish #6	fish #7	fish #8	fish #9	fish #10
Weight (mg)	202	199	204	142	161	147	160	193	243	152
Length (mm)	30	31	29	26	28	28	27	26	32	29

Haplorchis cysts **Body**

caudal (combine both sides)		0	0	0	0	0	0	0	0	0	0
head	L	0	0	0	0	0	0	0	0	0	0
	R	0	0	0	0	0	0	0	0	0	0

Centrocestus cysts **Gill Findings**

Mature (left gills only)	L	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0
Immature (left gills only)	L	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0

Monogenea	L	1,0,0,1	0, 0, 0, 0	0, 0, 0, 0	1,1,0,1	0,0,1,1	2,5,2,0	0,0,1,0	0,1,0,0,	2,0,0,0,	1,0,0,0
Myxobolus sp.	L	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0
Other	L										

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/859

Memorandum: July 31, 2017

To: Lindsay Campbell, San Marcos Aquatic Resource Center
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the Fountain darters (SNARRC Case Number 17-55)

On June 28, 2017, staff at the Southwestern Fish Health United (SFHU) received 5 Fountain darters (*Etheostoma fonticula*) from San Marcos ARC. The fish were originally collected from the wild on May 30-31 and moved into quarantine. They are on unfiltered flow through well water, with siphoning of tanks occurring at a minimum, weekly. This group of fish has been experiencing exophthalmia in some fish, many noted on mortalities. The only treatments these fish have received are a formalin treatment on intake and then retreatment with formalin on June 8 or 9. They received a 1% salt treatment on June 15.

Although exophthalmia was the concern, staff was unable to identify a good clinical example at the time of sampling, therefore, only mildly affected fish were collected. Three fish were sampled whole in Z-fix for histopathological evaluation. The kidney of one fish was cultured for bacteriology and parasitology and 2 fish were sampled for virology

Results:

Viral cell cultures revealed CPE on the BF2 cell line which was later confirmed with PCR to be due to Large Mouth Bass Virus (LMBV). Bacterial cultures on BHIA grew a culture tentatively identified as *Aeromonas hydrophila*. Parasitology was negative for pathogens. Histopathology identified necrosis of the hyaline cartilage with questionable intralesional microorganisms in the eye and skull. Although these organisms appeared to be myxozoa, they did not stain with Giemsa or Luna and PCR using pan-myxozoa primers was negative. Microsporidia were identified in the liver.

Final Diagnosis: LMBV, Undetermined exophthalmia etiology

The *Aeromonas hydrophila* is a common finding in water and is not likely to be clinically relevant. The LMBV was found in this lot previously and although certainly a diagnosis of note, is not likely the cause of the exophthalmia. The unidentified organisms are an interesting finding but preliminary testing did not result in identification. The staff has been asked to preserve any future fish they find that are clinical and these may be submitted at a later date for further evaluation. Although not the purpose of the submission, it is of note that despite the finding of LMBV in these fish, they do not appear to be exhibiting any clinical signs associated with the virus at this time.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 17-55 for any follow-up correspondence.

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/856

Memorandum: July 21, 2017

To: Lindsay Campbell, San Marcos ARC
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the wild salamanders (SNARRC Case Number 17-58)

On June 27, 2017, SNARRC staff obtained 10 salamander skin swabs from San Marcos ARC for chytrid testing. These swabs came from wild salamanders that are now being held at San Marcos ARC. Two samples came from Salado Springs salamanders (*Eurycea chisholmensis*), three samples came from San Marcos salamanders (*Eurycea nana*) and five came from Texas blind salamanders (*Eurycea rathbuni*). Specifics on the sampling date and location can be found on the attached data sheet. Samples were submitted in duplicate to be tested for *Batrachochytrium dendrobatidis (*Bd*)* and *Batrachochytrium salamandrivorans* (Bsal). Duplicate samples were labeled "A" and "B". Only the A samples were tested. All "B" samples will be stored in our -80 freezer in case of the need for any further testing. Unless otherwise requested, these samples will be tossed at the end of the fiscal year.

Results:
All salamanders were negative for Bsal. The Salado Springs salamanders were negative for Bd. Samples 198, 200 and 205 were positive for Bd. The remaining samples were negative for Bd. Specific results can be found on the attached data sheet.

Final Diagnosis: *Batrachochytrium dendrobatidis (Bd)*

The finding of Bd in these animals is not surprising as it has been previously detected in these positive species. Historically, these species have not shown any clinical signs related to the fungus. However, any future movements of these animals should be done with care, as group housing has likely resulted in many of the previously negative animals to become positive. The Salado Springs salamanders should be kept separate from the other salamanders since they were negative. If positive populations are moved back into the wild, they could transmit the pathogen to new waters. Therefore, no movement of these animals is recommended unless the receiving body of water has already been determined to be Bd positive.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 17-58 for any follow-up correspondence.

SALAMANDER SWABS SENT TO FISH HEALTH on 6/27/17

Vial # (a/b)	Date swabbed	Collection Location / TANK	Species	TL(mm)	Lesions?	Notes/Observations	Test results (Bd)
198	4/24/2017	Spring Lake (San Marcos River)	San Marcos (EN)	55	No	Under Lirceolus lure @ Diversion	+
199	5/5/2017	TXSTU	Texas Blind (ER)	33	No	Something red in abdomen (food?)	-
200	5/9/2017	Johnson's Well	Texas Blind (ER)	71	No	Some blood on nose, darker spine	+
201	5/12/2017	Johnson's Well	Texas Blind (ER)	71	No	In tank w/ 200, very thin back legs, longer gills	-
202	6/16/2017	Diversion	San Marcos (EN)	55	No	Some blood around pelvis/cloaca + food in gut	-
203	6/16/2017	Diversion	San Marcos (EN)	65	No	Some redness/blood around pelvis	-
204	6/22/2017	Sessoms Creek	Texas Blind (ER)	36	No	This sal was caught 5/5/17 but was too small to safely swab at the time	-
205	6/23/2017	Diversion	Texas Blind (ER)	71	No	Broken back appears healed, swims fine	+
Salado 1	-	Salado TX	Salado Springs	N/A	N/A		-
Salado 2	-	Salado TX	Salado Springs	N/A	N/A		-

United States Department of the Interior
U.S. Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/858

Memorandum: July 31, 2017

To: Lindsay Campbell, San Marcos Aquatic Resource Center
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the San Marcos salamanders (SNARRC Case Number 17-59)

On June 24, 2017, four San Marcos salamanders (*Eurycea nana*) were submitted directly to WADDL for histopathological evaluation. SNARRC had been alerted to an issue with these salamanders about one month prior. Staff report that the females were rupturing eggs out of the body cavity. Although staff reports this has been occurring for several years, there was a sharp increase in the percentage of affected individuals since January, with nearly 20 mortalities since then. When females are observed bloated, they are moved to hospital tanks and are treated with 1-2% salt treatments weekly. Although the salt treatment appears to help the clinical signs, the mortality of females once clinical signs are noted has been nearly 100%. Therefore, the salt appears to be slowing down the mortalities, but not stopping them. The staff was advised to save any fresh mortalities in 10% NBF for histopathological evaluation. Three specimens were selected and sent directly to WADDL.
Salamanders are kept in a flow through system with well water and their tanks receive weekly siphoning. At the time of submission, the DO was 8mg/L, temperature was 22C, pH was 7.1 and no ammonia, nitrites or nitrates were detected.

Results:
Histopathology indicated presence of a multifocal oophoritis and coelomitis associated with extracellular acid-fast bacilli bacteria (probable mycobacteriosis) as well as intralesional microsporidian spores in one salamander. In addition, there was an epaxial myositis and hepatitis also associated with presumed mycobacteria. Several salamanders had orthokeratotic hyperkeratosis with intralesional chytrid fungal thalli that was morphologically consistent with *Batrachochytrium dendrobatidis* on the skin of the feet. A phasmid-type nematodiasis was seen in the small intestine of one salamander

Final Diagnosis: Coeolomitis/oophoritis due to Mycobacteriosis. Concurrent infections with Microsporidia and Chytrid

The most concerning finding was that of mycobacteria in association with oophoritis and coelomitis in two of the submitted animals. Mycobacteria can be ubiquitous in the environment, therefore, complete sterilization and restocking of the tanks with newly collected animals may lead to the same problem. Amphibians can

maintain subclinical infections of Mycobacteria when they are not immunocompromised. However, in situations of high stress, such as those with overcrowding, poor water quality or other stressors, the mycobacteria can cause systemic problems. Given the threatened status of this species, complete eradication of the captive population may not be in the best interest of the species. However, given the chronic and untreatable nature of the illness, all clinically affected animals should be humanely euthanized and a thorough evaluation of the husbandry and welfare of the remaining animals examined to determine methods to decrease stress moving forward. This includes not only meeting the physical needs of the animals, but also the social, environmental, and behavioral needs and may require adjustment of holding methods, densities, feeding regimes, tank set up etc.

The finding of microsporidia may be a complicating factor. It was only noted in one of the three salamanders and has been seen associated with oophoritis in other closely related species. However, as the primary concern at this time is the mycobacteria, it is recommended that the facility focus on improving the mycobacterial infection rate in the collection prior to addressing any secondary issues. The secondary findings may well be associated with stress as well and correcting one factor may benefit several issues.

The finding of chytrid was expected as these salamanders had tested positive on previous swabs. They appear to tolerate the mild infection with little further associated clinical signs.

It should be noted that Mycobacteria is a zoonotic organism. Therefore, animal care staff should take precautions when working with the animals and use proper PPE such as gloves, bleach all associated equipment (Virkon is NOT effective against Mycobacteria), and take care not to work with the animals or water if they have any open wounds.

The complete histopathology report is attached to this report. If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 17-59 for any follow-up correspondence.

Washington Animal Disease Diagnostic Lab

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Martha Keller
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Case#: **2017-9519**
Report Date: 27 Jul 2017
Received: 25 Jul 2017
Owner: San Marcos National Fish
Animal: 17-59
Species: San Marcos salamander
Breed:
Sex/Age: ,

Histopathology Report

The formalin-fixed bodies of 4 San Marcos Salamanders (*Eurycea nana*) are briefly decalcified and serially transversely sectioned for histologic examination.

- ENUD4-3; 7/11/17; female; slides 1 and 2
- Hosp EN1-5 from ENWS4-2; 6/30/17; male; slide 3
- ENW53-upper; 6/20/17; female; slides 4 and 5
- ENW51-upper; 6/21/17; slides 6 and 7

ENUD4-3; 7/11/17

Kidney: In a single small cross section tubules are minimally ectatic and few tubular epithelial cells have intracytoplasmic eosinophilic droplets.

Skin, foot: There is multifocal mild to moderate orthokeratotic hyperkeratosis with intralesional fungal thalli. Thallus forms include internally septate colonial thalli, zoosporangia with discrete 1-2 micron zoospores and flask-shaped thalli with a prominent discharge tube.

There are moderate coelomic cavitary and tail adipose tissue stores. Sections of liver, heart, skeletal muscle, stomach, small intestine, pancreas, oviduct, ovary (large vitellogenic follicles), brain, spinal cord, eye, pituitary gland, gill and skin are histologically unremarkable. BH Gram, Fite's acid-fast and Luna stains are negative for microorganisms.

EN1-5 from ENWS4-2, 6/30/17

There are moderate to advanced autolytic changes that hinder histologic evaluation. There are moderate coelomic cavitary and tail adipose tissue stores. There is active spermatogenesis in the testis.

ENW53-upper; 6/20/17

Ovary and coelom: There are numerous large vitellogenic follicles that fill the coelomic cavity. Multifocally, follicles are disrupted by infiltrates of moderate numbers of large macrophages with foamy cytoplasm. Macrophages frequently have pale staining intracytoplasmic filamentous and beaded bacilli bacteria that are lightly BH Gram-positive and Fite's acid-fast positive. Moderate numbers of similar bacilli are present extracellularly. Macrophagic infiltrates and bacilli multifocally extend into the adjacent coelomic cavity and coelomic adipose tissue.

Liver: There is moderate multifocal melanomacrophage hyperplasia. A Fite's acid-fast stain is negative.

Small intestine: Focally, there is an intramucosal nematode parasite with a small bacillary band (probable aphasmid-type).

Kidney: There is mild multifocal tubular ectasia and occasional tubules have intraluminal bright red granular casts (? pigment casts).

Skin, foot: There is minimal multifocal orthokeratotic hyperkeratosis with intralesional fungal thalli as described above.

There are small to moderate coelomic and tail adipose stores. Sections of stomach, spleen, oviduct, skeletal muscle, gill and spinal cord are histologically unremarkable.

ENW51-upper; 6/21/17

Skeletal muscle, epaxial, level of kidney: The musculature is focally disrupted by aggregates of acid-fast bacilli and occasional macrophages as described above for the ovary/coelomic cavity of salamander ENW53.

Coelomic cavity: There are rare intracoelomic aggregates of macrophages with intracytoplasmic Fite's acid-fast bacilli as described above. Multifocally, there are pools of intracoelomic eosinophilic granular material (protein-rich fluid) with few admixed foamy macrophages and microsporidial spores (see ovary description below).

Kidney: There is mild multifocal tubular ectasia.

Ovary: The coelomic cavity is filled with numerous large vitellogenic follicles. Multifocally, follicles are disrupted by infiltrates of foamy macrophages. Macrophages surround small packets of 3-4 micron diameter oval spores with a refractile capsule. Spores are strongly Fite's acid-fast and lightly Luna stain positive (microsporidia). In other sections packets of similar spores are admixed with follicular yolk platelets in the absence of associated inflammatory cell infiltrates. No acid-fast bacilli are observed with Fite's acid fast stain.

Liver: There is a large focal nodular aggregate of macrophages (granuloma) with small numbers of lightly BH Gram-positive and strongly Fite's acid-fast positive filamentous beaded bacilli bacteria.

Skin, foot: There is minimal multifocal orthokeratotic hyperkeratosis.

Sections of brain, pituitary gland, heart, gill, intestine, and oviduct are histologically unremarkable.

HISTOLOGIC DIAGNOSES:

1. Oophoritis and coelomitis, granulomatous, multifocal, moderate with intrahistiocytic and extracellular acid-fast bacilli bacteria (probable mycobacteriosis); salamander ENW53

2. Epaxial myositis, granulomatous, focally-extensive, moderate with intralesional acid-fast bacilli bacteria (probable mycobacteriosis); salamander ENW51

3. Hepatitis, granulomatous, focal, moderate with intralesional acid-fast bacilli bacteria (probable mycobacteriosis); salamander ENW51

4. Coelomitis, granulomatous, multifocal, minimal with intralesional acid-fast bacilli bacteria (probable mycobacteriosis); salamander ENW51

5. Oophoritis, granulomatous, multifocal, moderate with intralesional microsporidian spores (ovarian microsporidiosis); salamander ENW51

6. Orthokeratotic hyperkeratosis, multifocal, minimal to moderate with intralesional chytrid fungal thalli (morphologically consistent with *Batrachochytrium dendrobatidis*; chytridiomycosis); skin of feet, salamanders ENUD4-3 and ENW53

7. Aphasmid-type nematodiasis, segmental, minimal; small intestine, salamander ENW53

COMMENTS:

There were several histologic findings of potential population health concern in these salamanders.

Ovarian and coelomic cavitary inflammation (oophoritis and coelomitis) potentially relating to the clinical history of eggs rupturing from the body cavity and deaths in gravid females was identified in 2 of the submitted animals (ENW53 and ENW51). In ENW53 oophoritis was associated with acid-fast bacteria suggesting a mycobacterial infection (see below) and in ENW51 there were spores morphologically consistent with a microsporidian parasite. I have seen very similar microsporidians as a cause of oophoritis in a closely-related salamander species (*Eurycea sosorum*), but the specific identity of the organism has not been determined. Ultrastructural and molecular studies to attempt to speciate these organisms may be of interest.

Probable mycobacteriosis was identified in both ENW53 and ENW51 and appeared to be the primary etiology for the oophoritis in ENW53 with extension into the coelomic cavity. The myositis observed in ENW51 could be related to the observation of ruptured body cavities. Mycobacterial infections in amphibians are usually caused by environmentally ubiquitous aquatic organisms and outbreaks (as might be occurring in this situation) and can be associated with factors such as overcrowding, high environmental organic loads or in animals immunosuppressed/stressed for other reasons. Mycobacterial cultures are in process from coelomic fluid from other affected animals (see WADDL 17-9604). If desired, we can also attempt to identify the etiologic agent by PCR and DNA sequencing from the paraffin blocks in this case.

Histopathology Report

Chytridiomycosis of toes and feet has also been observed in *Eurycea sosorum* and appears to be self-limiting with the possible exception of an increased prevalence of toe/foot loss and malformations. These species of neotenic salamander have limited cutaneous keratinizing epithelium which restricts the extent of infection and minimizes mortality in infected populations.

The nematodiasis in salamander ENW53 is interpreted as an incidental finding.

The cause of death could not be determined for salamanders ENUD4 (autolyzed tissues) or EN1-5.

Phone Contact: An email with preliminary findings was sent to Dr. Keller on 26 July 2017

WORK PENDING: None

Pathologist: Dr. Allan Pessier
Report authorized by: Dr. Allan Pessier, Senior Pathologist

United States Department of the Interior
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In Reply Refer To:
FWS/R2/FR-SFHU/868

Memorandum: September 5, 2017

To: Lindsay Campbell, San Marcos Aquatic Resource Center
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for San Marcos salamander #2 (SNARRC Case Number 17-62)

On July 25, 2017, one San Marcos salamander (*Eurycea nana*) was examined by Martha Keller at SMARC. This salamander was put into isolation due to abdominal distention in association with egg production. This has been an ongoing issue with numerous San Marcos salamanders for many months. Many females have had their abdomens burst prior to death. These salamanders have a history of being diagnosed with microsporidia. This particular salamander was anesthetized with 500mg/L of MS-222 and a coelomic tap was performed to obtain a fluid sample. The sample obtained was yellow and cloudy. The sample was placed on a culturette for bacterial/fungal culture as well as in a vial for cytological examination. The salamander recovered from anesthesia without incident.

Results:
Cytology of the fluid showed a cell count of 93% macrophages, 6% neutrophils, and 1% basophils. Occasional eosinophils were also observed. The neutrophils displayed karyolysis and moderately basophilic cytoplasm (rule-out toxic change). Multiple small ovoid structures with eccentrically located basophilic material and thin, refractile walls, cytologically suggestive of fungal organisms, are found intracellularly within several macrophages and degenerate cells. Occasional macrophages also display linear, non-staining structures that are cytologically suggestive of *Mycobacterium*. Culture of the swab for *Mycobacterium* grew an isolate that was subsequently identified vial molecular diagnostics as *Mycobacterium marinum*.

Final Diagnosis: Bacterial coelomitis with *Mycobacterium marinum*

While a primary cause of a fungal or other type of bacterial coeolomitis/oophoritis cannot be ruled out, the confirmation of *Mycobacterium marinum* is of great concern. *Mycobacterium marinum* is a ubiquitous pathogen in water. There is no current pharmacological treatment for the pathogen. Many organisms can tolerate low levels of the pathogen without displaying clinical signs. However, in stressful situations, such as those that can be found in aquaculture situations, the bacteria can cause significant morbidity and mortality. Typical recommendations for systems infected with the pathogen include euthanasia of all organisms, bleaching of the system, and restocking. As these salamanders are a threatened species and quite likely to be caught in the wild already infected with the pathogen, this route of control will likely be unrewarding.

Alternatively, it is recommended that careful reconsideration of the husbandry of these animals be undertaken to include evaluation of their tanks, environment (water quality, noise, vibrations, tank enrichment items, photoperiod etc), nutrition, etc. Keeping the animals in a low stress environment is crucial in preventing the *Mycobacteria* from becoming problematic. In the interim, it is recommended that all animals that present with clinical signs such as abdominal distension be humanely euthanized rather than allowing them to suffer until death. Continued sampling of deceased individuals in fixative is recommended for further histopathological evaluation. It must be noted that *Mycobacterium* is a zoonotic pathogen. All staff that work with these animals should be made aware of the zoonotic potential and should use proper PPE when working with these animals.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 17-62 for any follow-up correspondence.

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In Reply Refer To:
FWS/R2/FR-SFHU/869

Memorandum: September 5, 2017

To: Lindsay Campbell, San Marcos Aquatic Resource Center
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for San Marcos salamander #1 (SNARRC Case Number 17-63)

On July 25, 2017, one San Marcos salamander (*Eurycea nana*) was examined by Martha Keller at SMARC. This salamander was recently put into isolation due to abdominal distention in association with egg production. This has been an ongoing issue with numerous San Marcos salamanders for many months. Many females have had their abdomens burst prior to death. These salamanders have a history of being diagnosed with microsporidia. This particular salamander was anesthetized with 500mg/L of MS-222 and a coelomic tap was performed to obtain a fluid sample. The sample obtained was clear. The sample was placed on a culturette for bacterial/fungal culture as well as in a vial for cytological examination. The salamander recovered from anesthesia without incident.

Results:
Cytology of the fluid showed a cell count of 96% macrophages, 2% lymphocytes, 1% neutrophils, and 1% basophils. No infectious organisms or overtly neoplastic cells were seen. Culture of the swab grew very few colonies of a *Microbacterium* sp.

Final Diagnosis: Undetermined

In contrast to a similarly affected salamander (SNARRC Case #17-62), this salamander's coelomic tap was unremarkable. This salamander had been recently affected while the other salamander's condition was more chronic. This could explain the difference in the findings. The few colonies of *Microbacterium* are likely clinically insignificant. Continued sampling of deceased individuals in fixative is recommended for further histopathological evaluation. The finding of *Mycobacterium* in a similar case (17-62) is strongly suggestive that most of these salamanders have been exposed to the bacterium and may experience morbidity or mortality. Improvement in husbandry to reduce overall stress of these animals is highly recommended.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 17-63 for any follow-up correspondence.

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In Reply Refer To:
FWS/R2/FR-SFHU/862

Memorandum: August 23, 2017

To: Lindsay Campbell, San Marcos Aquatic Resource Center
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the Texas Blind Salamander "Godzilla" (SNARRC Case Number 17-64)

On July 25, 2017, one Texas Blind salamander (*Eurycea rathbuni*) was examined by Martha Keller at SMARC. This salamander has a well circumscribed raised mass on the dorsum, just proximal to the rear limbs. The mass borders are irregular, oval, and it measures 16.7 x 11.7 mm. The area has a whitish color to it on the surface compared to the rest of the body. Previous scrapes of the lesion/mass resulted in identification of fungal hyphae; therefore, staff was directed to attempt topical treatment using clotrimazole and silver sulfadiazine. These treatments were applied topically three times weekly and the medication was allowed to sit for one hour before returning salamander to its normal housing. Over the last two months of treatment, there has been no change in appearance of the mass. Other than the presence of the mass, the salamander is doing well.
In order to obtain better diagnostics, the mass surface was scraped using a blade and scrapings placed on two glass slides. Additional scrapings were placed in 95% ethanol and submitted for possible PCR evaluation.

Results:
Unfortunately, the glass slides arrived shattered and were therefore, unable to be read. Due to this, it was determined that no PCR could be recommended. Therefore, no useful data was obtained from these samples.

Final Diagnosis: Mass of unknown origin

Unfortunately no data was obtained with these samples. On a following visit, a fine needle aspirate may be the next best option to determine the origin of the mass. As treatment has been unsuccessful thus far, you may consider discontinuing treatment until a more accurate diagnosis can be obtained, especially if the salamander is doing well otherwise.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 17-64 for any follow-up correspondence.

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In Reply Refer To:
FWS/R2/FR-SFHU/863

Memorandum: August 23, 2017

To: Lindsay Campbell, San Marcos Aquatic Resource Center
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the Texas Blind Salamander "Godzilla" (SNARRC Case Number 17-65)

On August 15, 2017, one Texas Blind salamander (*Eurycea rathbuni*) was examined by Martha Keller at SMARC. This salamander was previously examined in July due to its well circumscribed raised mass on the dorsum, just proximal to the rear limbs (see Case 17-64). Previous attempts to scrape the mass were unsuccessful due to the slides breaking in transit. However, based on the appearance of the mass, it was decided to try a fine needle aspirate instead. A 22g needle was used to sample the mass in several areas. Glass slides of the aspirate were submitted directly to WADDL.

Results:
Slides were poorly cellular, consisting of rare erythrocytes on a background of adipocytes. No infectious organisms or overtly neoplastic cells were identified.

Final Diagnosis: Lipoma

Lipomas are benign fatty tumors that generally do not cause any health issues unless they grow to a size that can compromise normal function. In this case, it is reported that Godzilla has had this mass since 2014 and it has changed little over time. This diagnosis appears consistent with that clinical observation. This species of salamander does have subcutaneous fatty deposits over the dorsum in this region, so this is a location where a lipoma could form. Measurements of the mass have been taken and it is recommended that staff continue to monitor the size of the mass every 6 months or sooner if it appears the mass is changing in appearance. Based on these findings, there are no further concerns with the mass and it is not contagious to other salamanders. If not already done so, it would be recommended to discontinue any topical treatments.
There is no explanation for the color change on the surface of the mass. As the mass has corrugated edges, it is possible that external dermal infections could develop more easily in association with the mass. Therefore, continued observation of this animal will be important.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 17-65 for any follow-up correspondence.

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In Reply Refer To:
FWS/R2/FR-SFHU/870

Memorandum: September 19, 2017

To: Lindsay Campbell, San Marcos Aquatic Resource Center
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the San Marcos salamanders (SNARRC Case Number 17-68)

On August 29, 2017, three formalin-fixed San Marcos salamanders (*Eurycea nana*) were submitted directly to the Washington Animal Disease Diagnostic Laboratory (WADDL) from San Marcos ARC. All three of these salamanders had been clinically ill prior to their death. The first salamander experienced significant abdominal distention in association with egg production. The distension was so severe, the abdomen ruptured; however, the animal did not die. Staff excised the protruding egg mass from the abdomen and the wound healed with time and 3x weekly salt treatments. However, eventually, the animal bloated again and died, and the body was fixed in formalin.
The second salamander was the same individual that received a coelomic tap in July and cultured positive for *Mycobacterium marinum* (SNARRC Case Number 17-62). This individual had also experienced significant abdominal distention in association with the production of eggs. The coloemic cavity of the salamander was tapped on 7/23/17 and it recovered without incident. However, it died on 7/31/17 and was kept in formalin.
The third salamander was the second individual that received a coelomic tap in July (SNARRC Case Number 17-63). The fluid was unremarkable at the time and bacterial culture only grew a small amount of *Microbacterium*. The animal developed fungus-like growth around the gills and face and abdominal distention returned. The animal eventually died on 8/10/17 and was kept in formalin.

Results:
On the first salamander, notable findings include microsporidia found within the ovarian follicles. Acid fast bacteria was also noted (probable *Mycobacterium*). The second salamander was similar with microsporidia and probable *Mycobacterium* noted in the ovary. The third salamander had similar microsporidia noted in the ovary but no acid-fast bacteria was noted. Colonization of a water mold was also noted in the third salamander, consistent with the observations of the staff. Interestingly, no other organs were seen affected with either the microsporidia or the *Mycobacterium*.

Final Diagnosis: Oophoritis and coeolomitis associated with intralesional microsporidia (3 of 3 animals) and acid-fast bacteria (2 of 3 animals) (likely *Mycobacterium*).

These findings are consistent with previous findings in this species. Interestingly, the microsporidia was present in all 3 clinically affected animals whereas the *Mycobacterium* was only noted in 2 of the 3 animals. This is suggestive that the primary concern may be the microsporidia causing the oophoritis and the presence of the *Mycobacterium* may be a secondary factor. While there are no published treatments for microsporidia in this species, it may be recommended to perform additional treatment trials in these animals. While there is no treatment for *Mycobacterium*, the ability to address a significant disease factor (microsporidia) in these animals may greatly reduce the chances of the *Mycobacterium* becoming a clinical factor.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 17-68 for any follow-up correspondence.

cc: Kenneth Ostrand, San Marcos Aquatic Resource Center

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In Reply Refer To:
FWS/R2/FR-SFHU/871

Memorandum: October 2, 2017

To: Lindsay Campbell, San Marcos Aquatic Resource Center
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the Fountain darters (SNARRC Case Number 17-69)

On August 29, 2017, 6 Fountain darters (*Etheostoma fonticula*) were submitted directly from San Marcos ARC to the Washington Animal Disease Diagnostic Laboratory for histopathological evaluation. The fish were originally collected from the wild on May 30-31 and moved into quarantine. They are on unfiltered flow through well water, with siphoning of tanks occurring at a minimum, weekly. This group of fish has been experiencing exophthalmia and an increase in mortalities compared to a similar population collected at an alternate site at the same time. Previous samples of fish were submitted to SNARRC in June (Case 17-55) for diagnostic evaluation, but no definitive cause for the mortalities was noted. At that time, fish tested positive for LMBV but it was already known that this population was positive for that virus. Due to continuing mortalities, it was recommended to submit additional samples for histopathology along with "normal" fish from the other population to use as a comparison.

Three fish from the affected population along with 3 clinically normal fish were submitted whole in formalin for histopathological evaluation.

Results:

Histopathology on the "normal" fish revealed an encysted trematode, however, this finding is consistent with necropsy findings in previous fish from the area and is not considered significant. No other abnormalities were seen on the normal group of fish. In the affected fish group, a mild dermatitis and an intestinal nematode was noted along with a very mild neprhocalcinosis in one fish. Inflammation surrounding the eye on one fish was considered significant, but no associated etiology was determined and no microorganisms were seen. As the other fish did not have similar lesions, it is difficult to determine whether this finding is associated with the low-grade mortality.

Final Diagnosis: Undetermined

Unfortunately, no etiology was noted for the poor performance in these fish. Although these fish have been previously shown to be positive for LMBV, the clinical signs are not consistent with that pathogen. As these fish are unable to be moved to the main facility due to the presence of this pathogen, and they continue to have a higher than normal mortality rate, humane euthanasia of the lot may be considered rather than continue to have their numbers decrease over time. The clinical normal group of Fountain Darters that remains in quarantine can be moved without concern, as there is no indication of any problems in that group of fish.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 17-69 for any follow-up correspondence.

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In Reply Refer To:
FWS/R2/FR-SFHU/872

Memorandum: October 3, 2017

To: Marco Pedulli, Uvalde NFH
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the Fountain darters (SNARRC Case Number 17-70)

On September 11, 2017, 6 Fountain darters (*Etheostoma fonticula*) were submitted directly from Uvalde NFH to the Washington Animal Disease Diagnostic Laboratory for histopathological evaluation. The fish were originally collected from the wild on May 30-31 and moved into quarantine. They are on unfiltered flow through well water, with siphoning of tanks occurring at a minimum, weekly. This group of fish has been experiencing an increase in mortalities compared to a similar population collected at an alternate site at the same time. This group of fish was originally split into two groups and sent to both Uvalde and San Marcos. They have performed poorly at both facilities. Previous samples of fish were submitted from SMARC to SNARRC in June (Case 17-55) for diagnostic evaluation, but no definitive cause for the mortalities was noted. At that time, fish tested positive for LMBV but it was already known that this population was positive for that virus. Due to continuing mortalities, it was recommended to submit additional samples for histopathology along with "normal" fish from the other population to use as a comparison.

Three fish from the affected population along with 3 clinically normal fish were submitted whole in formalin for histopathological evaluation.

Results:

All fish submitted were noted to be moderately autolyzed which severely limits histopathological analysis. The only other finding of note was a moderate hepatocellular necrosis, but it was not associated with any evidence of parasites, bacteria, or viral disease.

Final Diagnosis: Undetermined

Unfortunately, no etiology was noted for the poor performance in these fish. A significant complicating factor is that all the fish were in moderate states of autolysis. This is likely due to these fish being found dead rather than being fixed after euthanasia. Fresher submissions from SMARC from this same group of fish did not establish an etiology for the low grade mortality either. Given the poor performance in this group and the presence of LMBV which makes it unable to move them out of quarantine, humane euthanasia of the remaining lot could be considered. If future submissions are considered, it would be recommended to select moribund or clinically affected fish and humanely euthanize them immediately prior to fixation to eliminate any chance of autolysis.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 17-70 for any follow-up correspondence.

cc: Patricia Duncan, Uvalde NFH

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In Reply Refer To:
FWS/R2/FR-SFHU/875

Memorandum: November 9, 2017

To: Marco Pedulli, Uvalde NFH
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the wild salamanders (SNARRC Case Number 18-01)

On October 2, 2017, 200 salamander skin swabs from 100 salamanders were received from Uvalde NFH. These swabs came from wild salamanders that were captured and moved into quarantine at Uvalde NFH. All samples were San Marcos salamanders (*Eurycea nana*). Samples were submitted in duplicate to be tested for *Batrachochytrium dendrobatidis (*Bd*)* and *Batrachochytrium salamandrivorans* (Bsal). Swabs were tested using PCR. Duplicate samples were labeled "A" and "B". Only the A samples were tested. All "B" samples will be stored in our -80 freezer in case of the need for any further testing. Unless otherwise requested, these samples will be tossed at the end of the fiscal year.

Results:
All 100 salamander samples (100%) were negative for Bsal. Additionally, 44/100 samples (44%) were positive for Bd, 56/100 (56%) were negative. An individual breakdown of the results is attached.

Final Diagnosis: *Batrachochytrium dendrobatidis (Bd)*

The finding of Bd in these animals is not surprising as it has been previously detected in this species. Historically, this species have not shown any clinical signs related to the fungus. However, any future movements of these animals should be done with care, as group housing has likely resulted in many of the previously negative animals to become positive. If they are moved back into the wild, they could transmit the pathogen to new waters. Therefore, no movement of these animals is recommended unless the receiving body of water has already been determined to be Bd positive.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 18-01 for any follow-up correspondence.

cc: Patricia Duncan, Uvalde NFH

VIAL #	DATE	COLLECTION LOCATION	SPECIES	TL (mm)	LESIONS PRESENT	DESTINATION	Bd result	Bsal result
236	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	47	N	UVALDE	+	-
237	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	56	N	UVALDE	-	-
238	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	61	N	UVALDE	-	-
239	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	55	N	UVALDE	-	-
240	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	59	N	UVALDE	-	-
241	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	51	N	UVALDE	+	-
242	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	52	N	UVALDE	+	-
243	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	64	N	UVALDE	-	-
244	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	54	N	UVALDE	-	-
245	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	30	N	UVALDE	-	-
246	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	32	N	UVALDE	-	-
247	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	48	N	UVALDE	+	-
248	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	41	N	UVALDE	-	-
249	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	47	N	UVALDE	-	-
250	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	58	N	UVALDE	+	-
251	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	50	N	UVALDE	+	-
252	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	57	N	UVALDE	-	-
253	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	44	N	UVALDE	+	-
254	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	38	N	UVALDE	+	-
255	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	47	N	UVALDE	-	-
256	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	50	N	UVALDE	-	-
257	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	61	N	UVALDE	+	-
258	19-Sep-17	CATFISH MOTEL/RIVERBED	E. nana	45	N	UVALDE	+	-
259	19-Sep-17	DEEP HOLE	E. nana	46	N	UVALDE	+	-
260	19-Sep-17	DEEP HOLE	E. nana	46	N	UVALDE	+	-
261	19-Sep-17	DEEP HOLE	E. nana	55	N	UVALDE	-	-
262	19-Sep-17	DEEP HOLE	E. nana	36	N	UVALDE	-	-
263	19-Sep-17	DEEP HOLE	E. nana	58	N	UVALDE	+	-
264	19-Sep-17	DEEP HOLE	E. nana	58	N	UVALDE	-	-
265	19-Sep-17	DEEP HOLE	E. nana	50	N	UVALDE	+	-
266	19-Sep-17	DIVERSION	E. nana	45	N	UVALDE	+	-
267	19-Sep-17	DIVERSION	E. nana	65	N	UVALDE	+	-

268	19-Sep-17	DIVERSION	E. nana	57	N	UVALDE	+	-
269	19-Sep-17	DIVERSION	E. nana	57	N	UVALDE	+	-
270	19-Sep-17	DIVERSION	E. nana	40	N	UVALDE	+	-
271	19-Sep-17	DIVERSION	E. nana	61	N	UVALDE	+	-
273	19-Sep-17	DIVERSION	E. nana	47	N	UVALDE	-	-
274	19-Sep-17	DIVERSION	E. nana	52	N	UVALDE	+	-
275	19-Sep-17	DIVERSION	E. nana	61	N	UVALDE	+	-
276	19-Sep-17	DIVERSION	E. nana	52	N	UVALDE	+	-
277	19-Sep-17	DIVERSION	E. nana	42	N	UVALDE	+	-
278	19-Sep-17	DIVERSION	E. nana	46	N	UVALDE	+	-
279	19-Sep-17	DIVERSION	E. nana	45	N	UVALDE	-	-
280	19-Sep-17	DIVERSION	E. nana	36	N	UVALDE	-	-
281	19-Sep-17	DIVERSION	E. nana	49	N	UVALDE	-	-
282	19-Sep-17	DIVERSION	E. nana	48	N	UVALDE	+	-
283	19-Sep-17	DIVERSION	E. nana	46	N	UVALDE	-	-
284	19-Sep-17	DIVERSION	E. nana	56	N	UVALDE	+	-
285	19-Sep-17	DIVERSION	E. nana	42	N	UVALDE	-	-
286	19-Sep-17	DIVERSION	E. nana	57	N	UVALDE	+	-
287	19-Sep-17	DIVERSION	E. nana	52	N	UVALDE	+	-
288	19-Sep-17	DIVERSION	E. nana	34	N	UVALDE	-	-
289	19-Sep-17	DIVERSION	E. nana	58	N	UVALDE	+	-
290	19-Sep-17	DIVERSION	E. nana	52	N	UVALDE	+	-
291	19-Sep-17	DIVERSION	E. nana	59	N	UVALDE	+	-
292	19-Sep-17	DIVERSION	E. nana	44	N	UVALDE	+	-
293	19-Sep-17	HOTEL	E. nana	50	N	UVALDE	-	-
294	19-Sep-17	HOTEL	E. nana	55	N	UVALDE	-	-
295	19-Sep-17	HOTEL	E. nana	63	N	UVALDE	-	-
296	19-Sep-17	HOTEL	E. nana	53	N	UVALDE	-	-
297	19-Sep-17	HOTEL	E. nana	47	N	UVALDE	-	-
298	19-Sep-17	HOTEL	E. nana	44	N	UVALDE	-	-
299	19-Sep-17	HOTEL	E. nana	55	N	UVALDE	-	-
300	19-Sep-17	HOTEL	E. nana	31	N	UVALDE	-	-
301	19-Sep-17	HOTEL	E. nana	31	N	UVALDE	-	-
302	19-Sep-17	HOTEL	E. nana	56	N	UVALDE	-	-

303	19-Sep-17	HOTEL	E. nana	58	N	UVALDE	-	-
304	19-Sep-17	HOTEL	E. nana	36	N	UVALDE	-	-
305	19-Sep-17	HOTEL	E. nana	39	N	UVALDE	-	-
306	19-Sep-17	HOTEL	E. nana	64	N	UVALDE	-	-
307	19-Sep-17	HOTEL	E. nana	62	N	UVALDE	-	-
308	19-Sep-17	HOTEL	E. nana	57	N	UVALDE	-	-
309	19-Sep-17	HOTEL	E. nana	56	N	UVALDE	-	-
310	19-Sep-17	HOTEL	E. nana	60	N	UVALDE	-	-
311	19-Sep-17	HOTEL	E. nana	44	N	UVALDE	-	-
312	19-Sep-17	HOTEL	E. nana	56	N	UVALDE	-	-
313	19-Sep-17	HOTEL	E. nana	45	N	UVALDE	-	-
314	19-Sep-17	HOTEL	E. nana	47	N	UVALDE	-	-
315	19-Sep-17	HOTEL	E. nana	57	N	UVALDE	+	-
316	19-Sep-17	HOTEL	E. nana	60	N	UVALDE	+	-
317	19-Sep-17	HOTEL	E. nana	65	N	UVALDE	-	-
318	19-Sep-17	HOTEL	E. nana	64	N	UVALDE	+	-
319	19-Sep-17	HOTEL	E. nana	55	N	UVALDE	+	-
320	19-Sep-17	HOTEL	E. nana	51	N	UVALDE	-	-
321	19-Sep-17	HOTEL	E. nana	44	N	UVALDE	+	-
322	19-Sep-17	HOTEL	E. nana	56	N	UVALDE	-	-
323	19-Sep-17	HOTEL	E. nana	47	N	UVALDE	+	-
324	19-Sep-17	HOTEL	E. nana	57	N	UVALDE	-	-
325	19-Sep-17	HOTEL	E. nana	56	N	UVALDE	-	-
326	19-Sep-17	HOTEL	E. nana	49	N	UVALDE	-	-
327	19-Sep-17	HOTEL	E. nana	58	N	UVALDE	+	-
328	19-Sep-17	HOTEL	E. nana	51	N	UVALDE	-	-
329	19-Sep-17	HOTEL	E. nana	34	N	UVALDE	+	-
330	19-Sep-17	HOTEL	E. nana	42	N	UVALDE	-	-
331	19-Sep-17	HOTEL	E. nana	47	N	UVALDE	+	-
332	19-Sep-17	HOTEL	E. nana	39	N	UVALDE	-	-
333	19-Sep-17	HOTEL	E. nana	.	N	UVALDE	-	-
334	19-Sep-17	HOTEL	E. nana	40	N	UVALDE	+	-
335	19-Sep-17	HOTEL	E. nana	58	N	UVALDE	-	-
336	19-Sep-17	HOTEL	E. nana	56	N	UVALDE	-	-

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/876

Memorandum: November 13, 2017

To: Lindsay Campbell, San Marcos ARC
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the wild salamanders (SNARRC Case Number 18-02)

On October 2, 2017, SNARRC staff obtained 132 salamander skin swabs from 66 salamanders, from San Marcos ARC for chytrid testing. These swabs came from wild salamanders that are now being held at San Marcos ARC. Samples came from five Comal Springs salamanders (*Eurycea* spp), nine Texas blind salamanders (*Eurycea rathbuni*), and 52 San Marcos salamanders (*Eurycea nana*). Specifics on the sampling date and location can be found on the attached data sheet. Samples were submitted in duplicate to be tested for *Batrachochytrium dendrobatidis (*Bd*)* and *Batrachochytrium salamandrivorans* (Bsal). Duplicate samples were labeled "A" and "B". Only the A samples were tested. All "B" samples will be stored in our -80 freezer in case of the need for any further testing. Unless otherwise requested, these samples will be tossed at the end of the fiscal year.

Results:
All salamanders were negative for Bsal. No Texas blind salamanders (0%) were positive for Bd. 3/5 (60%) of Comal springs samples were positive for Bd. 20/52 (38.5%) of the San Marcos salamanders were positive for Bd. Individual results can be found on the attached data sheet.

Final Diagnosis: *Batrachochytrium dendrobatidis (Bd)*

The finding of Bd in these animals is not surprising as it has been previously detected in these positive species. Historically, these species have not shown any clinical signs related to the fungus. However, any future movements of these animals should be done with care, as group housing has likely resulted in many of the previously negative animals to become positive. The Texas blind salamanders should be kept separate from the other salamanders since they were negative. If positive populations are moved back into the wild, they could transmit the pathogen to new waters. Therefore, no movement of these animals is recommended unless the receiving body of water has already been determined to be Bd positive.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 18-02 for any follow-up correspondence.

cc: Ken Ostrand, San Marcos ARC

Vial #	Date	Collection Location	Species	TL (mm)	Lesions?	Bd	Bsal
206	6/28/2017	Diversions	EN	45	N	+	-
207	7/6/2017	Comal - Spring Run 1	ESPP	43	N	+	-
208	7/6/2017	Comal - Spring Run 2	ESPP	45	N	+	-
209	7/6/2017	Comal - Spring Run 3	ESPP	65	N	+	-
210	7/6/2017	Comal - Spring Run 4	ESPP	45	N	-	-
211	7/21/2017	Rattlensake Cave	ER	73	N	-	-
212	7/21/2017	Rattlensake Cave	ER	72	N	-	-
213	7/27/2017	Diversions	EN	53	N	+	-
214	7/27/2017	Diversions	ER	22	N	-	-
215	8/3/2017	Comal - Spring Run3	ESPP	55	N	-	-
216	8/9/2017	Johnsons Well	ER	76	N	+	-
217	8/14/2017	Primers Fissure	ER	67	N	-	-
218	8/16/2017	Johnsons Well	ER	80	N	-	-
219	9/12/2017	TXSTU Well	ER	70	N	-	-
220	9/19/2017	Spring Lake-Hotel	EN	42	N	+	-
221	9/19/2017	Spring Lake-Hotel	EN	41	N	+	-
222	9/20/2017	SL Dam - below the falls	EN	29	N	-	-
223	9/20/2017	SL Dam - below the falls	EN	30	N	-	-
224	9/20/2017	SL Dam - below the falls	EN	34	N	-	-
225	9/20/2017	SL Dam - below the falls	EN	47	N	-	-
226	9/20/2017	SL Dam - below the falls	EN	57	N	-	-
227	9/20/2017	SL Dam - below the falls	EN	43	N	-	-
228	9/20/2017	SL Dam - below the falls	EN	58	N	-	-
229	9/20/2017	SL Dam - below the falls	EN	45	N	-	-
230	9/20/2017	SL Dam - below the falls	EN	35	N	-	-
231	9/20/2017	SL Dam - below the falls	EN	51	N	-	-
232	9/20/2017	SL Dam - below the falls	EN	48	N	-	-
233	9/20/2017	SL Dam - below the falls	EN	40	N	-	-
234	9/20/2017	SL Dam - below the falls	EN	64	N	-	-
235	9/20/2017	SL Dam - below the falls	EN	46	N	-	-
337	9/20/2017	SL Dam - below the falls	EN	50	N	-	-
338	9/20/2017	SL Dam - below the falls	EN	58	N	-	-
339	9/20/2017	SL Dam - below the falls	EN	55	N	-	-
340	9/20/2017	SL Dam - below the falls	EN	62	N	+	-
341	9/20/2017	SL Dam - below the falls	EN	57	N	-	-
342	9/20/2017	SL Dam - below the falls	EN	59	N	-	-
343	9/20/2017	SL Dam - below the falls	EN	52	N	+	-
344	9/20/2017	SL Dam - below the falls	EN	58	N	+	-
345	9/20/2017	SL Dam - below the falls	EN	47	N	-	-
346	9/20/2017	SL Dam - below the falls	EN	61	N	-	-
347	9/20/2017	SL Dam - below the falls	EN	61	N	-	-
348	9/20/2017	SL Dam - below the falls	EN	53	N	-	-
349	9/20/2017	SL Dam - below the falls	EN	55	N	+	-
350	9/20/2017	SL Dam - below the falls	EN	50	N	-	-
351	9/20/2017	SL Dam - below the falls	EN	49	N	-	-

352	9/20/2017	SL Dam - below the falls	EN	55	N	+	-
353	9/20/2017	SL Dam - below the falls	EN	69	N	+	-
354	9/20/2017	SL Dam - below the falls	EN	50	N	-	-
355	9/20/2017	SL Dam - below the falls	EN	43	N	-	-
356	9/20/2017	SL Dam - below the falls	EN	72	N	-	-
357	9/20/2017	SL Dam - below the falls	EN	47	N	+	-
358	9/20/2017	SL Dam - below the falls	EN	63	N	+	-
359	9/20/2017	SL Dam - below the falls	EN	44	N	+	-
360	9/20/2017	SL Dam - below the falls	EN	59	N	+	-
361	9/20/2017	SL Dam - below the falls	EN	40	N	+	-
362	9/20/2017	SL Dam - below the falls	EN	58	N	+	-
363	9/20/2017	SL Dam - below the falls	EN	56	N	-	-
364	9/20/2017	SL Dam - below the falls	EN	53	N	-	-
365	9/20/2017	SL Dam - below the falls	EN	52	N	+	-
366	9/20/2017	SL Dam - below the falls	EN	43	N	-	-
367	9/20/2017	SL Dam - below the falls	EN	65	N	+	-
368	9/20/2017	SL Dam - below the falls	EN	35	N	+	-
369	9/20/2017	SL Dam - below the falls	EN	64	N	+	-
370	on 9-27, collec	TXSTU Well	ER	55	N	-	-
371	on 9-27, collec	SLWO (Outflow)	EN	42	N	-	-
372	d on 9-27, colle	Unknown	ER	42	N	-	-

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/880

Memorandum: December 4, 2017

To: Marco Pedulli, Uvalde NFH
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final Report for the Fountain darters (SNARRC Case Number 18-11)

On November 9, 2017, 3 Fountain darters (*Etheostoma fonticula*) were submitted directly from Uvalde NFH to the Washington Animal Disease Diagnostic Laboratory for histopathological evaluation. The fish were originally collected from the wild on May 30-31 and moved into quarantine. They are on unfiltered flow through well water, with siphoning of tanks occurring at a minimum, weekly. This group of fish has been experiencing an increase in mortalities compared to a similar population collected at an alternate site at the same time. This group of fish was originally split into two groups and sent to both Uvalde and San Marcos. At that time, fish tested positive for LMBV but it was already known that this population was positive for that virus. They have performed poorly at both facilities. Previous samples of fish were submitted from Uvalde (Case 18-07) for diagnostic evaluation, and unusual histopathological findings were observed although the testing is not complete and no final report has yet been issued. WADDL requested additional samples of moribund fish. Unfortunately, no further moribund fish were noted by staff. Therefore, 3 random fish were sampled and euthanized and submitted to WADDL.

Results:

All fish submitted were noted to be free of histologic lesions although all fish appeared to have a very mild gastritis.

Final Diagnosis: Undetermined

Unfortunately, no etiology was noted for the previous poor performance in this group of fish. The mild gastritis identified is likely not clinically significant, especially given the recent improved performance in this group of fish. As mentioned previously, testing of the previous lot of fish is still

ongoing and a determination of the illness may still be forthcoming. The final results on that group of fish will be forwarded as soon as possible.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-11 for any follow-up correspondence.

cc: Patricia Duncan, Uvalde NFH

United States Department of the Interior
Fish and Wildlife Service
Southwestern Native Aquatic Resources and Recovery Center
Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/884

Memorandum: December 28, 2017

To: Linda Moon, San Marcos Aquatic Resource Center
From: Martha Keller, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center (SNARRC)

Subject: Final Report for the Fountain darters (SNARRC Case Number 18-19)

On December 27, 2017, five Fountain darters (*Etheostoma fonticula*) were submitted live to the fish health unit from San Marcos ARC for parasitological evaluation only. The fish were originally collected from the wild in the Comal River on November 1 & 2 and moved into SMARC quarantine. The number of *Centrocestus* on clinically normal fish was enumerated shortly after their capture by the fish health unit as a normal process (Case 18-13). Once in quarantine however, this group of fish showed increased mortalities with swollen gills and rapid ventilations. Samples of clinically affected fish were submitted to SNARRC in late November (Case 18-15) for diagnostic evaluation, but no definitive cause for the mortalities has been yet determined. However during that examination, it was noted that the fish had an extremely high number of *Centrocestus* compared to the previous submission. This group of fish tested also positive for LMBV but it was already known that this population was positive for that virus. Due to continuing mortalities, additional samples of moribund and deceased fish were submitted for histopathology (Case 18-16) and results are still pending.
Due to the finding of high numbers of *Centrocestus*, it was recommended that a Praziquantel treatment be administered to these fish. Although it was not considered a primary cause of illness, the high numbers could be playing a factor in the severity of the illness. The dose for appropriate treatment of the *Centrocestus* is currently unknown for this species. A dose of 2 ppm as a static bath for 24 hours, the standard treatment for Asian tapeworm, was the dose prescribed. In order to determine if this dose is appropriate, it was recommended that a small sample of treated fish be submitted to the fish health unit for evaluation of the gills to see if the numbers of *Centrocestus* was significantly reduced post-treatment.

Results:

Previous findings in these fish showed that the gills primarily contained immature *Centrocestus* numbering anywhere from 1-24 cysts per gill arch. All fish had at least one cyst. Today's

enumeration once again showed primarily immature *Centrocestus* present, ranging from zero to 13 cysts per gill arch. One fish had only one cyst noted total and another had zero cysts noted.

Final Diagnosis: Undetermined effectiveness of the treatment

It is difficult to directly compare the effectiveness of the treatment as we are comparing different fish each time. However, finding fish with little to no cysts at all is not common when normally examining these wild fish. And in today's examination 2/5 fish had only one or zero cysts present. It is suggestive that the treatment had some effect. Given that these are metacercaria and not adults, they may not drop off the fish immediately, even when killed by the treatment. Given that the treatment did not appear to negatively affect the fish, further studies would be recommended examining fish both pre and post treatment and also using control fish that remain untreated. However, future studies may warrant waiting for about a month before re-examining the treated fish to allow the metacercaria more time to drop off. Only through proper evaluation can an appropriate treatment dose be identified. Coordination with the fish health unit is recommended in order to assess these fish appropriately moving forward.
SMARC staff reports no change in the mortality level of these fish post-treatment. The mortality was not suspected to be associated with the *Centrocestus*, but the high levels were likely not helping the situation. Samples from the moribund fish are still being tested and results will be forwarded once obtained. For now, proper husbandry including providing a low stress environment is recommended. This includes providing the fish with shelter to hide in, good water quality, proper nutrition, appropriate lighting, low sound and vibrations, and tank cleaning that is minimal but still effective enough to maintain proper hygiene.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-19 for any follow-up correspondence.

cc: Lindsay Campbell, Ken Ostrand, San Marcos ARC

Larval development of Comal Springs dryopid beetles (*Stygoparnus comalensis*)

LITERATURE REVIEW AND PROPOSED METHODOLOGY

Prepared for:
Edwards Aquifer Authority

Prepared by:
Randy Gibson, Regional Invertebrate Zoologist
Dr. Lindsay Campbell, Refugia Managing Biologist
U.S. Fish & Wildlife Service
San Marcos Aquatic Resources Center

&

McLean Worsham, Senior Invertebrate Zoologist
BIO-WEST, Inc.

March 22, 2017

U.S. Fish & Wildlife Service
San Marcos Aquatic Resources Center
Southwest Region

BIO-WEST

Introduction

The Edwards Aquifer Habitat Conservation Plan (EAHCP) calls for the establishment of captive refuge populations of Edwards Aquifer (EA) Covered Species associated with their Incidental Take Permit inhabiting both subterranean and spring outflow habitats. The San Marcos Aquatic Resources Center (SMARC) operated by the United States Fish and Wildlife Service (USFWS) has been awarded the opportunity to establish and maintain captive refuge populations of EA species of concern; many of which have been cultivated successfully in captivity at SMARC for several years. Some of the species of concern still pose several substantial questions concerning refuge cultivation; particularly the invertebrate species. Recognizing this deficit, research into life-history and captive propagation of the Comal Springs dryopid beetle (*Stygoparnus comalensis*) is proposed to commence in 2017.

Roles and Responsibilities

As the prime contractor for the EAHCP refugia contract, SMARC will provide consultation, oversight and review activities for this 2017 Captive Propagation applied research project. In particular, Dr. Lindsay Campbell and Mr. Randy Gibson will serve as Co-Principal Investigators for this supervisory role. Additionally, as part of on-going refugia activities surrounding the maintenance and collection of standing stocks, SMARC biologists will carry out collection duties and routine technical assistance. Finally, SMARC will provide all facilities, utilities and equipment for 2017 experimentation. As a subcontractor on the USFWS refugia team, BIO-WEST will be responsible for task execution, analysis, and reporting. To perform these duties, BIO-WEST will provide a senior invertebrate zoologist and aquatic technician to work on station at SMARC.

Literature Review

Stygoparnus comalensis is a federally endangered species (USFWS 1997) that is adapted to subterranean habitats associated with Edwards Aquifer spring systems. *Stygoparnus comalensis* have been recovered from a limited number of perennial Edwards Aquifer springs (Comal, Fern Bank, and San Marcos springs). Since the species was discovered, it has rarely been encountered; less 80 adults have been collected or observed since the species was discovered in 1992 despite extensive sampling making it perhaps the rarest of the EA covered species. *Stygoparnus comalensis* is characterized by having vestigial eyes, lacking pigment, and wingless adults. *Stygoparnus comalensis* is also the type species of the genus *Stygoparnus* which is a monotypic genus with *S. comalensis* being the only species (Barr and Spangler 1992). Therefore, the conservation of *S. comalensis* should be considered particularly important as there are no related lineages found anywhere else on earth. Study of this species is also complicated by the fact that there is likely no suitable surrogate species.

Like other dryopid beetles, adult *S. comalensis* are fully aquatic and similar to adult riffle beetles in general ecology. They inhabit relatively clean rivers and streams feeding on biofilm scraped from surfaces and are relatively slow moving and incapable of swimming. Respiration is through a plastron, a gas film produced by area of dense water repelling hairs (Brown 1987, Resh et al. 2008). The life span of this species is unknown; however, some wild caught adults have survived in captivity 11-21 months (Barr and Spangler 1992, Fries et al. 2004).

Dryopid larvae typically inhabit moist terrestrial soils along stream banks, presumably feeding on roots and decaying vegetation, while adults are fully aquatic (Brown 1987, Ulrich 1987). However, *Stygoparnus comalensis* is unique among all other dryopids as the only species with fully aquatic larvae. Like the closely allied elmids, larvae utilizing retractable posterior gills for respiration (Brown 1987) making S. comalensis the only dryopid to have both aquatic larvae and adults. Aside from this, little else is known about the life history and development of the *S. comalensis*. There is no information on deposition sites for eggs, clutch size, incubation times or size of eggs. Additionally, there is insufficient information on larval development, although it is hypothesized that it requires up to 2-5 years before pupating like other dryopid species (Ulrich 1986). However, a single *S. comalensis* larva produced at SMARC grew from 2 to 10 mm in 9 months, suggesting larval development may require only one year for *S. comalensis*, although it is unknown how old the larvae was at 2 mm in length.

Thus far, captive-produced and wild-caught larvae have failed to pupate in captivity. Barr and Spangler (1992) hypothesized that the larvae may require a moist terrestrial habitat exposed above the waterline on the ceilings of spring orifices for development and pupation. With so little known about this species, it is vital that more information about the life history of this species is collected in order to maintain effective refuge populations.

Methods

The primary charge of EAHCP refugia is to preserve the capacity for the HCP Covered Species to be re-established at Comal and San Marcos springs, if ever necessary. This charge dictates that refugia research focuses on captive propagation. As limited life history or captive propagation information is available for *S. comalensis*, a large portion of this early research in 2017 will be exploratory or observational. Basic testing of everything from collection techniques, to housing apparatus, to flow, substrate, or other environmental stimuli. A key research objective moving forward is to build upon 2017 exploratory research that appears promising, by designing subsequent hypothesis driven, quantitative studies as appropriate in the future.

Task 1: Collection of test subjects

Adult *S. comalensis* will be collected from cotton lures and drift nets set in Sessoms Creek, Comal spring runs, and Landa Lake as needed in 2017 for the completion of study activities. Cotton lures will be set and allowed to develop biofilms for four weeks before being retrieved and checked for invertebrates following Gibson et al., (2008) and Hall (2016). Adults will be removed and transported back to the SMARC where they will be maintained initially within custom aquatic holding units (similar to picture to the right) and fed detrital material and matured biofilms on colonized cotton lures. Adults will be held in quarantine for 14 days before entering into research observations so they can acclimate to captivity.

Task 2: Exploratory Research

The historic difficulty in collecting *S. comalensis* individuals, limited information on captive propagation, and available literature for this genus leads to the following overarching key assumptions:

- Sufficient organisms will be collected for exploratory research.
- Ability to keep individuals alive in captivity.
- Life span amenable to accomplishing tasks in 1 year.

Additionally, each chronological Task 2 subtask is inherently dependent on the level of success in the previous activity. Both the key assumptions and subtask associations will be tracked throughout the project and any concerns reported to EAA upon identification.

2.1 Sexual dimorphism

Following the methods of the Comal Springs riffle beetle life history study (BIO-WEST 2016), sex determination will be attempted by gently placing adults under a dissecting microscope both dorsal and ventral pictures will be taken of various aspects of morphology. Images will be analyzed for evidence of sexually dimorphic external features.

Schedule: March through July

Anticipated results: photo diagrams of male/female sexually dimorphic external features.

2.2 Egg production and incubation

Depending on the outcome of the study of sexual dimorphism, adults will be bred in groups or pairs (i.e. if sex can't be determined then adults will be bred in large groups to ensure that members of the opposite sex are available to mate; however, if sex can be determined then adults will be bred in pairs). Assuming sex determination is successful, a single male-female pair of adults will be held in flow-through containers having both aquatic and terrestrial habitat provided with a variety of substrates (cotton cloth, wood, leaves, nylon mesh, etc.). Replication will depend on the availability of organisms but ideally, n=4 per treatment group. Substrates in each replicate will be checked weekly (at a minimum) and egg laden substrates will be removed and maintained in separate flow-through containers. If numbers suffice, analysis of variance (ANOVA) will be run to determine if there is a statistical preference for substrate(s) on which eggs are laid.

Once eggs are produced they will be monitored until they hatch by checking twice weekly (at a minimum). We will assess differences in the hatching success, amount of time eggs incubated, and survival rate of larvae immediately post hatching using a combination of regression analyses and one-way ANOVAs using the different treatment groups of the parents to determine which factors contribute to the greatest production of viable eggs.

Schedule: April through September

Anticipated results: egg production; egg morphology; incubation duration of eggs; better understanding of conditions that contribute to the project of eggs.

2.3 Larval development

1st instar larvae will be placed into individual holding chambers [flow through system with 75 µm or smaller pore size mesh until large enough to be moved to larger mesh size flow through systems (pictured to the right). An initial step of exploratory research will be to evaluate different types and configurations of flow-through chambers to determine which might be most effective for this species.

Once a chamber design is selected, it will then receive a variety of pilot treatment substrates until exploratory research results are able to provide insight into promising conditions for cultivation. Experimental replication will depend on the availability of larvae produced but will be evenly spread across experimental groups. Larvae will have development visually observed twice per week (at a minimum). Additionally, growth and/or molts will be recorded twice monthly (at a minimum) during 2017. To accomplish this, larvae will be wet mounted and have total body length and head capsule width measured using Olympus Cellcens camera system and measuring tool software at the standard shutter speed of 3.395 milliseconds. Growth and molt rate will be analyzed for significance via univariate analyses (e.g., ANOVA). Observations of aquatic and terrestrial substrate utilized by the larvae will be recorded daily/weekly and ANOVA tests run on preferences if warranted.

Schedule: July through December

Anticipated results: larvae production; instar morphology, better understanding of rate of larval development.

2.4 Pupation

Conditions that contribute to pupation are completely unknown for *S. comalensis*. Therefore, the conditions that best suit the development of larvae will be used as a starting point for exploratory research relative to pupation towards the conclusion of 2017. Pupation containers will provide substrate that is above and below the surface as many dryopid larvae are known to pupate in terrestrial habitats.

Schedule: October through December

Anticipated results: preliminary insight into pupation

Task 3: Analysis and Reporting

As typical for observational data, it is anticipated that a major portion of any analysis and explanation will be descriptive statistics and morphological documentation. Potential statistical analysis for exploratory research was briefly described above in each subtask as deemed applicable at this time. An interim report of the research activities conducted during 2017 be drafted during November 2017, finalized during December 2017, and submitted to EAA by December 31, 2017. The 2017 product is termed Interim in that it is anticipated that certain applied research components will be carried forward into 2018 in order to better understand and document the life cycle of this unique HCP Covered species.

Budget

As discussed in the roles and responsibilities section above, this project will be a Refugia Team effort. A detailed cost breakdown for BIO-WEST's involvement is documented in the table below. All SMARC costs for assistance in this effort will be covered through their established budget (Task 1 – 2017 Workplan) for maintenance and collection of standing stocks. Therefore, the total cost for the 2017 Larval Development of Comal Springs dryopid beetle study is $104,098.

BIO-WEST (01/01/2017)
DETAILED COST BREAKDOWN - EAHCP 2017 Refugia
Comal Springs Dryopid Beetle Life History Study

LABOR:

Position	Rate	TASK 2 - Applied Research	TOTALS Total Hours	TOTALS Cost
Project Principal	155.87	48	48	$ 7,481.76
Biologist II	75.26	636	636	$ 47,865.36
Senior Administrative	66.72	6	6	$ 400.32
Technician II	51.31	924	924	$ 47,410.44
Total Labor		1,614	1,614	**103,157.88**
TRAVEL				
Per diem: Hotel and Meals				$ -
Mileage ($.535 per mile)	0.535	500	500	$ 267.50
Total Travel				**$ 267.50**
DIRECT COSTS:				
Equipment: water quality sondes, flow meters, etc.				$ -
Supplies		636		636
Phone / Fax / Copies		37		37
Total Direct Costs		673		**$ 673**
Total Estimated Cost			Total	**$ 104,098.00**
	TASKS	$ 104,098		

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Ulrich, G. W. 1986. The larvae and pupae of *Helichus suturalis* Leconte and *Helichus productus* Leconte (Coleoptera: Dryopidae). The Coleopterist Bulletin 40:325-334.

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BIO-WEST, Inc. Funding Application

Edwards Aquifer Habitat Conservation Plan (EAHCP) Refugia

U.S. Fish and Wildlife Service
Fish and Aquatic Conservation

Fish and Wildlife Management Assistance
Catalog of Federal Domestic Assistance (CFDA) Number: 15.608

Project Summary

In compliance with the Edwards Aquifer Habitat Conservation Plan (EAHCP), the U.S. Fish and Wildlife Service has contracted with the Edwards Aquifer Authority (Contract # 16-822-HCP) to provide a series of refugia, with back-up populations, to preserve the capacity for imperiled species to be re-established in the event of the loss of population due to a catastrophic event. Covered species include endangered fountain darter *Etheostoma fonticola*, endangered Comal Springs riffle beetle *Heterelmis comalensis*, endangered (possibly extinct) 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*, threatened San Marcos salamander *Eurycea nana*, petitioned Edwards Aquifer diving beetle *Haideoporus texanus*, petitioned Comal Springs salamander *Eurycea* sp., and petitioned Texas troglobitic water slater *Lirceolus smithii*. The objective of the prime contract is to house and protect adequate populations of these covered species and to conduct research activities to expand knowledge of their habitat requirements, biology, life histories, and effective reintroduction techniques. This funding opportunity specifically addresses research activities for the endangered Comal Springs dryopid beetle and the endangered Peck's cave amphipod and supporting services, as needed, for the overall EAHCP refugia program.

Statement of Need:
There are still several substantial questions and issues associated with many of these EAHCP taxa which currently impede the ability of resource managers to maintain captive populations. Research is needed to better understand juvenile development and maturation of Peck's cave amphipods and Comal Springs dryopid beetles. The USFWS San Marcos Aquatic Resources Center (SMARC) has held Comal Springs dryopid beetles and Peck's cave amphipods in captivity but has experienced difficulties in refugium establishment. Information is needed to increase and survival of amphipods and beetle pupation successfully into adults. A better understanding of life history and the environmental requirements of both species would improve the EAHCP funded refugia.

Project Goals and Objectives:

Peck's cave amphipods

Peck's cave amphipods can be held in captivity, but SMARC has experienced difficulties in refugium establishment with reduced survival of amphipods likely due to cannibalism. Additionally, very little is known about life history and the environmental requirements of this subterranean species. *Stygobromus pecki* is a federally endangered species (USFWS 1997) that is adapted to subterranean habitats associate with two Edwards Aquifer springs (Comal and Hueco springs). *Stygobromus pecki* belongs to a rather speciose genus with ≈140 described species; all of which are subterranean and found primarily in North America (Holsinger 1994). Three *Stygobromus* species (*S. russelli, S. bifurcatus, and S. flagellatus*) are known to occur sympatrically with *S. pecki*. Because little is known about the life history and development of any of these species, it is essentially impossible to determine species of juvenile *Stygobromus,* which is problematic for bio-monitoring efforts and other research projects.

Objectives are to determine:

1. how many molts must occur before it becomes possible to distinguish individuals from other *Stygobromus* species and better understand the morphology of each developmental stage,
2. how many molts must occur before sexual maturity is reached,
3. how many broods a female can produce in a year and how frequently and what typical brood size is,
4. and to better understand sexual dimorphism for the purpose of creating individual breeding pairs.

Comal Springs dryopid beetles

Stygoparnus comalensis is a federally endangered species (USFWS 1997) that is adapted to subterranean habitat associate with three perennial Edwards Aquifer springs (Comal, Fern Bank, and San Marcos springs). *Stygoparnus comalensis* is characterized by having vestigial eyes, lacking pigment, and wingless adults. *Stygoparnus comalensis* is also the type species of the genus *Stygoparnus* which is a monotypic genus with *S. comalensis* being the only species (Barr and Spangler 1992). Therefore, the conservation of *S. comalensis* should be considered particularly important as there are no related lineages found anywhere else on earth. Study of this species is also complicated by the fact that there is likely no suitable surrogate species.

Objectives are to determine:

1. conditions that contribute to the production of eggs,
2. where and how eggs are deposited and egg size and morphology,
3. how long eggs incubate before hatching,
4. how many larval instars there are and the rate of larval development,
5. the morphology of larval instars,
6. factors that contribute to pupation,
7. and the overall life span.

Project Activities, Methods and Timetable:

Peck's cave amphipods

Peck's cave amphipods will be collected by hand using aquarium nets in upwellings and from drift nets set in spring runs and Landa Lake in the Comal Springs system, Comal County, Texas. These amphipods will be transported back to the SMARC and maintained in custom-built aquatic holding units and fed flake fish feeds and dried sycamore leaves. They will be held in quarantine for 14 days before commencing research observations so acclimation to captivity can occur. Periodically, gravid females will be isolated in flow-through containers and observed until eggs hatch and neonates emerge from the females' marsupia. These young will be isolated in individual flow-through chambers and used for observations and measurements to determine growth curve and molts. Measurements will be taken monthly. Photos will be taken to examine and document morphological characterizations of each life-history stage.

Deliverables for 2017:

1. Growth rate curve in respect to time and molts.
2. Quantification of the number of molts and the duration between molts prior to reaching sexual maturity.
3. Estimate of life span and number of molts as adults.
4. Morphological characterization of each life-history stage.

Schedule:

March 2017:	Proposal submitted to Edwards Aquifer Authority for approval.
April 2017:	Collect wild *S. pecki* to supplement individuals already housed at SMARC for studies.
May-October 2017:	Document amphipod growth, molts, and morphological characteristics to the degree practicable. It is anticipated that longer than six months may be necessary to fully document development into adults.
November 2017:	Draft Interim report of project progress.
December 2017:	Finalize and submit Interim project report.

Comal Springs dryopid beetles

Adults will be collected from cotton lures and drift nets set in Sessoms Creek and Landa Lake. Cotton lures will be set and allowed to develop biofilms for four weeks before being retrieved and checked for invertebrates following Gibson et al., (2008) and EAA 2004-2016. Adults will be removed and transported back to the SMARC where they will be maintained within custom aquatic holding units and fed detrital material and matured biofilms on colonized cotton lures. Adults will be held in quarantine for 14 days before entering into research observations so they can acclimate to captivity. Sex determination will be attempted by gently placing adults under a dissecting microscope and taking pictures of the posterior ventral surfaces, looking for evidence of sexually dimorphic external features. If this is unsuccessful, then a group of adults will be held in a small clear container and observed for pairs displaying mating behavior (amplexus). Subsequently, adults will be held in flow-through containers having both aquatic and terrestrial habitat provided. Each container will have a single male-female pair and a variety of substrates (cotton cloth, wood, leaves, and nylon mesh). These substrates will be checked weekly and egg

laden substrates will be removed and reared in separate flow-through containers. If numbers suffice, analysis of variance (ANOVA) will be run to determine if there is a statistical preference for substrate(s) on which eggs are laid. The eggs will be checked weekly and first instar larvae removed, measured (total length, and head capsule width), and reared in separate containers. Larvae will be measured every 2 to 4 weeks and followed through development of all instars. Observations of aquatic and terrestrial substrate utilized by the larvae will be recorded daily/weekly and ANOVA test run on preferences if warranted.

Deliverables for 2017:

1) Insight into conditions that contribute to the production of eggs and the site of egg deposition,
2) egg incubation duration and rate of hatching success,
3) larval growth rate including the number and age or size for each instar,
4) morphological development for each larval instar, and
5) if observable, photo diagrams of male/female sexually dimorphic external features.

Schedule:

March 2017:	Proposal submitted to Edwards Aquifer Authority for approval.
March-August 2017:	Collect wild *S. comalensis* to supplement those housed at SMARC to potentially be used for in study.
April-October 2017:	Document adult fecundity, note habitat utilization, and analyze photographs for evidence of sexually dimorphic external features. Document egg gestation and survival and study larvae development to the degree practicable as it is anticipated that longer than seven months may be necessary to fully document development into adults.
November 2017:	Draft Interim report of project progress.
December 2017:	Finalize and submit Interim project report.

Stakeholder Coordination / Involvement:

BIO-WEST will be working jointly with SMARC in fulfilling this contract for the Edwards Aquifer Authority. BIO-WEST has worked closely with the Edwards Aquifer Authority for nearly 20 years and is well versed at conducting project activities, dissemination project results, and incorporating our results and reports into the overall framework of the Edwards Aquifer Habitat Conservation Plan.

Project Monitoring and Evaluation:

BIO-WEST will be led by Edmund L. Oborny, Jr. who will serve as the project principal. McLean Worsham will serve as BIO-WEST's principal investigator. BIO-WEST will work under the direction of Dr. Lindsay Campbell (Refugia Managing Biologist) and Mr. Randy Gibson (Regional Invertebrate Zoologist) at SMARC who will continually be evaluating progress regarding project activities, schedule, deliverables and budget. Dr. Ken Ostrand (SMARC Center Director) will also be overseeing BIO-WEST's adherence to meeting deadlines and producing deliverables on time and within budget.

2017 SCOPE OF WORK

The U.S. Fish and Wildlife Service (USFWS) San Marcos Aquatic Resources Center (SMARC), Uvalde National Fish Hatchery (UNFH), and BIO-WEST Incorporated (BIO-WEST) will provide refugia, salvage, reintroduction, and monitoring services in fulfillment of the Refugia Contract (Contract # 16-822-HCP) between the Edwards Aquifer Authority (EAA) and the USFWS. The established prime contract term is effective January 1, 2017 through March 1, 2029. Although it is recognized that this funding agreement is for 2017, it is anticipated that this cooperative agreement between BIO-WEST and USFWS will adhere to the same Prime contract period. This 2017 scope of work and associated cost estimate have been developed per the requirements of contract number 16-822-HCP with specific tasks and costs specific to the approved 2017 refugia workplan.

Task 1: Refugia Operations

Activities under this task are the sole responsibility of USFWS. BIO-WEST is available to assist at the discretion of USFWS pending written authorization and cost negotiation.

Task 2. Research

Research Plan for term of contract:

BIO-WEST will assist USFWS in preparing a draft Research Plan for the term of the contract to be submitted to EAA. Upon USFWS completion of the final Draft, BIO-WEST will provide a thorough review and comment.

Research Plan for 2017:

BIO-WEST's main task in 2017 will be working in conjunction with USFWS to execute a research plan specific to Peck's Cave amphipods (*Stygobromus pecki*) and Comal Springs dryopid beetles (*Stygoparnus comalensis*). Two research projects are proposed to be initiated in 2017: 1) Juvenile development and maturation of Peck's Cave amphipods, and 2) Larval development of Comal Springs dryopid beetles.

BIO-WEST Activities associated with these projects include:

1) Research Plan development
2) Conduct Research
3) Data Reduction, Analysis, and Reporting

All activities will be conducted in conjunction with USFWS. BIO-WEST will provide a Co-Principal Investigator and aquatic technician to support 2017 Refugia research.

Task 3. Species Propagation and Husbandry

BIO-WEST will assist USFWS in the development and refinement of Species specific Propagation plans (SOPs) for animal rearing and captive propagation. Existing draft SOPs for the fountain darter, Texas wild rice, San Marcos salamander, Texas blind salamander, Comal springs salamander, Peck's Cave amphipod, and Comal Springs riffle beetle will be updated to reflect new protocols that are instituted for each species throughout the year. Additionally, BIO-WEST will assist USFWS with development of SOPs for the Comal Springs dryopid beetle, Edwards Aquifer diving beetle, and Texas troglobitic water slater.

Finally, BIO-WEST will review and provide comments on the draft Captive Propagation Plans to be developed by USFWS for the Comal Springs rifle beetle, Peck's cave amphipod, and Texas blind salamander during 2017.

Task 4. Species Reintroduction

BIO-WEST will assist USFWS in the preparation of a draft Reintroduction Strategy for all Covered Species during 2017.

Task 5. Reporting

BIO-WEST will assist USFWS in the preparation, review and comment for the following refugia related documentation:

- Species specific Propagation plans (SOPs)
- Species specific Reintroduction plans
- 2017 EAHCP Annual Program reporting
- Summaries of any data analyses and research stand-alone documents
- Monthly electronic reports to HCP Chief Science Officer

Task 6. Meetings and Presentations

At the direction of the USFWS and/or EAA Chief Science Officer, BIO-WEST will prepare for and participate in the following refugia related meetings:

- EAA Board
  - End of year report
  - Present research results
- Implementing Committee
  - End of year summary
- Stakeholder Committee
  - End of year summary
- Science Committee
  - Methods for research projects
  - Present research results

DELIVERABLES

Monthly activities report with invoices	Monthly (by the 7th)
Research Study Plans for Task 2	February 28, 2017
Interim Research Reports	December 15, 2017
EAA HCP Annual report summaries and sections	December 1, 2017

BUDGET **SUMMARY**

Task 1:	REFUGIA OPERATIONS	none
Task 2:	RESEARCH	$198,284
Task 3:	SPECIES PROPAGATION HUSBANDRY	$20,000
Task 4:	SPECIES REINTRODUCTION	$20,000
Task 5:	REPORTING	$50,000
Task 6:	MEETINGS AND PRESENTATIONS	$10,000
	TOTAL	**$298,284**

Budget Form:

BIO-WEST (01/01/2017)
DETAILED COST BREAKDOWN - EAA Refugia - 2017

LABOR:

Position	Rate	TASK 1 - Refugia Operations	TASK 2 - Research	TASK 3 - Species Propagation Husbandry	TASK 4 - Species Reintroduction	TASK 5 - Reporting	TASK 6 - Meetings and Presentations	TOTALS Total Hours	TOTALS Cost
Project Principal	155.87		96	64	64	128	48	400	$ 62,348.00
Senior Researcher	138.16							0	$ -
Senior Biologist / Project Manager	119.75							0	$ -
Senior Ecologist	114.62							0	$ -
Biologist I	109.48							0	$ -
Ecologist I	102.65							0	$ -
GIS / Senior Editor	97.60							0	$ -
Ecologist / Statistician	94.09							0	$ -
Technical Editor	86.63							0	$ -
Biologist II	75.26		1,236	98	98	296	24	1,752	$ 131,855.52
Senior Administrative	66.72		12	6	6	12	2	38	$ 2,535.36
Biologist III	65.01							0	$ -
Technician I	58.16							0	$ -
Technician II	51.31		1,708	36	36	128		1,908	$ 97,899.48
Total Labor		0	3,052	204	204	564	74	4,098	**294,638.36**
TRAVEL									
Per diem: Hotel and Meals							150		$ 150.00
Mileage ($.535 per mile)	0.535		1,000	500	500	500	500	3,000	$ 1,605.00
Total Travel									**$ 1,755.00**
DIRECT COSTS:									
Equipment: boats, water quality sondes, flow meters, etc.									$ -
Supplies			1,250	84	84	86	111		$ 1,615.00
Phone / Fax / Copies			75	50	50	50	50		$ 275.00
Total Direct Costs		0	1,325	134	134	136	161		**$ 1,890.00**
Total Estimated Cost								Total	**$ 298,283.36**
	TASKS	$0	$198,283	$20,000	$20,000	$50,000	$10,000