2018 EAHCP Refugia Work Plan

Introduction

The U.S. Fish and Wildlife Service (USFWS) San Marcos Aquatic Resources Center (SMARC) and 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.

This annual work plan and associated cost estimate have been developed per the requirements of contract number 16-822-HCP for the Implementation of the Refugia Program under the EAHCP. The tasks and subtasks that follow provide the details for the services to be performed in 2018, which provide for the maintenance of a refugia population of the Covered Species (Table 1) including the salvage, propagation, and restocking of the species, if species-specific habitat triggers occur and species are extirpated.

Table 1: Eleven species identified in the EAHCP and listed for coverage under the ITP.

Common Name	Scientific Name	ESA Status
Fountain darter	*Etheostoma fonticola*	Endangered
Comal Springs riffle beetle	*Heterelmis comalensis*	Endangered
San Marcos gambusia	*Gambusia georgei*	Endangered*
Comal Springs dryopid beetle	*Stygoparnus comalensis*	Endangered
Peck's Cave amphipod	*Stygobromus pecki*	Endangered
Texas wild-rice	*Zizania texana*	Endangered
Texas blind salamander	*Eurycea rathbuni*	Endangered
San Marcos salamander	*Eurycea nana*	Threatened
Edwards Aquifer diving beetle	*Haideoporus texanus*	Petitioned
Comal Springs salamander	*Eurycea* sp.	Petitioned
Texas troglobitic water slater	*Lirceolus smithii*	Petitioned

*The San Marcos gambusia was last collected in the wild in 1983, and may already be extinct.

Long-term Objective

A series of refugia held at the SMARC and UNFH will preserve the capacity for the Covered Species to be re-established at the Comal and San Marcos rivers in the event of the loss of population due to a catastrophic event such as the loss of spring flow or a chemical spill.

Background: Section 5.1.1 of the EAHCP requires the EAA to provide a series of refugia, with back-up populations, to preserve the capacity for these species to be re-established in the event of the loss of population due to a catastrophic event.

The concept of refugia is to house and protect adequate populations of the Covered Species and to conduct research activities to expand knowledge of their habitat requirements, biology, life histories, and effective reintroduction techniques. Actions and funding contained within this work plan will be limited to the Covered Species listed in the EAHCP and those associated

species that have significant impact on the Covered Species such as predators, competitors, pathogens, parasites, food, cover, and shelter.

2018 Assumptions

As work plans are developed almost a year prior to implementation, it is possible that methods described herein may be contingent on the status of the current year's activities or authorization from the HCP process.

- Target numbers for the standing and refugia stocks to be housed at both the UNFH and SMARC are established by the USFWS-EAA Refugia Contract (Contract # 16-822-HCP).
- Species capture and mortality rates will be similar to historic values.
- Mortality rates of specimens held in captivity will be similar to historic values.
- Target species collection numbers from the 2017 work plan are reached.
- Construction and renovation will not be interrupted or unexpectedly delayed due to weather, equipment, procurement related delays, or other unforeseen issues.
- Staffs remain employed at the two Service facilities throughout the performance period.

Target for 2018 (Deliverables and Methods by Task):

Task 1. Refugia Operations

Standing Stocks The standing stocks at the SMARC and UNFH will be considered standing stocks under the executed contract (Contract # 16-822-HCP) and will be held in Service facilities until EAA specific Refugia and Quarantine facilities are complete and functional. USFWS staff will take all appropriate steps to collect and maintain standing/refugia stocks at their respective target captive population size in order to provide refugia for all the Covered Species. Table 2 displays the target species numbers.

Table 2. Species target refugia numbers and census.

Species	Standing Stock	Refugia Stock	Salvage Stock	SMARC census (1/1/2018)	Anticipated SMARC census (12/31/2018)	UNFH census (1/1/2018)	Anticipated UNFH census (12/31/2018)
Fountain Darter (Comal)	1000	1000 including specimens within the standing stock	2000	408	400	66	100
Fountain Darter (San Marcos)	1000	1000 including specimens within the standing stock	2500	610	600	147	500
Texas Wild-Rice	430	430 including specimens within the standing stock	1500	240	232	67	121
Texas Blind Salamander	500	500 including specimens within the standing stock	500	47	60	0	15[1]
San Marcos Salamander	500	500 including specimens within the standing stock	500	267	300	180	250
Comal Springs Salamander	500	500 including specimens within the standing stock	500	47	70	4	30
Peck's Cave Amphipod	500	500 including specimens within the standing stock	500	173	250	45	100
Comal Springs Riffle Beetle	500	500 including specimens within the standing stock	500	191	175	51	100
Comal Springs Dryopid Beetle	500	500 including specimens within the standing stock	500	13	*	2	*
Edwards Aquifer Diving Beetle	500	500 including specimens within the standing stock	500	0	*	0	*
Texas Troglobitic Water Slater	500	500 including specimens within the standing stock	500	25	*	0	*

[1]transfer of Texas blind salamanders to UNFH is contingent upon completion of facilities construction and tank system set-up

*catch rates and hatchery survival are uncertain given the rarity of the species

Collection: In 2018, we will collect Covered Species as required to reach and maintain target standing and refugia stock numbers as shown in Table 2. Species collections will be coordinated with other ongoing HCP activities (e.g. Biological Monitoring Program) so that collections for refugia do not impact other efforts adversely. Species specific collections will be carried out through a variety of passive and active collection methods. Prior to collections, Hazard Analysis Critical Control Point (see Appendix A 2017 Work Plan) will be conducted to minimize aquatic

invasive species transfer. Collection efforts will be documented and reported to EAA. Captured specimens will be divided between the SMARC and UNFH facilities in order to ensure redundancy and to expedite the obligation to establish and maintain two refugia populations at separate locations. All species will be held in respective quarantine areas until their health has been assessed. Once it is determined that specimens are free from pathogens, parasites, and invasive species they will be incorporated into the general refugia population. USFWS will share reports, including test results, produced as part of the quarantine process. Species-specific collection plans generally follow those detailed within the 2017 Work Plan; however, collection efforts vary based upon collection and knowledge gained during the 2017 collection efforts. The following sections briefly describe planned 2018 collection, maintenance, and propagation efforts for each species.

Please note that we anticipate that once construction on new buildings is completed (at each facility) collection efforts will be slowed or briefly suspended so that staff can focus on setting up new systems in the buildings and begin moving refugia populations to those systems.

Fountain Darters:

Collection: Fountain darters will be collected primarily using dip nets and SCUBA divers in deeper locations (greater than wading depth) to obtain and maintain target numbers (N = 1,000 per river). Approximately 20% of the fountain darters collected annually succumb to natural mortality. If unusual mortality events occur, they will be thoroughly investigated and summary reports will be conveyed to the EAA as part of the monthly reports. As a result, fish collections will target additional fish so that as individuals perish the remainder within the captive population should not decrease below the target number between collection events. Specimens will be collected along a longitudinal gradient. Approximately equal proportions of fish from upper and lower reaches in the Comal (upper = above Landa Lake dam; lower = below Landa Lake dam) and San Marcos (upper = Spring Lake, Middle = Spring lake dam to Rio Vista dam, lower = below Rio Vista dam to Capes dam) rivers will be collected.

Due to the detection of largemouth bass virus in the Comal fountain darter throughout the Comal River habitat all Comal fountain darters will be maintained in quarantine facilities in consideration of other species located on the two stations. Collection numbers of Comal fountain darters will be reduced and 2018 target census numbers lower because of space limitation until new facilities are built and systems up and running.

Fountain darters will be collected primarily during the spring and fall to minimize thermal stress during capture and transport. As part of quarantine procedures, a subset of fish (N = 60) will sent to Dexter Fish Health Unit or equivalent facility for pathogen (bacteria, virus, and parasite) testing prior to specimen incorporation into the general refugia population following standardized methods outlined within USFWS and AFS-FHS (2016) and AFS-FHS (2005); reports will be provided to EAA.

Maintenance: Water quality (i.e. temperature, pH, dissolved oxygen, total dissolved gasses) will be monitored and recorded weekly. Fountain darters will be fed live foods reared or purchased. Ponds will be utilized to produce zooplankton and amphipods. Ponds will be managed to maintain idealized zooplankton assemblages and densities. Amphipods will be collected from other managed ponds and raceways (see Cantu et al. 2009). Black worms will be purchased when necessary along with other food resources (i.e. blood worms, black worms, brine shrimp,

etc.) if the need arises. Food items are not routinely examined for pathogens. However, if they are suspect and tested for pathogens all diagnostic results will be conveyed to the EAA within monthly reports.

Propagation: Standing and refugia stocks for each river will be maintained to discourage reproduction unless HCP triggers occur. Fish will be maintained by their geographical locations. If reintroduction is warranted, subsets from each geographical location will be communally spawned. Subset groups will be culled to an equal number of progeny prior to release.

Texas wild rice:

Collection: Texas wild rice tillers will be collected from specific San Marcos River reaches, with a break during summer months when wild rice does not fare well due to heat stress (Fig. 1). In 2018 collections for SMARC will target stands that are not already part of the refugia population or require supplementation. Collections for UNFH will continue to build their refugia numbers and representative locations. The refugia populations will reflect the wild populations in both their respective proportion and genetic diversity that was historically documented within San Marcos River (Table 3; Wilson et al. 2016). During tiller collection, the GPS coordinates, area coverage, and depth of the stand or individual plant will be recorded so the exact location of the clone is known. For larger stands, tillers will be collected at the beginning, middle and end of the stand, or every 20% of the stand's total length for the largest stands. Tiller collection will be done by wading and the use SCUBA gear. Texas wild rice seeds from the river will also be collected monthly or when available and stored at both facilities. Seed stocks will be replaced every six months when seeds are available. Please note that during the 2017 Texas wild rice survey no plants were found in Section E I, J, and K. Plants were found in sections G and H.

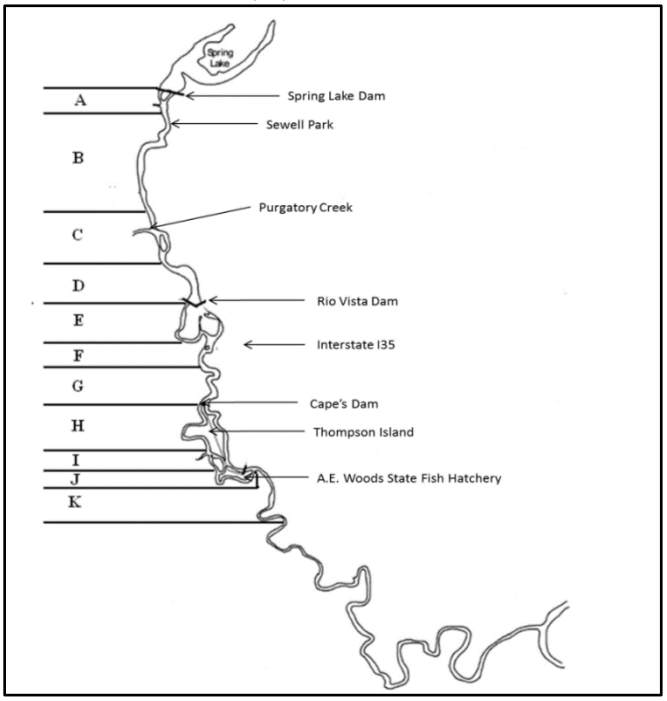

Figure 1 Letters define designated San Marcos River reaches where Texas wild rice is collected for refugia populations.

Maintenance: Once tillers have been successfully rooted they will be tagged and maintained so that their collection location is known.

Propagation: Plants will be maintained so sexual reproduction does not occur within the refugia population, unless HCP triggers occur. If reintroduction is warranted, seeds and tillers from each geographical location will be produced. Plants produced from seeds and tillers would be transplanted back within their original geographic location.

Table 3. The number of Texas wild rice plants needed at the SMARC and UNFH to obtain the total target number of 430. Each San Marcos River reach is denoted by a letter and the proportion of specimens needed per reach is estimated from Wilson et al. (2016). Based on Wilson et al. (2016) no plants will be collected from sections I, L, M (, shaded-out). No plants were observed in sections E, I, J, and K (*) during 2017; these sections will be re-evaluated in 2018. Projected numbers are based on an anticipated mortality of 20% for newly acquired plants and 10% for mature refugia stock**.

River Section	Census Jan 2018	Number of plants targeted in 2018	Anticipated 2018 EOY Census
SMARC			
A	21	10	27
B	107	5	101
C	41	5	41
D	6	5	10
E*	5	0	5
F	25	5	27
G	5	3	7
H	3	3	5
I**	-	-	-
J*	8	0	7
K*	2	0	2
L**	-	-	-
M**	-	-	-
UNFH			
A	11	15	22
B	23	25	41
C	10	15	21
D	10	0	9
E*	0	0	0
F	13	10	20
G	0	5	4
H	0	5	4
I**	-	-	-
J*	0	0	0
K*	0	0	0
L**	-	-	-
M**	-	-	-

Texas blind salamanders:

Collection: Texas blind salamanders will be collected through the use of nets and traps. Traps will be deployed quarterly for approximately 12 consecutive days with traps checked every 2-4 days to collect Texas blind salamander specimens from Primers Fissure, Johnson's well, Rattlesnake cave, and Rattlesnake well (Table 5). To avoid oversampling these habitats, only 1/3 of salamanders observed from each of these locations will be collected during quarterly sampling events. Concurrently, salamanders will also be collected from a driftnet on Diversion Springs in Spring Lake fished continuously throughout the year. Periodically collections will be made from Spring Lake Outflow with a driftnet. Specimens from these two sites will all be kept, given the assumption that any Texas blind salamander leaving a spring orifice that enters a stream or lake environment will ultimately succumb to predation. These sites will be checked for specimens up to three times per week where applicable. All specimens will be transported live and maintained in the SMARC and UNFH refugia. When not being checked by Texas State staff, we will also check nets on Sessom Creek and Texas State Artesian Well; when these nets are being check by Texas State staff live Texas blind salamanders are transferred to SMARC according to their permits.

Maintenance: Specimens will be maintained by collection location. As part of quarantine, all salamanders of each species will be non-lethally cotton swabbed. These 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 testing will occur in batches where groups of five swabs will be pooled for analysis as opposed to individual analysis. Duplicate individual swabs will be retained in case further testing is warranted. All salamanders will be held in quarantine for at least 30 days and until test results have returned. Chytrid (Bd) fungus has caused mortalities in amphibian species; however, some species appear to have innate immunity. Previous tests of wild caught salamanders at SMARC (both Texas Blind and San Marcos) have almost always tested positive for Bd. 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. Salamander tank and system maintenance such as acid washing and system sterilization will occur annually or as needed to ensure proper system function. Water quality will be monitored and recorded weekly. Salamanders will be fed live foods reared or purchased. Ponds will be utilized to produce amphipods. Amphipods will be collected from other managed ponds and raceways (see Cantu et al. 2009). Black worms will be purchased when necessary along with other food resources (i.e. blood worms, brine shrimp, etc.) if the need arises.

Propagation: Standing and refugia stocks will be maintained to encourage reproduction. Salamanders will be marked by their geographical locations. All progeny will be maintained separately by generations. If reintroduction is warranted, an attempt will be made to produce offspring from each geographical location.

San Marcos salamanders:

Collection: San Marcos salamanders will be collected up to quarterly from below Spring Lake dam and with SCUBA teams in Spring Lake (Table 5). The drift net on Diversion Springs will

be checked routinely and specimens will be kept from this location. Collection efforts will be coordinated with the HCP Biological Monitoring Program. All specimens will be transported live and maintained in the SMARC and UNFH refugia. Approximately 30% of the San Marcos salamanders collected annually succumb to natural mortality. As a result, salamander collections will target additional specimens so that as individuals perish the remainder within the captive population should not decrease below the target number between collection events.

Maintenance: As part of quarantine, all salamanders of each species will be non-lethally cotton swabbed. These 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 testing will occur in batches where groups of five swabs will be pooled for analysis as opposed to individual analysis. Duplicate individual swabs will be retained in case further testing is warranted. All salamanders will be held in quarantine for at least 30 days and until test results have returned. Chytrid (Bd) fungus has caused mortalities in amphibian species; however, some species appear to have innate immunity. Previous tests of wild caught salamanders at SMARC (both Texas Blind and San Marcos) have almost always tested positive for Bd. 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. Salamander tank and system maintenance such as acid washing and system sterilization will occur annually or as needed to ensure proper system function. Water quality will be monitored and recorded weekly. Salamanders will be fed live foods reared or purchased. Ponds will be utilized to produce amphipods. Amphipods will be collected from other managed ponds and raceways (see Cantu et al. 2009). Black worms will be purchased when necessary along with other food resources (i.e. blood worms, brine shrimp, etc.) if the need arises.

Propagation: Standing and refugia stocks will be maintained to discourage reproduction. If reintroduction is warranted, pairwise mating will be employed to produce offspring. Stocking will occur once juveniles have reached 30 mm total length.

***Comal Springs salamanders*:**

Collection: Comal Springs salamanders will be collected up to quarterly from Comal Spring runs 1-3 and Spring Island and surrounding areas (Table 5). Close coordination with the HCP biological monitoring program will take place to ensure that to the degree practicable, refugia collections do not overlap with specific HCP long-term monitoring locales. In the event overlap of sampling areas is unavoidable, Comal salamanders for refugia will be collected at a rate of no more than 10% of salamanders observed in those specific locales per daily sampling trip. A SCUBA team will be used for a portion of these collection efforts as necessary. Annual natural mortality will be recorded.

Maintenance: As part of quarantine, all salamanders of each species will be non-lethally cotton swabbed. These 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 testing will occur in batches where groups of five swabs will be pooled for analysis as opposed to individual

analysis. Duplicate individual swabs will be retained in case further testing is warranted. All salamanders will be held in quarantine for at least 30 days and until test results have returned. Chytrid (Bd) fungus has caused mortalities in amphibian species; however, some species appear to have innate immunity. Previous tests of wild caught salamanders at SMARC (both Texas Blind and San Marcos) have almost always tested positive for Bd. 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. Salamander tank and system maintenance such as acid washing and system sterilization will occur annually or as needed to ensure proper system function. Water quality will be monitored and recorded weekly. Salamanders will be fed live foods reared or purchased. Ponds will be utilized to produce amphipods. Amphipods will be collected from other managed ponds and raceways (see Cantu et al. 2009). Black worms will be purchased when necessary along with other food resources (i.e. blood worms, brine shrimp, etc.) if the need arises.

Propagation: Standing and refugia stocks will be maintained to discourage reproduction. If reintroduction is warranted, pairwise mating will be employed to produce offspring. Stocking will occur once juveniles have reached 30 mm total length.

Comal Springs riffle beetle:

Collection: Comal Spring riffle beetle collection will be reduced from the number of collections that occurred in 2017 with up to six targeted events in 2018 (Table 5). The reduced target census numbers in Table 2 reflect this reduction in effort. No collections will occur during months when HCP monitoring is scheduled. Riffle beetles will be collected with cotton lures. Cotton lures will be deployed in a variety of locations (Spring Runs 1, 2, 3, N = 5-15 lures per spring run; western shore of Landa Lake, N = 5 lures; Spring Island and associated Spring Lake habitats N = 15-20 lures) following EAHCP standard operating procedures (Hall 2016). Coordination with the HCP biological monitoring program will take place to ensure that to the degree practicable, refugia collections do not overlap with specific HCP long-term monitoring locales. In the event overlap of specific routine sampling locations is unavoidable, Comal Springs riffle beetles for refugia will be collected at a rate of no more than 25% of beetles observed per lure in those specific locales per daily sampling trip. Lures will be allowed to mature biofilms for four weeks. Riffle beetles will be collected during the fourth week and lures will be removed. Approximately 50% of the Comal Springs riffle beetles collected annually succumb to natural mortality. As a result, invertebrate collections will target additional specimens so that as individuals perish the remainder within the captive population should not decrease below the target number between collection events.

Maintenance: Specimens will not be maintained by collection location. Comal Springs riffle beetles will be maintained within custom built aquatic holding units and fed detrital matter and matured biofilms colonized on cotton lures.

Propagation: Propagation methods for this species are being developed.

Peck's Cave amphipod:

Collection: Peck's Cave amphipod collection will occur up to five times annually (Table 5).

Adult Peck's cave amphipods will be collected through the use of drift nets and hand collection. Drift nets will be deployed in a variety of locations (Spring Run 3, N = 2; Spring Island and associated Spring Lake habitats, hand collection). Approximately 50% of the Peck's Cave amphipod collected annually succumb to natural mortality. As a result, invertebrate collections will target additional specimens so that as individuals perish the remainder within the captive population should not decrease below the target number between collection events.

Maintenance: Specimens will not be maintained by collection location. Peck's Cave amphipods will be maintained within custom built aquatic holding units and fed commercial flake fish feeds.

Propagation: Propagation methods for this species are being developed as part of standard refugia operations.

Comal Springs dryopid beetle:

Collection: Comal Spring dryopid beetle collection will occur quarterly (Table 5). Dryopid beetles will be collected through the use of cotton lures concurrently with Comal Spring riffle beetle lure collections. In addition to cotton lures, wooden dowel rods will concurrently be tested as a lure technique for dryopid beetles. All lures (cotton or wooden) will be allowed to mature biofilms for four weeks. Dryopid beetles will be collected during the fourth week and lures will be removed. Bottle traps and experimental nets will also be deployed into Panther Canyon Well during April and September. These will be checked weekly for a month. We have ceased collection efforts of lures in Sessom Creek as these were not fruitful during 2017; a new design for Sessom Creek might be revisited at a later date.

Maintenance: Specimens will not be maintained by collection location. Comal Spring dryopid beetle will be maintained within custom built aquatic holding units and fed detrital matter and matured biofilms colonized on cotton lures.

Propagation: Propagation methods for this species are being developed as part of normal refugia operations and research projects.

Edwards Aquifer diving beetle:

Collection: Drift nets will be used to collect Edwards Aquifer diving beetle (Table 5). Drift nets will be set at a variety of locations where the species has been collected in the past (Sessoms Creek N = 1; Texas State University Artesian Well N = 1; and Diversion Springs N = 1). Drift nets will be deployed and checked weekly over the course of the year.

Maintenance: Specimens will not be maintained by collection location. Captured specimens will be transferred to the SMARC and housed in custom made aquatic holding systems. Initially the species will be fed small invertebrates (e.g. ostracods), given they are predators.

Propagation: Propagation methods for this species are to be determined and will be conducted as part of normal refugia operations.

Texas troglobitic water slater:

Collection: Drift nets will be used to collect the Texas troglobitic water slater (Table 5). We intend to set drift nets (Sessoms Creek; N = 1, Texas State University Artesian Well N = 1; and Diversion Springs N = 1 to 2) weekly as necessary. Drift nets will be checked weekly over the course of the year. We will also employ new lure designs developed for well and cave environments. The lures will be allowed to mature a biofilm for four to six weeks. The success or failure of these trials will be recorded and assessed.

Maintenance: Captured specimens will be transferred to the SMARC and housed in custom made aquatic holding systems. Initially the species will be fed detrital matter and matured biofilms colonized on cotton lures.

Propagation: Propagation methods for this species are to be determined and will be conducted as part of normal refugia operations.

Table 5. A tentative schedule for all species sampling during 2018. Collections listed here are subject to change due to extenuating circumstances such as weather, coordination with external partners, and completion of construction projects. EEA and partners will be notified of sampling dates as they become known or changed. Not included in this table are Texas wild rice seed collections given the unpredictable nature of sexual reproduction.

Edward's Aquifer Species Collection Plan 2018			
Date (month)	**Interval**	**Location**	**Target Species**
Continuous	Check nets T and F every week	Diversion Springs	Texas Blind salamander, San Marcos salamander, Edward's Aquifer diving beetle, and troglobitic water slater
January	Check every T & F for 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
January	Beginning of month, check and reset lures	Spring Runs	Comal Springs riffle beetle, Comal Springs dryopid beetle
February	Check every T & F for 2 consecutive weeks	Primer's Fissure & Johnson's Well	Texas blind salamander
February	Beginning of month, check and reset lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Spring dryopid beetle
February	1-day sampling event, hand pick	Landa Lake	Peck's Cave amphipod
February	1-day sampling event	San Marcos River	Texas wild rice
March	1-2 day sampling event	Spring Lake and below dam	San Marcos salamander
March	Beginning of month, retrieve lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle
March	1-day sampling event	San Marcos River	Texas wild rice
March	1-day sampling event, hand pick	Landa Lake	Peck's Cave amphipod

April	Check every T & F for 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
April	1-day sampling event	San Marcos River	Texas wild rice
April	Throughout month	Panther Canyon	Comal Springs dryopid beetle
April/May	Reset lures after biomonitoring	Spring rungs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle
May	Check every T & F for 2 consecutive weeks	Primer's Fissure & Johnson's Well	Texas blind salamander
May	1-2 day sampling event	Comal Springs	Comal Springs salamander
May	1-day sampling event	San Marcos River	Texas wild rice
May	1-day sampling event, hand pick	Landa Lake	Peck’s Cave amphipod
May	Check lures (4 weeks after set) and reset	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle
June	4-day sampling event	San Marcos River and Comal River	Fountain darters
June	Check and retrieve lures	Spring runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle
July	Check every T & F for 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
August	Check every T & F for 2 consecutive weeks	Primer's Fissure & Johnson's Well	Texas blind salamander
August	Beginning of month set lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle

August	1-day sampling event, hand pick	Landa Lake	Peck's Cave amphipod
September	1-2 day sampling event	Spring Lake and below dam	San Marcos salamander
September	Beginning of month, check and remove lures	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle
September	Throughout month	Panther Canyon	Comal Springs dryopid beetle
October	Check every T & F for 2 consecutive weeks	Rattlesnake Cave & Rattlesnake Well	Texas blind salamander
October	4-day sampling event	San Marcos River and Comal River	Fountain darters
October	1-day sampling event	San Marcos River	Texas wild rice
November	Check every T & F for 2 consecutive weeks	Primer's Fissure & Johnson's Well	Texas blind salamander
November	Beginning of month set lures, if needed	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle
November	1-day sampling event, hand pick	Landa Lake	Peck's Cave amphipod
November	1-day sampling event	San Marcos River	Texas wild rice
November	1-2 day sampling event	Comal Springs	Comal Springs salamander
December	Beginning of month, check and reset lures, if needed	Spring Runs, Landa Lake	Comal Springs riffle beetle, Comal Springs dryopid beetle
December	1-day sampling event	San Marcos River	Texas wild rice

Refugium Stocks:

Collection: Species collections will be ongoing until refugia stocks target numbers are obtained as shown in Table 2.

Maintenance: Maintenance will be conducted in a similar manner described for standing stocks.

Propagation: Texas blind salamander, Comal Springs riffle beetle, Comal Springs dryopid beetle, Edwards Aquifer diving beetle, and Texas troglobitic water slater may be propagated to further advance culture techniques. Propagation for stocking is not anticipated during 2018.

Salvage Stocks:

Collection: If HCP species-specific salvage triggers are reached in consultation with the EAA, the SMARC will accommodate salvaged organisms no more than two times during the 12-year period. If triggers for multiple species are reached simultaneously species collections during salvage operations will be prioritized based upon the perceived species-specific effect of reduced river and spring flow and habitat degradation (i.e. EAHCP triggers). Those species that are river obligate species (e.g. fountain darter and Texas wild rice) or that occupy spring orifice and interstitial ground water habitats (e.g. San Marcos and Comal Springs salamander, Peck's Cave amphipod, Comal Springs dryopid beetle) as opposed to those that reside solely within the aquifer (e.g. Edwards Aquifer diving beetle, Texas troglobitic water slater and Texas blind salamander) are presumed to be affected first as flows decrease.

Maintenance: Organisms collected during salvage operations would be maintained at the SMARC for a limited duration (up to one-year) or until their disposition was determined. Research may be suspended or terminated if space is required for salvaged organisms. Research may also be suspended if personnel are directed to collection and maintain salvage stocks.

Propagation: Likewise, production of species would be limited to no more than two times during the 12-year period once species extirpation is determined. Species produced at the SMARC would be held for a limited time (up to one year) or less if stocking is required. Research activities may be suspended or terminated if space is required to house cultured species. Research may also be suspended if personnel are directed to reproduce, maintain, or stock salvage stocks or standing stock progeny.

Construction/Renovation/Infrastructure/Facility:

Construction on the SMARC Refugia and Quarantine buildings will continue into 2018 with anticipated completion during summer 2018. SMARC staff inspector will continue weekly reports until construction completion.

SMARC staff will install tanks upon the construction completion. After systems are set up, covered species will be moved into the spaces.

The renovations at UNFH will be put out for contractor bids in early 2018. After the contract is

awarded construction will commence. It is anticipated that construction at UNFH will be completed by December 2018. UNFH staff will install tanks upon the construction completion. After systems are set up, covered species will be moved into the renovated spaces.

After construction is complete (at both sites) the SMARC Center Director will develop and maintain a list of warranty problems during the 1-year warranty period, forwarding items, as they occur, to the Contracting Officer (CO) and the USFWS Project Manager (COR).

As detailed within the EAA contract with the USFWS (Contract No. 16-822-HCP) all invoices from the USFWS to the EAA for the construction services shall be billed on the last business day of the month and sent monthly and shall provide an itemization of the expenses incurred and all supporting documentation.

All reasonable and practical security measures will be instituted by SMARC and UNFH staff to safeguard EAA refugia facilities, equipment, and species.

Anticipated Equipment Purchases 2018 not including construction and renovation materials:

U.S. Fish and Wildlife Service

	Task	Equipment	Quantity	Cost/Unit	Total	Total Task Budget Amount
1	Refugia Operations					$404,539
	SMARC Refugia & Quarantine bldg.					
		Fiberglass tanks	30	$ 3,000	$ 90,000	
		35 ton chiller	1		$ 72,532	
	UNFH Renovation Refugia & Quarantine					
		Fiberglass tanks	30	$ 3,000	$ 90,000	
		1 HP Chiller Units	9	$ 6,600	$ 59,400	
		35 ton chiller	1		$ 75,000	
		Generator	1		$ 17,607	
2	Research	Tanks			$ 1,000	$17,102
		PVC/Fittings/Hose			$ 7,000	
		Cameras/Scope/Software			$ 5,000	
		Misc. Supplies			$ 4,102	
3	Species Propagation and	N/A				$0
4	Species Reintroduction	N/A				$0
5	Reporting	N/A				$0
6	Meetings and Presentations	N/A				$0
Total					**$ 421,641**	

Staffing/Labor/Personnel:

The Supervisory Fish Biologists (SFBs) at both the SMARC and UNFH will continue in their duties including, but not limited to: supervising, mentoring, and training lower-graded employees, authorize purchases, oversee facility maintenance and repair, develop and implement

budgets, organize and maintain outreach materials and activities that relate to all contract activities. The SFBs will manage and coordinate propagation, culture, and field activities related to the refugia. The SFBs are expected to provide proper and efficient use of facilities and staff resources. The SFBs will work with the Center Director to ensure that contractual obligations are met in a timely manner. In coordination with the Center Director, they will prepare all the required written materials required for the reimbursable agreement reporting. Likewise, the SFBs will also prepare oral presentations to be used as briefing statements, outreach presentations, internal reports, work summaries, and technical presentations at professional meetings. The two SFBs will continue to work and communicate regularly with partners, Service personnel and other researchers to effectively meet Service and reimbursable agreement goals.

Under the management of a lead supervisory biologist at both facilities, it is expected that the three Biological Science Technicians will continue to assist with the collection, daily upkeep, maintenance, and propagation efforts for the nine species at the SMARC and UNFH. This includes maintaining experimental and culture production systems, keeping records along with entering, filing, and collating data. The incumbents will also generate basic summary statistics and graphic analyses of data and document program accomplishments through the composition of Standard Operating Procedures (SOPs), reports, and manuscripts.

Permitting:
Both the UNFH and SMARC operate under the USFWS Southwest Region's Federal Fish and Wildlife Permit for Native, Endangered, and Threatened Species Recovery (number TE676811-3) and the Texas Parks and Wildlife Scientific Research Permits (UNFH SPR-1015-222, SMARC SPR-0616-153).

Biosecurity:
Both the UNFH and SMARC operate under the SMARC BioSecurity Plan (2014) (Exhibit E of 16-822-HCP). Specimen Collection, Hazard Analysis Critical Control Points, Quarantine, & Specimen Transfer: San Marcos Aquatic Resources Center Standard Operating Procedure.

Task 2. Research

The Research Plan for 2018 will involve a series of activities ranging from 1) continuing and expanding upon on-going species-specific studies for *Stygoparnus comalensis*, *Stygobromus pecki*, and *Heterelmis comalensis*; 2) conducting research specific to captive propagation refinement for San Marcos salamanders; and 3) reexamining invertebrate collection methodologies concurrent with testing new designs. The following section describes the basic components of each of these proposed 2018 activities.

Continuation of Life History Studies:
Project 1:

Title: Life-history study of Comal Springs dryopid beetles (*Stygoparnus comalensis*).

Species: *Stygoparnus comalensis*

Principal/Co-PI: BIO-WEST, input by SMARC staff

Overview: Ongoing research initiated in 2017 is focused on producing eggs and larvae of

S. comalensis, determine where and how eggs are deposited. When successful, larval growth and habitat preferences will be investigated.

Objectives and Methods:

1. Identify sexual dimorphic characters.
2. Determine if eggs are oviposited above or below water.
3. Estimate fecundity (number of eggs per clutch).
4. Estimate incubation duration.
5. Identify larval habitat (submerged or emergent).
6. Begin documentation on larval growth rates.
7. Identify adult response to flow.

Due to the paucity of knowledge related to this species, basic observations are necessary in order to ask more directed questions. Furthermore, study aspects should be intended as non-lethal experiments. Additional collections will be required in order to conduct observations and experiments. An Oblique Plan Apparatus (OPA) was constructed in 2017 and a mating trial was initiated. Continued monitoring of this experiment and modification of the OPA or construction of a more effective monitoring device is anticipated. Construction of a variable flow mesocosom will be necessary for investigating environmental conditions favorable to adults and relevant to oviposition.

Expected Results: Identifiable characters for distinguishing sexes, a better understanding of environmental stimuli related to oviposition, identification of environmental requirements for hatching and larval growth. Documentation of larval development, egg incubations rates, and fecundity.

Project 2:

Title: Life-history study of Peck's Cave Amphipod (*Stygobromus pecki*).

Species: *Stygobromus pecki*

Principal/Co-PI: BIO-WEST, input by SMARC staff

Overview: Ongoing research initiated in 2017 is focused on better tracking of individual growth of known species. Investigation on the size class at which *S. pecki* can be identified and characters for separating immature stages of *S. pecki* from sympatric congeners for various size classes will be investigated. Investigations on the possibility of environmental factors that may influence sex ratios will be initiated.

Objectives and Methods:

1. Estimate how many molts or what size class sexual maturity is reached.
2. Estimate fecundity.
3. Detect differences between immature sympatric congeners.
4. Estimate growth rates.
5. Investigate factors effecting sex ratios.
6. Estimate egg incubation rates.

New collections will be necessary to establish a common garden that can fully support the proposed investigations. Modification and continuation of existing operations will proceed. New mesocosms will be constructed to support treatment subjects for feeding trials.

Expected Results: It is anticipated that estimates of fecundity, egg incubation rates, and early growth rates will be established. The size at which immature stages of *S. pecki* can be distinguished form sympatric congeners and a suite of characters that can be used for separating species will be documented. Insights into how feeding may influence sex ratios may raise new questions regarding growth rates, feeding schedules, and cannibalism.

Project 3:

Title: Continuation of Comal Springs riffle beetle (*Heterelmis comalensis*) life-history study.

Species: *Heterelmis comalensis*
Principal/Co-PI: BIO-WEST, input by SMARC staff

Overview: This project is the continuation and final reporting on Comal Springs riffle beetle (*Heterelmis comalensis*) life history studies started in 2016 with another funding source. The primary goal of the second year of study (2017) was to identify factors contributing to pupation and experimentation is ongoing. In addition, a new investigation on the interaction of flow conditions with food preference will also be initiated.

Objectives and Methods:

1. Continued monitoring of ongoing pupation experiments from 2017.
2. Identify the behavioral response of adults and larvae to varying flow conditions with food resource effects.

The construction of a variable flow variable flow mesocosom as described for Project 1 will be utilized for this species first since test subjects are more readily available and may be used in part as surrogates.

Expected Results: Conclusion of pupation rate investigation and improved information of environmental requirements for successful captive propagation.

Project 4:

Title: San Marcos Salamander propagation refinement

Species: *Eurycea nana*

Principal: Dr. Lindsay Campbell, Kelsey Anderson

Overview: The objective of the proposed study is to determine if reproduction can be reliably triggered in San Marcos salamanders with the non-invasive technique of separation and re-combination. Additionally, we will compare pairwise versus group tank reproduction success. If eggs are produced egg development will be documented.

Objectives and Methods:

Salamanders will be sexed and then separated in different tank systems by sex for at least one month. Next groups of male and females will be placed into the same tank system, but physically separated for two weeks; they will share water and be able to see each other. Salamanders will then be combined into either equal sex-ratio groups (i.e. 4 females/4 males, three replicates) or individual pairs (12 pairs) per tank system (three replicate systems) to initiate mating.

Expected Information gathered:

1. Average time to courtship behavior once combined
2. Average days to oviposition to occur after sexes combines
3. Average clutch size
4. Survival rate to hatch of eggs
5. Document egg developmental stages
6. Test for differences between pairwise vs group mating

Expected Results: The results of the study will be presented as a report to the EAA and potentially submitted as a journal article. If this technique is successful the Culture Propagation Manual for this species will be updated.

Task 3. Species Propagation and Husbandry

Development and refinement of SOPs for animal rearing and captive propagation: Continue to refine SOPs for all species as needed for updates to reflect new protocols that are instituted for each species throughout the year. As new information becomes available about genetic management, further develop draft Captive Propagation Plans for all species.

Task 4. Species Reintroduction

Reintroduction Plan for term of contract:
Further revise the draft Reintroduction Strategy presented in 2017.

Reintroduction Plan for 2018: None

Any anticipated triggers being prepared for: Given current weather predictions, spring flows, and the Edwards Aquafer water level none are anticipated during the 2018 performance period.

Task 5. Reporting

5.1 Species specific Propagation plans (SOPs): Refine throughout year as needed
5.2 Species specific Genetic Management plans: None during 2018
5.3 Species specific Reintroduction plans: Revise draft plan presented in 2017
5.4 2018 EAHCP Annual Program reporting 12/31/2018 – A year-end report of 2018 activities will be provided to the EAA no later than 1/31/2019.
5.5 Program reporting as required by ITP and TPWD. TPWD Scientific Research Permit Report will be conveyed to the EAA July 31, 2018.
5.6 Descriptions and photographs of procedures from collections to restocking – Photographs and documentation of collection and restocking will be included in the monthly report to

the EAA CSO along with the year-end report.

5.7 Summaries of any data analyses, research, or genetic analyses – Research projects and results of collection efforts will be provided to the EAA in the monthly reports, year-end documentation, and stand-alone documents (agreed upon by Center director and HCP CSO).

5.8 Description of terms and conditions of any permits received – As permits are received, their contents will be conveyed to the EAA.

5.9 Monthly electronic reports to HCP CSO: A monthly report of all activities will be provided to the HCP CSO. We anticipate providing the report by the 10th of each month for the previous month's activities.

Task 6. Meetings and Presentations

Planning or coordination meetings:

- Yearly planning meeting with SMARC and UNFH staff

- Public 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

Monitoring:

Monitoring will be conducted through the use of progress reports and site visits to the refugia as well as through collaborative management by the EAHCP CSO.

Budget: Projected 2018 budget.

U.S. Fish and Wildlife Service 2018			
Task		Task Budget Amount	Total Task Budget Amount
1 **Refugia Operations**			**$4,405,316**
SMARC Refugia & Quarantine Bldg.			
*Construction		$1,632,934	
Equipment		$162,532	
Utilities		$82,400	
UNFH Renovation Refugia & Quarantine Bldg.			
*Construction		$999,369	
Equipment		$242,007	
Utilities		$75,000	
SMARC Species Husbandry and Collection			
Fish Biologist (GS-12, 972 hrs)		$47,889	
Fish Biologist (GS-07, 1262 hrs)		$35,185	
Fish Biologist (GS-07, 1384 hrs)		$39,199	
Fish Biologist (GS-07, 1384 hrs)		$39,199	
Fish & Wildlife Administrator (GS-14, 186 hrs)		$14,226	
SMARC Staff (GS-11, 184 hrs)		$8,396	
Maintenance technician (WG-8, 694 hrs)		$19,432	
Diving		$7,000	
Weekend Walk Thru		$7,500	
Other Overtime		$2,000	
UNFH Species Husbandry and Collection			
Fish Biologist (GS-11, 1250 hrs)		$51,350	
Fish Biologist (GS-06, 1672 hrs)		$41,767	
Fish Biologist (GS-06-07, 1612 hrs)		$43,972	
Fish Biologist (GS-06-07, 1976 hrs)		$56,866	
Supervisory Fish Biologist (GS-12, 208 hrs)		$11,604	
Weekend Walk Thru		$5,400	
Other Overtime		$2,000	
Fish Health		$17,000	
SMARC Reimbursibles		$74,000	
UNFH Reimbursibles		$47,000	
Subtotal		*$3,765,227*	
Admin Cost Subtotal		*$640,089*	

2	**Research**			**$495,790**
	BIO-WEST: Dryopid beetle life history		$129,956	
	BIO-WEST: Peck's Cave amphipod life history		$135,435	
	BIO-WEST: Riffle beetle life history		$26,259	
	Captive propagation refinement salamanders		$115,000	
	Fish Biologist (GS-12, 572 hrs)	*28,154*		
	Fish Biologist (GS-07, 694 hrs)	*19,348*		
	Fish Biologist (GS-07, 572 hrs)	*15,947*		
	Fish Biologist (GS-07, 572 hrs)	*15,947*		
	Fish Biologist (GS-11, 250 hrs)	*10,270*		
	Fish Biologist (GS-06, 364 hrs)	*9,905*		
	Fish Biologist (GS-9, 261 hrs)	*9,921*		
	Fish & Wildlife Administrator (GS-14, 72 hrs)	*5,508*		
	Equipment		$17,102	
	Subtotal		*$423,752*	
	Admin costs for Task 2		*$72,038*	
3	**Species Propagation and Husbandry**		*$0*	**$0**
	Subtotal		*$0*	
4	**Species Reintroduction**		*$0*	**$0**
	Subtotal		*$0*	
5	**Reporting**			**$115,257**
	BIO-WEST		$20,320	
	SMARC Staff		$56,110	
	Fish Biologist (GS-12, 416 hrs)	*20,476*		
	Fish Biologist (GS-07, 104 hrs)	*2,900*		
	Fish Biologist (GS-07, 104 hrs)	*2,900*		
	Fish Biologist (GS-07, 104 hrs)	*2,900*		
	SMARC Staff (GS-11, 364 hrs)	*16,609*		
	Fish & Wildlife Administrator (GS-14, 135 hrs)	*10,325*		
	UNFH Staff		$22,080	
	Fish Biologist (GS-11, 100 hrs)	*4,108*		
	Fish Biologist (GS-06, 88 hrs)	*2,198*		
	Fish Biologist (GS-06-07, 104 hrs)	*2,830*		
	Fish Biologist (GS-06-07, 104 hrs)	*2,987*		
	UNFH Staff (GS-06, 338 hrs)	*9,957*		
	Subtotal		*$98,510*	
	Admin costs for Task 5		*$16,747*	
	Meetings and Presentations			**$26,898**
6	BIO-WEST		$9,890	
	SMARC staff		$13,100	
	Fish Biologist (GS-12, 120 hrs)	*5,908*		
	Fish Biologist (GS-07, 20 hrs)	*557*		
	Fish Biologist (GS-07, 20 hrs)	*557*		
	Fish Biologist (GS-07, 20 hrs)	*557*		
	Fish & Wildlife Administrator (GS-14, 72 hrs)	*5,521*		
	Subtotal		*$22,990*	
	Admin costs for Task 6		*$3,908*	
	TOTAL		**$5,043,261**	

**= Remainder of 2017 construction costs detailed within the 2017 work plan will be applied to 2018. This would occur through an amendment to the 2018 work plan. Budget totals for the construction and renovation projects at UNFH and SMARC are not anticipated to increase.*

Projected (2018) Budget Summarized by Task:

Task 1: $4,405,316
Task 2: $495,790
Task 3: $0
Task 4: $0
Task 5: $115,257
Task 6: $26,898

Projected (2018) Subcontractor Expenses Summarized by Task

Task 1: Dexter Fish Health Unit Dexter NM $17,000 (Health Diagnostics)
Task 2: BIO-WEST $291,650
Task 3: $0
Task 4: $0
Task 5: BIO-WEST $20,320
Task 6: BIO-WEST $9,889

Timeline of 2018 Milestones (List major deliverables)

January	Continue with species collection
	Subcontract drafted
	2018 Specific Research Study Plans Drafted
February	Subcontract executed
	2018 Specific Research Study Plans finalized
June-Aug	Construction completed on SMARC Refugia and Quarantine buildings
July	Submit and renew TPWD permit
September to	Draft Research Reports
December	Draft Annual report

Chad Furl, PhD Chief Science Officer Edwards Aquifer Authority

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Ken Ostrand, PhD Center Director SMARC, UNFH US Fish and Wildlife Service

Literature Cited

AFS-FHS (American Fisheries Society-Fish Health Section). 2005. Model Quality Assurance/Quality Control Program For Fish Health Laboratories, 2016 edition. Accessible at: http://afs-fhs.org/bluebook/bluebook-index.php.

Cantu, V., J. N. Fries, and T. A. Ryan. 2009. An apparatus for separating live amphipods from debris. North American Journal of Aquaculture 71:6-9.

Crawford, D. M., and D. C. Tarter (1979) Observations on the life history of the freshwater amphipod, *Crangonyx forbesi* (Hubricht and Mackin), in a spring-fed cistern in West Virginia. American Midland Naturalist 2: 320-325.

Culver DC, T Pipan (2009) The biology of caves and other subterranean habitats. Oxford University Press, New York.

Hall, R (Edwards Aquifer Authority). 2016. 2016 Comal Springs Riffle Beetle SOP Work Group: Attachment 2: Existing CSRB Cotton Lure SOP. Available at: http://www.eahcp.org/index.php/administration/work_groups/2016_comal_springs_riffle_beetle_sop_work_group

Wilson, W. D., J. T. Hutchinson, K. G. Ostrand. 2016. Genetic diversity assessment of in situ and ex situ Texas wild rice (*Zizania texana*) populations, an endangered plant. Aquatic Botany 136:212-219.

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

USFWS and AFS-FHS (U.S. Fish and Wildlife Service and American Fisheries Society-Fish Health Section). 2016. Standard procedures for aquatic animal health inspections. *In* AFS-FHS. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2016 edition. Accessible at: http://afs-fhs.org/bluebook/bluebook-index.php.

Edwards Aquifer Refugia
Research Goals and Plan

By
Dr. Lindsay Campbell
San Marcos Aquatic Resources Center
500 East McCarty Lane
San Marcos, Texas 78666

May 23, 2018

INTRODUCTION

Building on the research conducted at San Marcos Aquatic Resources Center (SMARC), starting in 1985 with the endangered fountain darter and continuing to present day with added species covered under the Edwards Aquifer Habitat Conservation Plan (EAHCP), we propose the following Research Goals and Plan to guide studies within the Edwards Aquifer Refugia Program under contract #16-822-HCP. The main charge of the Refugia Program is to establish fully functional refugia for the ten covered species (Table 1). Fully functional refugia populations are those that can be predictably collected, maintained, and bred with statistical confidence. Thus, the focus of research conducted will center on advancing the topics of fully functional refugia populations focusing primarily on improving our ability to efficiently capture, breed, and maintain physically and genetically healthy populations for potential reintroduction into the wild. The Research Plan takes into account the species ranking priority (Table 1) and research topic ranking priority (Table 2) as defined within the Contract (Exhibit B Task 2).

Table 1 Ten species identified in the EAHCP and listed for coverage under the Edwards Aquifer Incidental Take Permit ranked by the presumed need for research that will result in fully functional refugia.

TABLE 1 **Species Federally Listed Endangered:**	**Priority Ranking**
Comal Springs Riffle Beetle (*Heterelmis comalensis*)	1
Comal Springs Dryopid Beetle (*Stygoparnus comalensis*)	1
Pecks Cave Amphipod (*Stygobromus pecki*)	1
Texas Blind Salamander (*Eurycea rathbuni*)	2
San Marcos Salamander (*Eurycea nana*)	2
Fountain Darter (*Etheostoma fonticola*)	3
Texas Wild Rice (*Zizania texana*)	3
Species Petitioned for Listing as Endangered	
Texas Cave Diving Beetle (*Haideoporus texanus*)	4
Texas Troglobitic Water Slater (*Lirceolus smithii*)	4
Comal Springs Salamander (*Eurycea sp.*)	4

Table 2 Research topics necessary to establish fully functional refugia.

TABLE 2 **Research Topics**	**Priority Ranking**
Collection Methods and Techniques	1
Species Husbandry	2
Species Propagation	3
Species Genetics	4
Species Reintroduction Methods	4

ROLE OF RESEARCH IN FULLY FUNCTIONAL REFUGIA POPULATIONS

To date, we have established fully functional refugia for two of the ten covered species currently being maintained (i.e., fountain darter and Texas wild rice). While strides are being made for the other species, a large degree of uncertainty remains around critical elements until further research can be completed. In fact, there are many essential pieces of knowledge that are currently unknown for the majority of species, especially the covered invertebrate and salamander species (Table 3). For example, breeding of salamanders has taken place opportunistically at the station, but we currently cannot reliably reproduce offspring of the covered species or have documented methods to do so. For the macroinvertebrate species we need a fundamental understanding of the basic life-history and ecological requirements before developing subsequent research projects. Current knowledge for the covered species will require additional, and sequential, research to provide meaningful information for the establishment and operation of fully functional refugia (Table 3).

Table 3 Table of knowledge know for each covered species with a gradient from 5 to 0, where 5 means that documented procedures exist to 0 meaning no information currently exists in a form usable for refugia management.

Species	Collection	Husbandry	Propagation	Genetics	Reintroduction
Fountain darter	5	5	5	4	5
Texas wild rice	5	5	5	4	5
Texas blind salamander	4	5	2	1	0
San Marcos salamander	5	4	3	3	0
Comal Springs salamander	5	4	2	2	0
Comal Springs riffle beetle	5	3	2	2	0
Comal Springs dryopid beetle	3	2	0	2	0
Texas troglobitic water slater	1	1	0	0	0
Peck's cave amphipod	3	3	2	2	0
Edwards Aquifer diving beetle	1	0	0	0	0

The goals of our Research Plan are designed to investigate these essential knowledge gaps in order to establish fully functional refugia for the remaining species.

RESEARCH PLAN GOALS

1. Add two or more species to the list of "fully functional" refugia
2. Improve or establish collection techniques for invertebrates
3. Advance husbandry techniques; increase survival
4. Reliably reproduce species
5. Parameterize life histories of invertebrate species
6. Formulate genetic management plans for species
7. Design reintroduction plans or strategies for salamanders and invertebrates

One of our goals with research is to add at least two species, if not more, to this list of fully functional refugia over the course of the contract. We believe we are closest to adding the San Marcos salamander and Comal Springs riffle beetle at this time, and thus, will focus the main efforts of our research on these species at first. More specifically, we will focus on these topics first:

San Marcos salamander

1. Cues for fertilization and egg deposition

2. Egg and juvenile survival and growth
3. Wild population, standing stock, and offspring genetic diversity
4. Reintroduction technique

Comal Springs riffle beetle

1. Dietary requirements for maintenance of standing stocks
2. Increased survival rates of standing stocks
3. Increased pupation success
4. Wild population, standing stock, and offspring genetic diversity
5. Reintroduction techniques

While we intend to focus the bulk of our research on the above topics, we will continue to advance knowledge and research projects on other species concurrently. Much information can be gleaned from the other species with smaller research projects. We do not want to be hampered by a limiting "step" in one species while we could be going much further with another species. For example, continuing work on Peck's cave amphipod life history may advance us towards a fully functional refugia for this species faster than the Comal Springs riffle beetle, if we cannot find a way to increase Comal Springs riffle beetle successful pupation rates. Another example would be continuing to document development, maturation, and reproduction of Texas blind salamanders that we have in captivity.

Any research exclusively directed at those species considered to already have functional refugia would be deliberate. Such research would provide critical information for their respective Captive Propagation Plans, Genetic Management Plans, and Reintroduction Plans.

In collaboration with the EAA, USFWS will annually identify and prioritize research projects based on new knowledge obtained from the Refugia Program or other sources. In many cases research cannot be designed or planned until results from a previous study are known, especially information regarding basic life-history metrics of invertebrates. Specific consideration will be continually examined to align refugia goals and research needs with adaptive management strategies and current environmental conditions to meet newly identified concerns. Research projects may be suspended or terminated if higher priorities identified in the EAHCP conflict with the proposed research (e.g., space is required for salvaged organisms,

or, if space is required to house cultured organisms for reintroduction).

EAA-USFWS RESEARCH PROPOSAL PROCESS

Annually, research proposals for each project will be composed and submitted to the Edwards Aquifer Authority (EAA) for review and approval. The proposal will include research to be conducted by both internal (USFWS) and external (subcontracted) partners. Research proposals will contain the following components:

1. **Species**: species proposed for study
2. **Background:** this section defines the problem, presents pertinent information through a literature review, and develops projects objectives. If part of a multi-year project, the previous year's results will be summarized here if not already done through an interim report.
3. **Objectives:** this section clearly states project objectives.
4. **Expected Benefit to refugia:** This section clearly defines how the project objectives will aid in establishing a fully functioning refugia for the species.
5. **Materials and Methods:** this section includes detailed descriptions of methods and procedures intended to be performed to accomplish objectives.
6. **Investigator Responsibilities:** this section defines ownership of the tasks described in the Materials and Methods section. Tasks assigned to sub-contractors should be identified here.
7. **Schedule:** this section defines the dates for major benchmarks, and it states when draft, interim, and final reports are due.
8. **Budget:** this section provides a detailed description of all allocated monies predicted to conduct the study including anticipated subcontractor expenses.
9. **Intended Method of Dissemination:** this section states the method in which research results will be conveyed (i.e., report for EAA website, EAA Committee presentation, USFWS Refugia Annual Report, or manuscript composition for peer reviewed journal, Captive Propagation Plan, Genetics Management Plan, Reintroduction Plan).

Projects for the upcoming year will initially be described as part of the annual work plan due to the EAA by March 31 of the previous year (e.g. descriptions of 2019 projects will be included in

the 2019 Work Plan due to EAA on March 31, 2018). After the annual work plan is approved, USFWS staff will develop detailed research proposals (as described above) on the topics agreed upon in the work plan. The research proposals will be presented to EAHCP staff and members of the EAHCP Science committee during the Fall/Winter prior to the calendar year the projects are to begin. After comments by EAHCP staff and members of the EAHCP Science Committee are incorporated, written proposals will be submitted the EAA for approval. Once the research proposals have been finalized and approved by the EAA, the project may begin. It is anticipated that this process of developing, editing, and approving proposals will be completed prior to the beginning of the year the research takes place.

2018 Salamander Reproduction Research Report

Investigating San Marcos Salamander Reproduction in Captivity

Dr. Lindsay Campbell and Kelsey Anderson

Background

The San Marcos salamander (*Eurycea nana*) is an aquatic, federally threatened (USFWS 1980) plethodontid salamander endemic to the spring outflows in Spring Lake and just below Spring Lake dam in San Marcos, Texas (Tipton *et al*. 2012). The San Marcos salamander evolved under stable water quantity and quality conditions from springs supplied by the Edwards Aquifer (Chipppindale and Price 2005). These conditions have become less stable as increasing human population and subsequent community development has impacted both spring flow and water quality. Federal listing of this and other Edwards Aquifer dependent species was warranted due to decline in population sizes, low population numbers, and multiple threats to the species and their habitat, including changes in water flows, pollution, and habitat alteration due to human action (USFWS 1980).

The San Marcos Aquatic Resources Center has established a refugia population (captive assurance colony) to be used if animals would need to be reintroduced into the wild. A catastrophic event in the wild would trigger reintroduction, once the event had passed and the habitat stabilized. This is one of many conservation measures implemented for this federally listed species. Successful and predictable breeding in captivity is critical to the success of the refugia in case restocking of the species were needed. To produce a reliable reintroduction plan for the species breeding rates, egg survivorship rates, juvenile survivorship rates, and length of time needed to produce the number of individuals required for restocking must be known. In addition, the space and staff time needed for a full-scale production event needs to be estimated and taken into account. Currently, a reliable captive breeding method for the San Marcos salamander has not yet been established. Only two published studies have investigated reproduction methods in the San Marcos salamander (Fries 2002; Najvar et al. 2007). Fries (2002) examined simulated upwelling flows ranging from 1 $cm \cdot sec^{-1}$ to 5 $cm \cdot sec^{-1}$, but did not find significant results that upwelling flows effected reproduction. Najvar *et. al.* (2007) investigated captive pairwise breeding and found that 24 salamander pairs produced seven clutches of eggs (three to the same pair) over a period of nine months. Additional information on the reproduction strategies for this species is required to implement and maintain a successful refugia population.

Courtship behavior in plethodontid salamanders is lengthy and complex. It consists of multiple steps that must occur in sequence and can last several minutes or up to an hour. Given the complexity of this mating, Arnold (1977) suggested that success rates might be as low as 50% for some species of plethodontids. This behavior has been examined in several

plethodontid species (Arnold 1976; Houck 1980; Bechler 1988; Duellman and Trueb 1994; Houck *et al*. 1998; Kozak 2003), but no specific ethogram has been documented for San Marcos salamanders.

Despite past research efforts into the courtship behaviors and reproduction of these species, a reliable method to successfully breed plethodontid salamanders in captivity is still lacking. There does not appear to be a seasonal trigger for reproduction, as juvenile San Marcos salamanders are observed throughout the year in its native habitat (Najvar *et. al.* 2007). Some success was found with a similar species, the Barton Springs salamander (*Eurycea sosorum*), by using a non-invasive (as opposed to hormone injection) reproductive trigger technique of temporary sex separation followed by reunion (Cantu *et al.* 2016). In the current study, we used the general model presented in that paper, with modifications in separation times and the comparison of pair-wise versus group mating.

Objectives

The primary goal of this research was to compare breeding success (defined in terms of number of egg clutches laid) in paired versus group breeding tanks following the separation/reunion technique. We also sought to compare time to incipient reproductive behavior in paired versus group breeding tanks through the use of video observations. We hypothesized that grouping salamanders would encourage greater reproductive success by providing some degree of mate choice. Eggs produced were observed for hatching success and documenting egg developmental stages.

Methods

Adult San Marcos salamanders previously collected from the wild and held in refugia at the San Marcos Aquatic Resources Center (SMARC) were used in this study. Salamanders were anesthetized in a 500 mg/L Tricaine methanesulfonate (MS-222) solution to reduce handling stress. Each salamander was sexed via candling (Gillette and Peterson 2001) and marked with a small Visible Implant Elastomer (VIE) tag in the back hip (Red = female, green = male); this mark allowed researchers a quick way to identify sex with minimal or no handling.

Salamanders were separated by sex into three different partial re-circulation systems (n = 78 salamanders of each sex, including six excess salamanders in case of loss). Each of the three systems was a 1,135 L (300 gallon) insulated fiberglass tank plumbed with a pump and heater-chiller unit that was external to the building. Each system was independent, with no access or shared water between the sexes.

After an initial, complete separation period (70 d), salamanders were removed, selected for random distribution into experimental systems, measured for length, and weighed. We tested with ANOVA to make sure there was no difference in the lengths and weights of females and

males among the systems. One mis-marked female and one mis-marked male had been grouped with the opposite sex. After correction, the separation phase was extended for an additional 30 d. The randomly selected groups of each sex were tracked so the same group could be re-measured and weighed at the end of the extended separation period. Groups were placed into holding systems separated by a perforated screen. Salamanders were able to see each other through the screens and shared partially re-circulating water, allowing potential sensation of pheromones. Salamanders were partially separated for 14 d.

Following the two-week partial separation, salamanders within a system were randomly sorted into either pair (n = 12 tanks per system, Figures 1 & 2) or group breeding tanks (4 females/4 males, n = 3 tanks per system, Figures 1 & 3). Females were examined for gravidity and all females exhibited some degree of egg development. Pairs were housed in round 7.6 L (2 gallon) tanks with a small rock pile in the center of each tank to facilitate courtship behavior and provide cover for the salamanders. A small artificial plant was placed on top of the rock pile as a substrate for oviposition. Groups were housed in rectangular 76 L (20 gallon) tanks with three small rock piles down the centerline of each tank and artificial plants on top of each rock pile. All experimental tanks were painted with aquarium-safe epoxy paint to make the sides and bottoms opaque to prevent visibility of other tanks and to provide better traction to the salamanders. Previous observations of this species raised speculation that slick glass surface could influence movement, courtship, and spermatophore transfer (Wright 2001). Each tank had both a fresh chilled-well-water input and a recirculating water input at approximately 0.95 $L \cdot min^{-1}$ (0.25 $gallons \cdot min^{-1}$) each. This created different flow zones—high water flow under and near the inputs, lower flow areas in the middle of tanks, and flow refuge zones behind the habitat items.

Figure 1 Overhead view of tank set-up for the final phase of the experiment. Each system was on its own re-circulation through a heater-chiller unit to maintain water temperature (21C), in addition to fresh chilled well-water inputs. Cameras mounted above tanks on poles can also be seen.

Figure 2 Example of the set-up of the pair tanks with rock and artificial plants.

Figure 3 Example set up of a group tank with rocks and artificial plants.

A Lorex 4k Ultra HD NVR video surveillance system with low-light infrared capabilities was set up over the tanks to monitor and record courtship behavior. Time to incipient male courtship behavior (males approaching a female and rubbing mental glands on her head and body, Figure 4) and time to incipient female courtship behavior (female following the male into Tail-Straddle Walk, TSW) and differences between pair and group courtship were analyzed. The courtship of pair versus group breeding tanks was compared and evaluated. Kaplan-Meir estimators were calculated and differences between pair and group courtship curves were compared with a logrank test in R version 3.5.1 and RStudio.

Figure 4 Top-down view of rubbing behavior in Eurycea nana.

During the first two weeks of courtship, efforts were made to reduce unnecessary intrusion and disturbance of animals, so as not to interrupt mating activities. At the end of this

period, all habitat items were temporarily removed and it was discovered that several individuals had escaped from pair tanks. Upon video review, most salamanders did not escape until several days into the trial. However, one female escaped within 4 hours of the trial starting. Initially, escaped salamanders were recaptured and returned to their respective tank. Due to the disruptive nature of moving tanks and catching a salamander out of a section, male escapees were left to live in the underpart of the system and only females were retrieved. If more than one individual escaped and could not be differentiated, both were removed from the experiment. As a result of repeated escapes by the same individuals, mesh coverings were put on tanks.

Egg clutches produced during the experimental period were moved to an isolated nursery system for development. The number of days from courtship behavior to oviposition was recorded. Number of eggs per clutch, time to hatch, and number of eggs that survived to hatch were recorded. Visual developmental milestones will be documented for each clutch and one clutch was photographed to document the developmental process. Eggs that became infected with fungus or those did not develop were removed and documented

Results

No significant difference between salamander length (TL, mm) or weight (g) was detected with ANOVA. (females: length $F(2,73) = 2.36$, $p = 0.10$; weight $F(2,71) = 2.44$, $p = 0.09$; males: length $F(2,76) = 1.91$, $p = 0.16$; weight $F(2,76) = 0.55$, $p = 0.58$) among the three tank systems (Table 1).

Table 1 Average ± S.E. total length (mm) and weight (g) of salamanders measured at the start of the partial separation period.

	Length	Weight
Females	62.0 mm (± 0.63)	0.67 g (± 0.01)
Males	64.8 mm (± 0.71)	0.67 g (± 0.01)

Interestingly, under infrared camera vision eggs in females can be discerned, thus making tracking and differentiating of females and males easier under these camera settings.

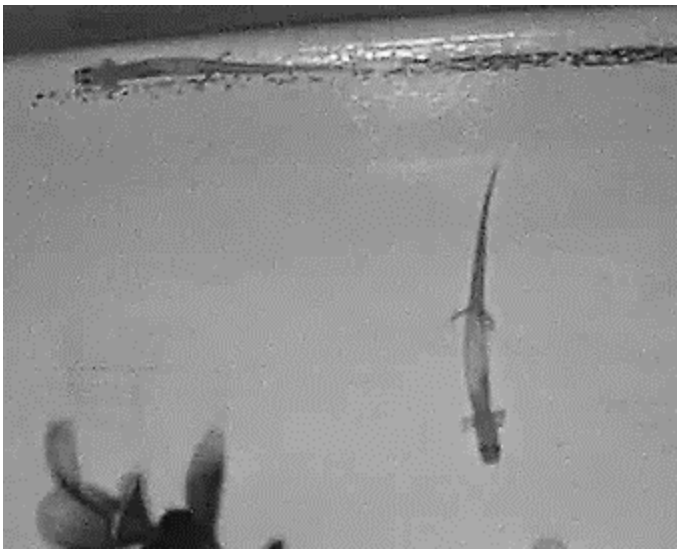

Figure 5 An example of being able to see eggs in two different females in a group tank under infrared vision. The lighter color in the center mass of he two salamanders are eggs. The individual in the center has mature ova.

Courtship Behavior

A logrank test of Kaplan-Meir estimators revealed that time to incipient reproductive behavior for males (the probability of male interest being shown) was significantly different, ($\chi^2_{(1,44)} = 28.4$; $p < 0.001$) between pairs and groups (Figure 6). In group tanks, male interest happened on

average 1 min 19 seconds after the sexes were combined compared to 33 min 33 seconds in pair tanks.

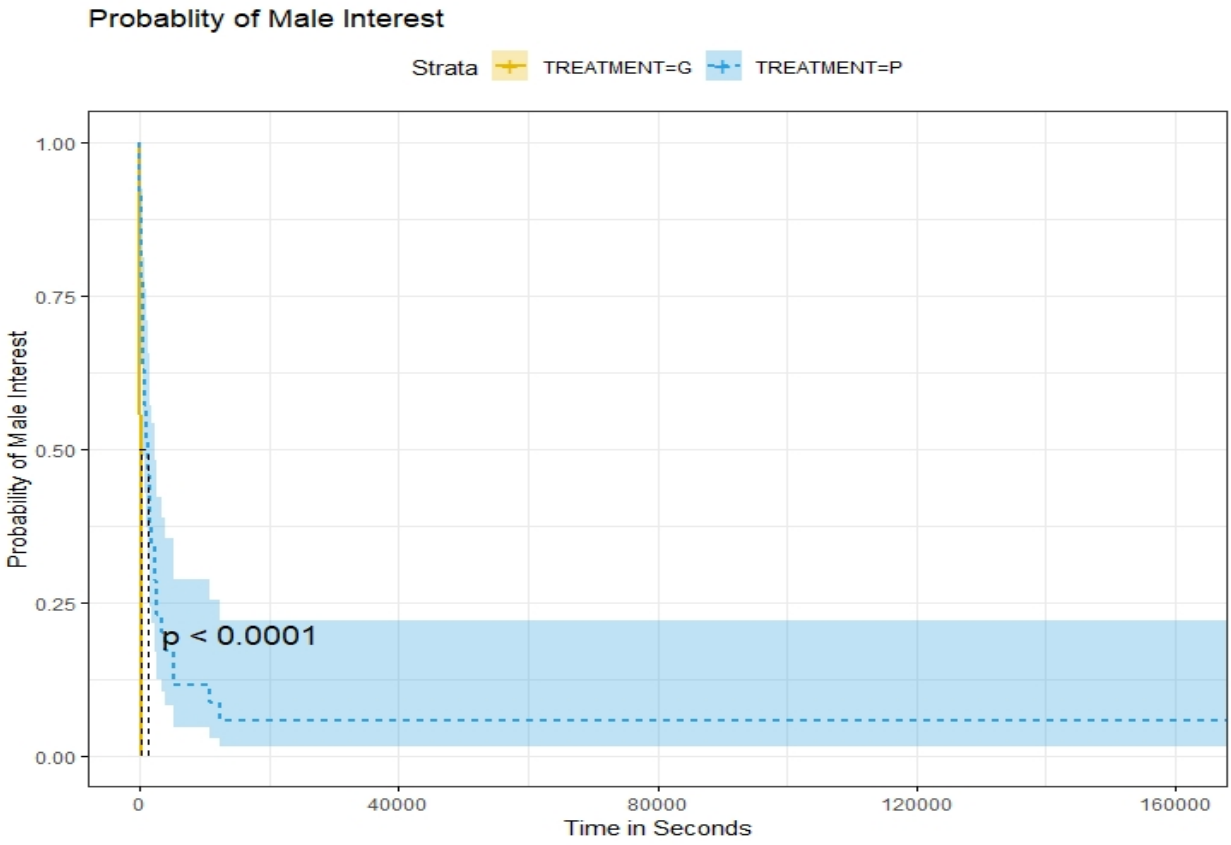

Figure 6 Kaplan-Meir curve estimators for time to first male interest.

A logrank test of Kaplan-Meir estimators revealed that time to incipient reproductive behavior for females (the probability of TSW having occured) was significantly different z ($\chi^2_{(1,44)} = 24.1$; $p < 0.001$) between pairs and groups. In group tanks, female interest and TSW happened on average 9 min 51 seconds after the sexes were combined compared to 4 hours 2 min 52 seconds in pair tanks, excluding those where TSW did not occur. One-third of pairs did not court to TSW during the first 48 hours.

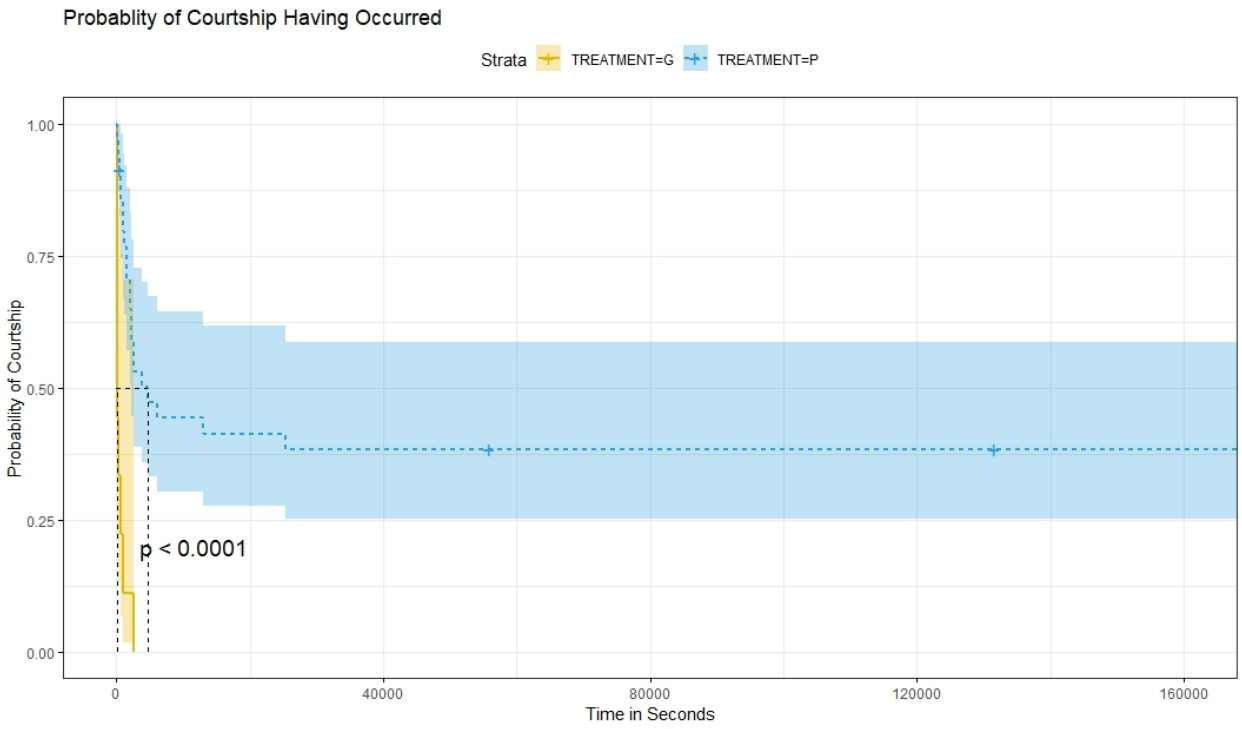

Figure 7 Kaplan-Mier curves of time to first TSW.

In group tanks, the average number of non-reciprocated attempts by males, before a courtship bout resulting in TSW, was 4.6 ± 2.1 SE (range 0-17). Overall, in pair tanks, the average number of non-reciprocated attempts was 22.5 ± 7.5 SE (range 0-175). However, considering only pairs where TSW was observed in 48 hours (i.e, excluding the pairs that did not court), the average number was 4.7 ± 1.2 SE (range 0-26). In pair tanks, where TSW was not observed, individuals either had very little interest in each other or males were very interested in a non-receptive female. In tanks where the male engaged in reproductive behavior but the female did not, the number of non-reciprocated attempts ranged from 75-175 in 48 hours. Further descriptive observational differences in courtship between pair and group tanks will be related in the Discussion section.

During the experiment, approximately 16 salamanders died between June 25-July 25, 2018. Samples were sent to USFWS Fish Health Unit for analysis further details can be found in the Discussion section.

Egg Development

A viable clutch was laid on July 29, 2018, by a female that was alone in a pair tank after the male had been removed for escaping. The oviposition occurred 59 days (not including July 29) after the pair was combined. The 33 eggs were moved to their own nursery tank on August 2, 2018. In total, 22 eggs made it to hatch for a survival rate of 66.7%. Another clutch of 7 eggs was laid in a pair tank on September 21, 2018, but none of these eggs developed.

We photographed the viable clutch of eggs throughout development. We do not attempt in this report to establish species averages for clutch size, time to hatch, and time to ovipostion as only one clutch was produced. Eggs developed at different rates during the period with the first salamander hatching 23 days after the clutch was laid and continuing over the next 11 days. In quality eggs, the ovum and three membranes can be clearly seen, two inner capsules and one outer capsule (Figure 8).

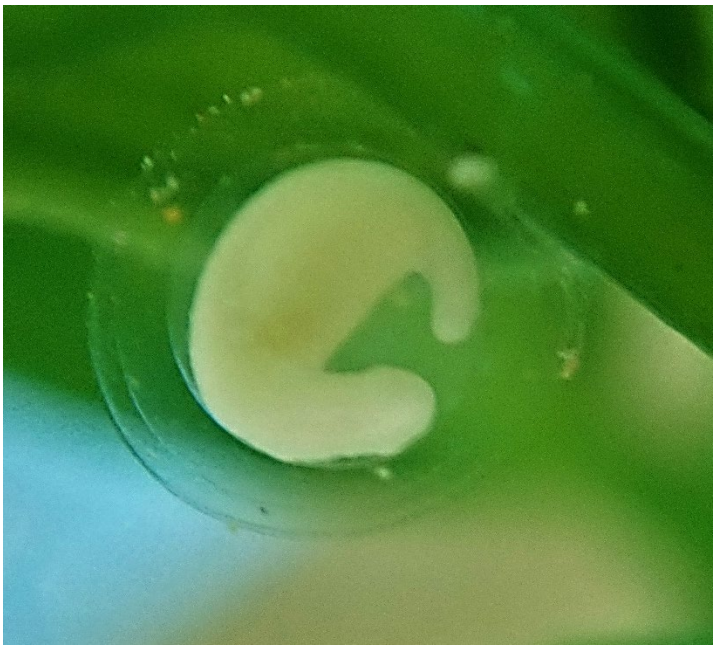

Figure 8 Developing egg with the three membranes clearly seen and intact.

Cell division and cleavage can be seen with the naked eye by day two. Eggs that began to fungus were removed so as not to spread fungus to other eggs. Notable developmental milestones, as defined by Duellman and Trueb (1986) and Hurney *et. al.* (2015), seen in the pictures are as follows: head formation (Stage 21-25), tail bud (Stage 26-29), gill folds/gills (Stage 31-37), limb buds/limbs (Stage 37-45), yolk sac, eye development, and hatching (Figure 9).

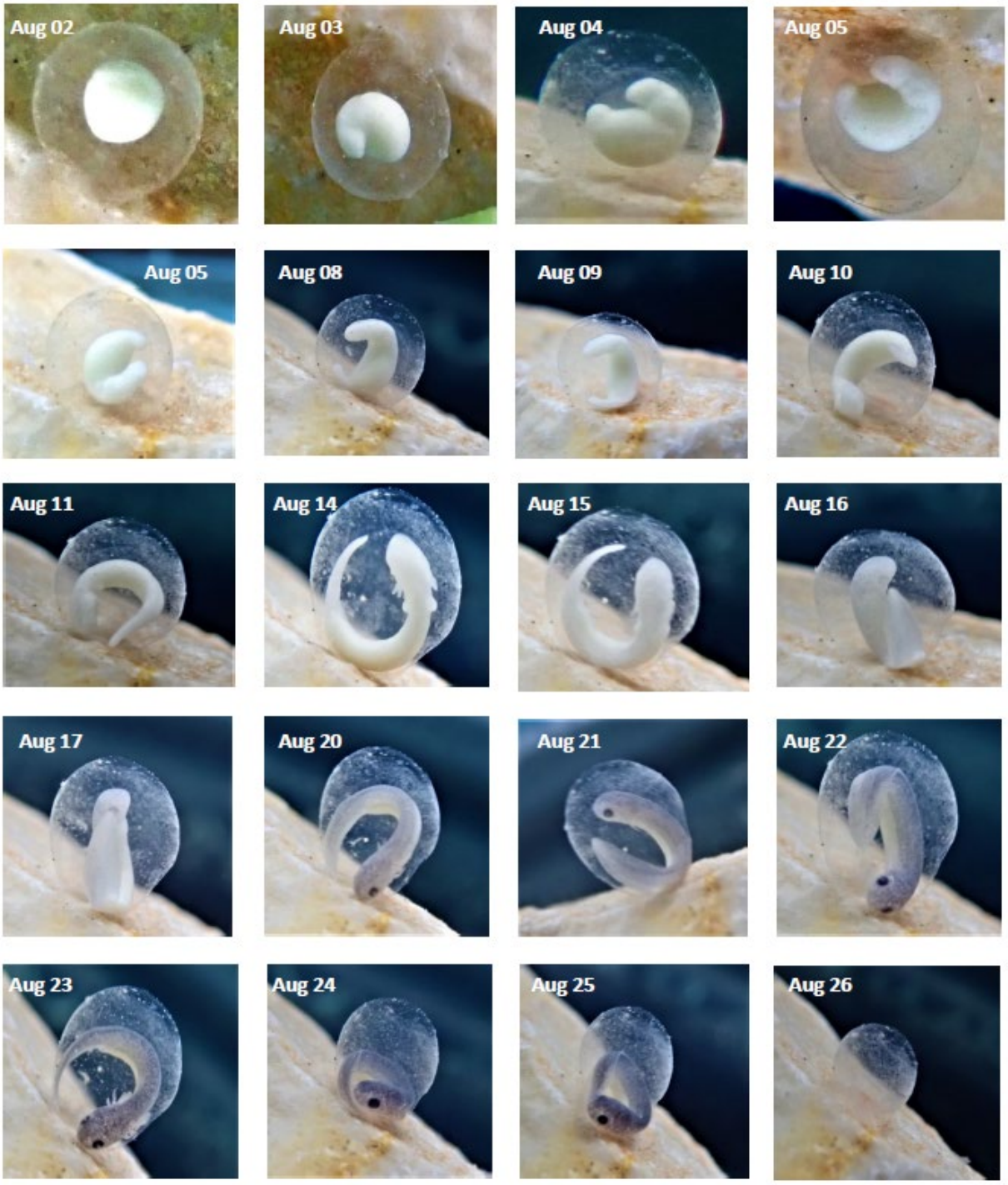

Figure 9 Progression of egg development of San Marcos salamander.

Discussion

Though we did not have the number of egg clutches produced that we had anticipated, much can still be gleaned from the study and video of courtship behavior. In Najvar *et. al.* (2007) salamander pair success rate of clutches produced ranged from 21%-29%. Reproduction in *Eurycea* species may be at a lower rate than anticipated as the study of the separation-trigger technique in Barton Springs salamanders (Cantu *et. al.* 2016) only resulted in 13 clutches of eggs laid from 60 pairs (22% success rate). The City of Austin reports the number of clutches of eggs laid in a year for Barton Springs salamanders, but to our knowledge does not report the rate of clutch deposition to the number of groups/pairs of salamanders; however, their breeding program may not be designed to report these types of statistics.

This study suggests that reproductive behavior may be more frequent in captivity if salamanders are held in larger tanks of grouped individuals rather than in pairs. However, this may not be feasible if a specific pedigree is required. Time to both male and female incipient reproductive behavior was significantly less for groups than pairs, and a third of the pairs did not exhibit full courtship and likely did not mate within the first 48 hours. While the optimum ratio of females to males should be investigated further, mating success is expected to be higher in the group setting. If one female is not receptive to a male, he can move on to another female that may be receptive instead of persisting with the same female. Groups also give some mate choice to the salamanders, which might increase receptivity. Competition and abundance of sexually mature animals plausibly assisted in the decreased time to courtship in group tanks. We observed what appeared to be inexperienced salamanders that did not seem to know how to correctly initiate courtship or lead in TSW, so group tanks might allow salamanders to learn from others, leading to greater success in the future.

From a facility standpoint, grouping salamanders allows for more efficient use of space and effort, especially when trying to produce a large number of offspring quickly, such as for a restocking event. Whereas this does not allow for assured paternity, females could be separated to individual tanks after mating to ensure that at least the maternal parent is known and a list of potential fathers could be associated with them. Potential half-sibling groups could then be proportionally selected or culled to achieve the genetic diversity best for a genetic management plan. If a pedigree of specific individuals is required, we recommend tanks filmed and reviewed for the first 12-24 hours so pairs that do not court could be noted and removed/replaced. Genetic management must be considered in reproduction events, as an overabundance of siblings is undesirable to restock the wild population. To account for this, sibling groups could be culled or only a few of each group selected for restocking so that a variety of genetic combinations could be introduced to the wild. Genetic management plans for this species should be developed to help determine the type or degree of pedigree needed for a restocking event.

While time to courtship milestones were faster in groups as opposed to pairs, similar patterns where observed. Individual courtship dances did not appear drastically different from

groups to pairs. However, in groups, courtship dances between multiple individuals occurred, and occurred most frequently within the first day (Figure 10). Comparatively to other noted courtship kinematic diagrams, courtship behavior of *Eurycea nana* followed the standard approach-rub, initiation of TSW, increased tail waggle of male, and theoretical deposition and pick up of spermatophore (though spermatophore disposition and pick-up not observed in this study, more below). *E. nana* males employ a tactic of near constant pursuit of females, often engaging in long bouts of following and rubbing. Male approach and rubbing of the mental gland occurred both in and out of cover for both treatments. Rubbing behavior was often intense, focusing primarily on the head of the female but also moving down the body and even to the tip of the tail. In some instances, for both groups and pairs, rubbing of the male was so forceful that females were flipped onto their backs and pushed. Tail fanning was observed in both sexes. TSW occurred in and out of cover with individuals often following the edges of the aquaria. The aggressive and relatively quick nature of courtship suggests that separation followed by reunion may be a good method to induce mating in this species.

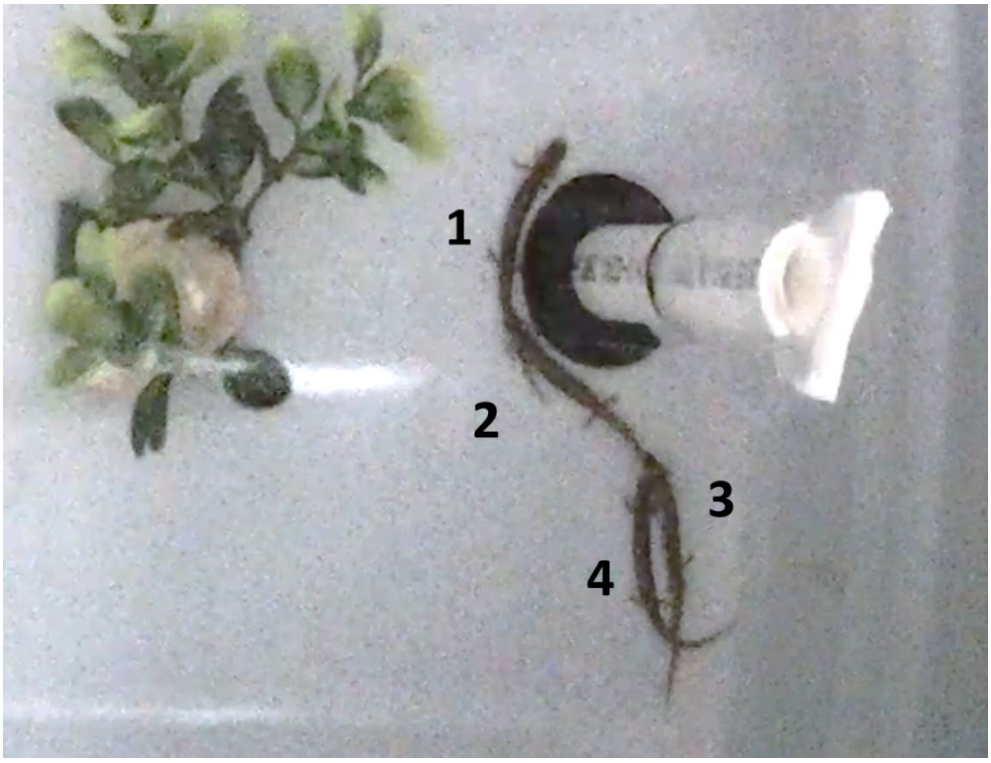

Figure 10 A chain of four salamanders engaging in courtship behavior in a group tank.

Reproductive behaviors, including approaches, rubs, tail fanning, and TSWs, involving multiple individuals occurred in group tanks. However, salamander gender could not always be detected group tanks from a top down view. Nevertheless, both inter- and intra-sex courtship

and TSW was observed. Interruption of pairs already engaging in TSW did occur. Sexual interference can include diverting a female or disrupting the spermatophore deposition of rivals by covering the deposited spermatophore or misleading them into an unprofitable deposition (Arnold 1977). Male on male courtship is not unique to plethodontids, though terrestrial forms exhibit far more elaborate behavior to encourage rival males to waste spermatophores (Duellman and Trueb 1986). In group tanks, where mating continued even into the two-week mark, males were seen guarding or defending a female they were attempting to court.

Over time the intense courtship behavior that was seen during the 24-48 hr after sexes were combined lessened but did not completely drop off by day 14 for either groups or pairs. With time, courtships transitioned to multiple non-reciprocated attempts by males, with fewer instances of female engagement. Instances of multiple animals engaging in simultaneous group courtship decreased with time. The observation that males continue to initiate courtship and conduct repeated bouts of TSW leads us to question whether they may be able to produce multiple spermatophore packets or regenerate them quickly. Further tests are warranted to estimate spermatophore production in males.

Neither spermatophore deposition by males nor retrieval by females was observed. This is not unexpected given the chosen camera angle. The primary goal of filming for this particular experiment was not to capture spermatophore deposition and retrieval, rather to document courtship behavior. Spermatophore deposition and retrieval has only been recorded a handful of times for all salamander species. The spermatophore packet is small and transparent. Successful retrieval by the female should rapidly follow the deposition by the male. The bodies of the salamanders would likely obscure the view of the packet from cameras filming from above. Additionally, while courtship and TSW occurred both in and out of cover, the frequency at which salamanders returned to habitat items suggest that any deposition or pick up may have been further obscured from view. Using a video system, a future study could use a transparent tank with cameras at multiple angles, especially from the sides and underneath in order to capture spermatophore transfer. With much fewer cameras (only filming one tank) the frame rate could be increased, under the same file storage capacity.

The egg clutch we observed in this study followed developmental patterns and rates of published eggs studies on other salamanders (Duellman and Trueb 1986) and salamanders of the same Family (Hurney et al 2015). More observations on clutches are needed before suggesting normal rates for San Marcos salamanders in captivity.

USFWS Fish Health testing showed that the salamanders that died during the experiment had microsporida, mycobacteria, and chytrid fungus on the limbs. Microsporidia and chytrid fungus have previously been found in this species with no current treatment known or protocols prescribed. Mycobateria is naturally occurring in the wild but presents as a serious problem when it manifests in large amounts in organisms. Stress can cause organisms to have a weakened immune system, allowing for the overgrowth of mycobateria and other harmful

manifestations. Salamanders samples from all three systems were positive for mycobateria. We did not feel that it would be useful to euthanize all of the remaining salamanders that appeared healthy (a common practice with other organisms where mycobateria is found). However, this group of salamanders will remain isolated from the rest of refugia stock so as not to chance a potential spread of disease if they are carriers. It is not known if any of these health findings could lead to reproductive dysfunction.

Future Recommendations

We found that TSW typically commences within an hour after combining the sexes for groups (and within 1-3 hours for most pairs). Whether in groups or pairs we recommend the removal of males after 48 hours, 72 hours at the most, because they continue to pursue non-reciprocated overtures to females. This was observed even at the two-week mark where we stopped recording video. A successful mating will most likely happen during the 48-hour time period and extending exposure of highly amorous males may cause undue stress on non-receptive females.

The two-week partial separation period may be superfluous. We suggest, rather, observation during the partial separation period for female behavior that suggests courtship amongst themselves as an indication that they are ready and receptive to be combined with males. This was observed in *E. sosorum* during the partial separation period (it was not specifically looked for in this experiment) and may serve as an indication that females are receptive to mating and ready to be combined with males.

We highly recommend that egg clutches be transferred to their own tank or to a system without adult animals. The live food that adults consume, such as amphipods or blackworms, also predate on eggs and leaving eggs in a tank also exposes them to possible cannibalism by adults. Eggs should also be monitored for fungus and an egg that is fungused should be removed as quickly as possible, so as not to spread spores to other eggs. Best practices would also have UV sterilized water flowing through a "nursery" system to decrease chances of other pathogens being introduced.

With only one viable clutch of eggs produced in the study, we believe that there is a problem within either the system design or the salamanders themselves. Reproduction has not been produced for the rest of our population of San Marcos salamanders, which are housed in mixed sex tanks. In 2019, we plan to follow up on this study, further investigating potential causes for the lack of reproduction. One possibility may be to remove the males after an initial courtship period, possibly allowing for conditions more conducive for oviposition. However, we will investigate other avenues in conjunction with this so as not to repeat the experiment with a different group of animals. We plan to analyze egg content and physiological make up of salamanders held on station to those in the wild to see if there are any differences. We will use Gore-sorber modules to test water quality (similar to the tests run at our well sites) in the Refugia

tanks for endocrine disruptors or detrimental abiotic factors. San Marcos salamanders at this facility have a history of egg rupture from the body cavity. Preserved samples of these ruptures (that have not been analyzed) will be dissected to determine if eggs were fertilized. Further actions will be planned after obtaining results from these initial investigations.

Works Cited

Arnold, S.J. 1976. Sexual behavior, sexual interference, and sexual defense in the salamanders *Ambystoma maculatum*, *Ambystoma tigrinum*, and *Plethodon jordani.* Ziet Tierpsychology 42:247–300.

Bechler, D.L. 1998. Courtship behavior and spermatophore deposition by the subterranean salamander, *Typhlomolge rathbuni* (caudata, plethodontidae). The Southwestern Naturalist 33:124-126.

Chippindale, P.T., and A.H. Price. 2005. Conservation of Texas spring and cave salamanders (Eurycea). Pp. 193–197 In Amphibian Declines: Conservation Status of United States Species. Lannoo, M. (Ed.). University of California Press, Berkley, California, USA.

Cantu, V., J.C. Crow, and K.G. Ostrand. 2016. A Comparison of Two Non-Invasive Spawning Methods to Genetically Manage Captive Barton Springs Salamanders, Eurycea sosorum. Herpetological Review 47:59–36.

Duellman, W.E. and L. Trueb. Biology of Amphibians. New York: McGraw-Hill Publishing Company. 1994.

Fries, J.N. 2002. Upwelling flow velocity preferences of captive adult San Marcos salamanders. North American Journal of Aquaculture 64:113-116.

Gillette, J.R., M.G. Peterson. 2001. The benefits of transparency: candling as a simple method for determining sex in Red-backed Salamanders (*Plethodon cinereus*). Herpetological Review 32: 233–235.

Houck, L.D. 1980. Courtship behavior in the plethodontid salamander *Desmognathus wrighti*. American Zoological Society 20:25A.

Houck, L.D., Bell, A.M., Reagan-wallin, N.L., and Feldhoff, R.C.. 1998. Effects of experimental delivery of male courtship pheromones on the timing of courtship in a terrestrial salamander, *plethodon jordani (caudata: plethodontidae*). Copiea 1:214–219.

Hurney, C.A., S.K. Babcock, D.R. Shook, T.M. Pelletier, S.D. Turner, J. Maturo, S. Cogbill, M.C. Snow, K. Kinch. 2015. Normal table of embryonic development in the four-toed salamander, *Hemidactylium scutatum*. Mechanisms of Development, 136: 99-110.

Kozak, K.H. 2003. Sexual isolation and courtship behavior in salamanders of the *Eurycea bislineata* species complex, with comments on the evolution of the mental gland and pheromone delivery behavior in the plethodontidae. Southeastern Naturalist 2(2):281-292.

Najvar, P.A., Fries, J.N., and J.T. Baccus. 2007. Fecundity of San Marcos salamanders in captivity. The Southwestern Naturalist 52:145-147.

Tipton, B.L. Hibbits, T.L., Hibbits, T.D., Hibbits, T.J., and T.J. Laduc. 2012. Texas Amphibians A Field Guide. University of Texas Press, Austin, Texas.

United States Fish and Wildlife Service. 1980. Endangered and Threatened Wildlife and Plants; Listing of the San Marcos Salamander as Threatened, the San Marcos Gambusia as Endangered, and the Listing of Critical Habitat for Texas Wild Rice, San Marcos Salamander, San Marcos Gambusia, and Fountain Darter. Federal Register 45:47355-47364.

Wright, K. 2001. Amphibian medicine and captive husbandry. Krieger Publishing Company, Malabar, Florida, USA.

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Life-History Aspects of *Stygobromus pecki*

Prepared by:
Ely Kosnicki, PhD – Senior Invertebrate Ecologist
Eric Julius – Aquatic Ecologist
BIO-WEST, Inc.
1405 United Drive, Suite 111
San Marcos, TX 78666

Prepared for:
The Edwards Aquifer Authority
900 E. Quincy Street
San Antonio, TX 78215

BIO-WEST

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Purpose statement

The objectives of this project were to provide a better understanding of the life histories of the Peck's Cave Amphipod (*Stygobromus pecki*) and to contribute information towards the improvement of captive propagation of this species that is in-line with the goals and objective of the Edwards Aquifer Habitat Conservation Plan. More specifically, the goals of this project were to better understand sexual dimorphism, estimate growth rates, estimate how many molts or how long it takes individuals to reach maturity, gather information related to fecundity, gain a better understanding of mating behavior, and gain a better understanding of feeding preferences.

Studies conducted

- Common garden mortality
    - The mortality rates of communal tanks of *Stygobromus* spp. were documented.
- Egg incubation and immature growth
    - Brooding females were individually tracked and observed to estimate egg incubation times. Newly released neonates from the brooding females were individually tracked and observed to estimate how many days were spent in each instar.
    - Wild-caught immature subjects were reared in the laboratory, individually tracked, and measred to estimate how many days were spent in each instar.
- Number of instars until maturity and sexual dimorphism
    - A separate set of wild-caught specimens were preserved, separated by size class, and slide mounted. The apical process of the peduncle of uropod 1 was used to identify mature males; additionally, the occurrence of brooding plates was used to confirm some individuals as female. Morphological character states were used to grade individuals into an instar based on three quantitative methodologies: principal components analysis, cluster analysis, and the length of the antenna 2 peduncle.
    - The average estimated number of days reared wild-caught subjects spent in each instar was used to calculate how many days it took individuals of the slide mounted specimens to reach maturity, based on their instar estimates.
- Mating trials
    - Three female and male pairs of *S. flagellatus* were placed into separate chambers and allowed to interact. Females were inspected for signs of eggs or brood plates, routinely after trials.
- Feeding studies
    - Ten female and male *S. russelli* were fed an increased amount of food and inspected for brooding females.
    - Fifty adult *S. flagellatus* were used to examine feeding preference for five different food items to see if there was a food preference.

Main findings

- Common garden mortality
    - Survivorship of captive individuals appears to be influenced by the amount of habitat heterogeneity provided to their mesocosm.
- Egg incubation and immature growth
    - The average egg incubation time was 49.7 ± 12.7 days with ca. 24% hatching; mortality of hatchlings was 100% after ca. a month, likely due to rearing in static arrays and excessive handling.

    - Wild-caught laboratory reared subjects were estimated to molt ca. every 50 days.
- Number of instars until maturity
    - Morphological character states of slide-mounted specimens were used to grade 58 individuals into 8 instars with mature specimens appearing as soon as instar 6 but more instances at instar 8 and it was estimated to take ca. 387 ± 28.0 days to reach maturity.
- Mating trials
    - These trials were unsuccessful.
- Feeding studies
    - *S. russelli* fed increased amounts did not survive effectively due to apparent poor water quality.
    - *S. flagellatus* were found to choose fish flakes over other food items and appeared to use chemoreception to detect this high-protein food item. Free-swimming food items were preyed upon and appeared to be detected by mechanoreception.

Executive summary

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. Some of the species of concern still pose several substantial questions concerning refuge cultivation; particularly the invertebrate species. *Stygobromus pecki* is a federally endangered species that is adapted to subterranean habitats associated with Edwards Aquifer springs. *S. pecki* belongs to a rather speciose genus with > 135 described species; however, little is known about the life history and the environmental requirements of this species.

Common gardens of *S. pecki*, *S. flagellatus,* and *S. russelli* were kept in flow-through aquarium-style containers, holding about 15 L of water and outfitted with nylon mesh, leaves, and rock as habitat. Each garden was censused ca. once a month to search for brooding mothers and track mortality. Our common garden of *S. pecki* decreased from 31 individuals to eight over the course of 274 days. The rate of decrease began to flatten around 10 individuals suggesting that fewer individuals should be kept within a confined space. Due to high amounts of mortality observed within the first five censuses, the other common gardens were moved to a smaller volume flow-through tube holding about 0.5 L of water utilizing PVC-shavings as habitat. Survivorship appeared similar to that of *S. pecki* and it is surmised that the amount of habitat, rather than the quantity of water is important for reducing captive mortality.

Twenty-six brooding females of *Stygobromus* were observed from the SMARC refuge and BIO-WEST common gardens, producing a total of 139 eggs. From the common garden of *S. pecki* alone, five out of 19 females produced broods consisting of 28 eggs over the course of this study. There was no relationship between female size and number of eggs produced. High egg mortality was observed, presumably due to female stress. It is recommended that brooding females should be removed to separate containers to minimize stress and to give neonates a nursery to inhabit after being released from the marsupium.

The average egg incubation time for *S. pecki* was 49.7 ± 12.7 days with ca. 24% developing to free-swimming neonates. The average body length of F1s was 2.87 ± 0.16 mm; however, these were never observed to molt and no individual survived past 32 days. It is surmised that a combination of the use of

the suspended static arrays and excessive handling was responsible for early mortality. We recommend that first instars are given adequate space with plentiful habitat heterogeneity in a flow-through system.

Due to the lack of laboratory production of young, early instar *Stygobromus* subjects were wild caught and brought to the laboratory for growth analysis. Thirty individuals tracked successively over a minimum of 60 days were analyzed for time between molts. Starting instar was determined by best professional judgement for each individual by comparing graphs of their body measurements to measures of other wild-caught specimens and a body length benchmark of 2.87 mm for the first instar. It was estimated that individuals molt about every 50 days.

A separate set of wild-caught *Stygobromus* specimens were graded into size classes based on 0.5 mm increments in body length. Fifty-eight individuals were slide mounted and a set of 34 character states consisting of measures or counts were compiled. Individuals that reached size-class 6.5 mm were found to have fully developed sexually identifiable characters and this body size was used as a benchmark for determining sexually maturity. Three instar estimation methods were employed and averaged to estimate the instar or developmental stage of each individual. The first method separated instars among the 58 individuals by finding the inflection points of a smooth spline of the ranked principal component 1 of their character states. The second method used the gap-statistic to determine the best number of instars from a hierarchical cluster analysis of the character states. The third method utilized inflections points similar to method one, but only for the length of the antenna 2 peduncle. At least 8 instars or developmental stages were delineated by this method for the 58 specimens and it was determined that it takes individuals about 387.5 ± 28.0 days to reach maturity, based on the instar estimates of individuals that were determined to be sexually mature and the average amount of time estimated between instars of the wild-caught laboratory reared subjects (50 days).

A HIGH-feeding experiment was conducted with 10 female and male *S. russelli* subjects exposed to 2-3 times normal amounts of food. Although 20% of the females developed broods within a four-month period of time, mortality was 80% and was largely attributed to poor water quality conditions, resulting in excessive decompositions from the increased food resources.

A food preference experiment was conducted by giving starved *S. flagellatus* a choice between two food items. Subjects chose commercially available fish flakes over conditioned leaf, a living but restrained *Hyalella* sp., and a plastic strip (control). No other food source was found to be preferable to another. Subjects exposed to free-swimming *Hyalella* sp., and no other food items, did not show any predation behavior unless allowed to remain in the chamber with the prey item overnight. Subjects exposed to free-swimming *Lirceolus* sp., and no other food items, consumed the prey two out of five trials that ran for ca. 20 mins. These results suggest that predation by *Stygobromus* is a result of mechanoreception due to direct contact with prey items; however, behavior in obtaining the fish flakes suggests they have chemoreception toward high protein food sources.

Many questions remain unanswered regarding basic life-history aspects of *S. pecki* and a great deal more applied research is needed in order to establish a fully functioning refuge for this species. With the current knowledge gained from these studies, we recommend that more housing is provided to accommodate the refuge stock. In addition to added mesocosms, we recommend that increasing habitable surface area is increased. Currently utilized substrates appear useful, but alternative bio-media should be considered. We also recommend that brooding mothers should be removed to more stress-free environments and that future work should specifically investigate reproductive aspects of the captive rearing program.

Background

Stygobromus pecki is a federally endangered species (USFWS 1997) that is adapted to subterranean habitats associated with Edwards Aquifer springs. *S. pecki* belongs to a speciose genus with > 135 described species (retrieved 22-Oct-2017, from the Integrated Taxonomic Information System on-line database, http://www.itis.gov), all of which are subterranean and found primarily in North America (Holsinger 1967; Holsinger 1994). There are at least 10 species known from Texas (Holsinger 1967) and 3 of these (*S. bifurcatus, S. flagellatus, and S. russelli*) are known to be sympatric with *S. pecki* (Holsinger 1967, Gibson et al. 2008, Ethridge et al. 2013). However, a recent study on molecular phylogeny and population genetics of the genus within this region indicated that there may be additional species that have gone undetected morphologically (Ethridge et al. 2013).

S. pecki is well supported as a monophyletic taxon (Ethridge et al. 2013) and consists of several subpopulations with sufficient gene flow to prevent isolation (Ethridge et al. 2013, Lucas et al. 2016). Species determination is primarily based on adult characters; basis of pereopods 5-7 not expanded and without developed lobes, 5 apical spines of the uropod 3 ramus, and a telson without lateral spines. Furthermore, Holsinger (1967) and Kosnicki et al. (2019) noted there are major problems with *Stygobromus* taxonomy due to continuous growth and variation of distinguishable morphological character states. At present, we are unaware of any publications on separating species at immature stages of development. Because other species of *Stygobromus* are known to occur with *S. pecki* and because we may not know the full extent of *S. pecki*, developing a better understanding of immature stages of *S. pecki* should be of importance for conservation, life-histories study, and refugia efforts.

There is little information on the life history of *S. pecki*. In general, it appears that subterranean amphipods (like other subterranean species) have a much slower rate of development and reproduction than epigean species. Most epigean species of amphipods have multiple generations per year, while subterranean amphipods typically take at least a year to mature (Crawford and Tarter 1979). However, the subterranean hyporheic amphipod, *Niphargus aquilex aquilex*, has been shown to have the capacity to produce up to two generations per year (Gledhill and Ladle 1969).

It has been shown in a number of amphipod species that sex ratios fluctuate (Crawford and Tarter 1979). Crawford and Tarter (1979) and Bollache and Cezilly (2004a) suggested that the greater abundance of males during the breeding season corresponds to females having synchronous pre-copulatory molts. Sex ratios may also become distorted due to the mechanism of sex determination; experimental evidence indicates that sex is determined in amphipods by a set of alleles distributed across several chromosomes (Bulnheim 1978). Furthermore, it has been shown that certain pairings can lead to exclusively male or female offspring (Sutcliffe 1992). Environmental factors have also been shown to affect or covary with sex (Sutcliffe 1992; Watt and Adams 1993; McCabe and Dunn 1997). Infection with microsporidians (Bulnheim and Vávra 1968) and chemical pollutants (Gross et al. 2001) have also been shown to affect sex ratios or the development of sexual characteristics in amphipods. Evidence suggests that amphipods are subject to environmental sex determination (ESD). Temperature and daylight have been shown to be seasonally linked to ESD of an epigean species *Gammarus duebeni* where immatures growing at higher temperatures and during longer daylight tend to be larger and develop as males, with a critical period of development at about 3 weeks after leaving the marsupium (Bulnheim 1978). However, this should not be the case for troglobitic species that reside within more uniform temperatures and are not exposed to sunlight. It is possible that exposure to food resources may be important with regard to developing larger body sizes that may results in males. Holsinger

(1967) noted that differences in sex sizes of *Stygobromus* varied from species to species. Successful housing of *S. pecki* in a refugia should require thorough understanding of reproduction and life history as related to sex determination and sex ratios.

Amphipods are thought to only be able to mate after the female molts, because only then is the cuticle of the females' exoskeleton flexible enough to allow the release of eggs through the genital pores into the marsupium (Bollache and Cezilly 2004a). Because females are only momentarily receptive to mating, males typically guard a female (via amplexus) prior to her molting, insuring that he fathers offspring; however, this has never been observed in *S. pecki*. Molt cycles in males also appear to have a role in reproductive timing. Males approaching a molt tend to avoid entering into amplexus, likely because they will inevitably have to release the female upon molting, thus never successfully copulating with the female because such an effort would be a wasted investment in a mating (Bollache and Cezilly 2004a). Mate guarding comes at a cost to males by hindering their ability to forage, thereby reducing lipid stores and hindering growth (Robinson and Doyle 1985). In response to this energetic cost, males tend to avoid amplexus unless they have sufficient amounts of stored lipids and glycogen to wait out female molt cycles and only if the female is expected to molt before the male (Plaistow et al. 2003). It is likely that proper nourishment is necessary to offset the nutritional costs of amplexus. In addition, there appears to be significant cannibalism in captive populations of *S. pecki* (R. Gibson, pers. obs.). However, keeping both males and females well-fed may reduce the tendency of cannibalistic interactions to occur. A study initiated on 21-Dec-2015 with *S. pecki* with pairings over a six-week period of time, resulted in 44.4% cannibalism with larger individuals acting as the predators (Worsham unpublished). The results of that study suggest that confined pairs may be more successful if they are relatively equal in size; however, there is question to whether or not the subjects were correctly sexed before being paired.

Bollache and Cézilly (2004b) proposed that there is greater competition among males for access to larger females, because larger females are more fecund. Consequently, larger males preferentially out-compete smaller males for larger females. Thus, there appears to be size associated pairing between females and males, at least in some species of amphipods (Bollache and Cézilly 2004b; Franceschi et al. 2010; Worsham et al. 2017). Furthermore, pairs in amplexus with smaller females tend to have greater swimming efficiency (Adams and Greenwood 1983).

Work at SMARC noted *S. pecki* females may have multiple broods of ca. 10 young each and neonates are ca. 2 mm in length upon hatching (Fries et al. 2004). Once hatched, it is unknown how many molts are undergone before young become sexual mature; however, F1's produced at SMARC reached lengths of 9 mm in 14 months and produced offspring the following year. It is also unknown how frequently and how many molts adults undergo once reaching sexual maturity. Although the life span of *S. pecki* is unknown, wild-caught adults have been reared in captivity at SMARC for at least 2.7 years with dried leaves and tropical fish flakes as the nutrient sources.

Study objectives

- Gain a better understanding of sexual dimorphism
- Estimate egg incubation rates
- Estimate growth rates
- Estimate how many molts or how long it takes individuals to reach maturity
- Investigate mating behavior
- Gain a better understanding of feeding preferences

Methods

Specimen acquisition
Specimens were acquired using multiple methods and collection events. On February 6, 2018, 30 *S. pecki* adults were collected from Comal Springs and combined with the one individual remaining in the common garden at SMARC that was already in operation from 2017 activities. This garden was checked ca. once a month for brooding females and to take a census of the population in order to track mortality rates. Brooding females encountered during routine inventories from refuge populations, were given to BIO-WEST researchers to track egg incubation times and growth of released neonates. On February 25, 2018, a drift net was placed at the mouth of Spring #7 of Comal Springs to collect drifting immature *Stygobromus* spp. specimens for laboratory rearing; during this collection, four *S. pecki* were preserved and used for instar estimations. Another eight *S. pecki* were collected from Comal Springs during May and July for instar estimations. Preserved specimens from past collections housed at SMARC were also used for the instar estimation study. Collections of *S. flagellatus* and *S. russelli* were routinely collected from the Diversion Spring at San Marcos Springs by SMARC staff and were given to BIO-WEST researchers for surrogate species experimentations. On July 7, 2018, 72 immature *Stygobromus* subjects were collected from Spring #7 of Comal Springs to supplement the immature growth study.

Common garden mortality
One common garden of *S. pecki* and two gardens of *S. flagellatus* and *S. russelli*, each, were maintained for egg incubation and immature growth studies (see section below). Gardens consisted of flow-through aquaria, containing ca. 15 L of water fed from Edwards Aquifer wells. Habitat consisted of limestone rock, nylon mesh, and leaves. Throughout this study, all *Stygobromus* were fed tropical fish flakes two times per week (product# F30K krill/plankton/spirulina from Pentair), unless indicated otherwise.

As part of monthly checks, we recorded the numbers of males and females remaining in each garden, and in this way, we were able to estimate mortality and survivorship of each of the five gardens. On July 25, 2018 both common gardens of *S. flagellatus* and *S. russelli* were transferred to a flow-through tube housing, constructed of 2-inch diameter PCV pipe to a length of eight inches with 150 micrometer mesh capped at the ends. The internal volume was ca. 0.6 L and was packed with PVC shavings as a bio-media substrate instead of the traditional nylon mesh which tended to bunch up. PVC shavings are stringy, promoting a high-surface area, and were thought to be a good simulate for an interstitial environment. The change was made in order to see if this design would reduce mortality but since it was untested *S. pecki* was maintained in the original flow-through aquarium.

Egg incubation and immature growth
About every two weeks cultures of adult *S. pecki*, *S. russelli*, and *S. flagellatus* were checked for the presence of females brooding eggs in their marsupium. Brooding females were removed and placed into "brooding chambers" (**Fig. 1**) and developmental stage of the eggs were documented as: "fresh", "linear embryos", "neonates", or "free swimming immatures". These females were checked every one to two weeks and the embryological development and number of eggs was documented. Once neonates hatched from eggs and were released from the marsupium, the mother was returned to her culture or origin. Brood size, incubation, and hatching rates for each species was recorded. Neonates released from captive females and juvenile wild-caught *Stygobromus* collected from Spring #7 (collected on July 7, 2018) were individually placed into a "suspended static array" (K-cup) following the methods of Nowlin et al. (2015). Periodically, individuals were measured by gently wet mounting and photographing under magnification using an Olympus Cellsens camera system and software. A calibrated scale bar was superimposed on each photograph and Digimizer software (www.digimizer.com) was used to make

measurements. The length of the body, antenna 1, and antenna 2 were recorded for each photograph date. Individuals were originally photographed ca. every two weeks; but was later reduced to every four weeks due to high mortality.

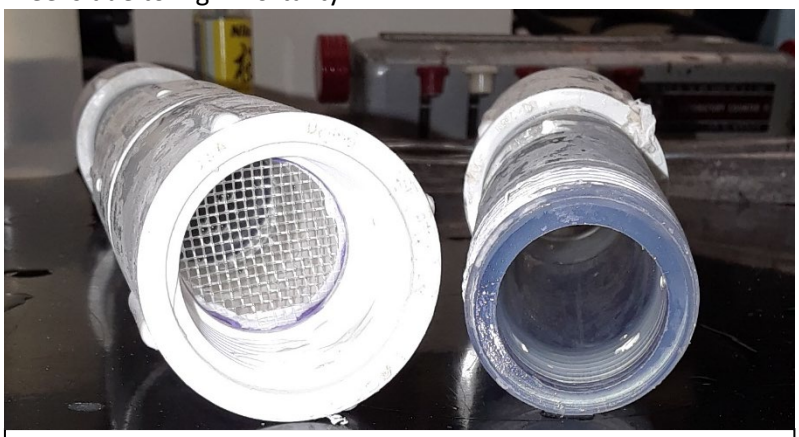

Figure 1. Brooding chamber.

The StygoBanD database was constructed with Microsoft Access® (2016) to keep track of mothers, their broods, and released neonates. Measurements and observations were housed within the StygoBanD and queries were written to track length of time between inspections. Bench sheets were used to maintain a hard copy of all measurements and observations (**Appendix A)**.

Wild-caught individuals from Spring #7 were independently graded into starting instars using best professional judgement (BPJ) of two BIO-WEST biologists based on measurements taken from the data set that was used for determining the number of instars until maturity (see below). Graphs used for BPJ considered the average starting size of F1 released neonates as a benchmark for the size of the first instar and successive instars were delineated after that point (**Appendix B**). Occurrence of individual molts was estimated by BPJ of two BIO-WEST biologists by visually comparing graphs of body measures among photo dates (**Appendix C**). Additional information such as regrowth of an appendage was also used. The number of days between photographs were a molt occurred was used to estimate how long each individual spent in an instar and this data was constituted the "immature growth dataset". The number of days between molts was averaged among all individuals to represent the average number of days between molts for each representative instar. The final estimate of instar duration (days between molts) was used to calculate length of time to maturity.

Number of instars until maturity and sexual dimorphism

Stygobromus caught from Spring #7 on February 6, 2018, and other sources were photographed and measured as described above in the *Egg incubation and immature growth* section. Total body length was used to grade individuals into 0.5 mm size classes. There were at least five individuals representing each size class up to size class 7.0 mm (**Fig. 2A**). Additional specimens representing size classes > 7.0 mm were also included, but were more difficult to obtain and so these size classes were under-represented.

During the process of delineating individuals into size classes, the apex of the uropod 1 peduncle was inspected for the presence of an apical process (**Fig. 2B**). Individuals with an apical process were noted as males (Holsinger 1967). Individuals without an apical process were considered immature until a "discernable size class" could be allocated to mature specimens based on the confidence that males could always be recognized for a specific size class. Individuals not having an apical process at the "discernable size class" and above were considered females. The presence of females with brooding plates were also taken into consideration when determining the "discernable size class" upon which sexes could be reliably delineated.

Figure 2. (**A**) Example of the total body length used to divide specimens into size classes for instar estimations; (**B**) apical process of male uropod 1 peduncle.

Size class graded specimens were dissected and slide mounted so that finer measures could be obtained in relation to maturation. A set of 34-character states were enumerated for each individual (**Appendix D**). Characters were chosen to represent evidence of successive growth between instars (e.g. lengths, number of spines).

Principal Components Analysis (PCA) was performed on the character set of all size classed specimens, excluding body size and size class. Ordination of the first two components was used to visualize morphological progressions among size classes. The first principal component was also used to estimate instar groups by ranking individuals based on principal component 1, then the second derivative of a smooth spline was used to find inflection points where the rate change of the rate of change was equal to zero. We only selected an instar break where the curve of the second derivative descended from positive to zero, opposed to ascending form negative to zero. Groups of individuals between inflection points were considered to belong to the same instar. PCA was conducted with the base R software *prcomp* function whereas the spline and derivatives were calculated using the *features* package for R software version 3.4.1 (R Development Core Team 2017).

A second method to identify instars used hierarchical cluster analysis of individuals based on the same set of 34-character states. The gap statistic (Tibshirani et al. 2001) was used to find the number of groups and each group was considered an instar class. Analyses were performed with the *hcut* and *clusGAP* functions of the *cluster* package invoked by the *factoextra* package for R software version 3.4.1 (R Development Core Team 2017).

A third method for assigning instars was conducted by utilizing the length of the antenna 2 peduncle of each individual. Individuals were ranked by the length of their antenna 2 peduncle and instars were determined by the second derivative of a smooth spline as described for the first method. The antenna 2 peduncle was selected as a single character because it appeared to display low variability and was thought to experience damage less often compared to other appendages.

The three instar estimation methods were averaged for each individual to assign it into a “final instar” number. The number of days estimated between instars calculated from the “immature growth dataset”

was multiplied by the "final instar" number to estimate the length of time it took each individual to reach each instar. The "final instar" number for individuals that were determined to be sexually mature was used to estimate how long it takes individuals to reach sexual maturity.

Mating trials

Stygobromus flagellatus mating trials were conducted in a dark room at SMARC. Three small plastic containers were modified into flow-through mating chambers. Cameras with infrared night vision were connected to a recording device and each camera was positioned over the top of a mating chamber to record subject behavior. The outside of each container was painted black for better viewing of the subjects.

Three separate chambers populated with a pair of *S. flagellatus* (one female and male). Subjects were allowed to interact, undisturbed from May 10-15, 2018. After the trial was complete, females were inspected for signs of eggs or brood plates, then placed individually in flow-through tubes for later examination. Videos were examined using VLC media player at an increased frame rate during periods of the pair's inactivity, and slowed during periods of observable interaction.

Feeding studies

HIGH-feeding treatment

To test if nourishment may affect body size and or may influence sex determination of immatures, we constructed a flow-through aquarium-style common garden containing about four liters of water and nylon mesh. Ten female and male pairs of *S. russelli* were placed into the mesocosm and fed greater than double the amount of tropical fish flakes, compared to the other common gardens described above. This HIGH-feeding common garden was also supplied with biofilm-conditioned leaves and cotton. The HIGH-feeding common garden was censused ca. every month to inspect for brooding females.

Food preference

To test if *Stygobromus* is an obligate predator, facultative shredder, or scavenger, we conducted a study to examine feeding preference. Fifty adult *S. flagellatus* collected from the Diversion Spring were starved for on average of 16 days (one-week minimum) before randomly placed into a feeding treatment. Food sources consisted of 1) free-swimming *Hyalella* sp., 2) restrained *Hyalella* sp., 3) free-swimming *Lirceolus sp.* donated from SMARC stock cultures, 4) tropical fish flake, 5) tropical fish flake leached for 24 hours, 6) conditioned sycamore leaves, and 7) a thin plastic film to act as a non-food control. *Hyalella* sp. were collected by hand from the Comal river in New Braunfels Texas. Treatments consisted of 1) *Hyalella* sp. verse control, 2) *Hyalella* sp. verse conditioned leaf, 3) *Hyalella* sp. verse fish flake, 4) fish flake verse control, 5) fish flake verse conditioned leaf, 6) conditioned leaf verse control, 7) control verse control, 8) free-swimming *Hyalella* sp., 9) free-swimming *Lirceolus* sp., and 10) diluted flake.

The treatment container consisted of a plastic bowl filled with two liters of water. Two circles were drawn on the bottom of the bowl to serve as "food zones" for food items and controls. Bases for food items consisted of 25x3 mm strips of aluminum foil, which were tightly folded flat around a 22x22 mm glass cover slip. A 3x3 mm duct tape square was affixed to the center of the base with a small drop of hot glue with the adhesive side facing outward. This design served to affix food items and keep them within the food zones. Biofilm-conditioned sycamore leaf, tropical fish flake, and controls were cut into 3x3 mm squares and were affixed to the duct tape. Restrained *Hyalella* sp. were patted dry with a paper towel before being affixed to the tape as a means of immobilization.

A single *S. flagellatus* was added to the bowl and allowed to acclimate in a darkened room; a headlamp with a red 630 nm LED lamp was used as a source of light for observation. After the subject was acclimated, the trial was initiated by placing the food types within the food zones, simultaneously. All subjects were allowed 20 minutes of "foraging time". Timestamps and durations spent in a food ring, inactive, or actively feeding were recorded. Trials with free-swimming prey items were conducted under the same conditions, except that prey were introduced to the center of the bowl using a pipette. Subjects were used for one trial, each. Paired t-test was used to test for food preference for trials with two food items. Percentage response was made for free-swimming prey and diluted fish flakes.

Results and Discussion

Common garden mortality

Our common garden of *Stygobromus pecki* was initiated on February 12, 2018 with 31 individuals (19 female) and was last censused on November 13, 2018, retaining eight individuals (six female). At this mortality rate, (accounting for individuals removed and added to the garden) a power function indicated that the living conditions could support < 10 individuals, indefinitely (**Fig. 3**); though, the last three censuses suggested the population would slope closer to zero over the next several months. Consequently, the common gardens of the other two species had similar trajectories (**Appendix E**). These results suggest density dependent effects on survival. The living space in the aquarium-style common garden accounted for considerably more volume than the flow-through tube gardens; however, it is possible that the realized living space may be relegated to the area of physical substrate and that these areas were similar between the two garden types. Increased survivorship might be enhanced by increasing the amount of heterogeneous space within the aquarium-style common garden; however, more work is needed to gain a better understanding of the optimum captive habitat requirements. Future investigations should consider accounting for the total area of physical substrate in determining optimum conditions for survival.

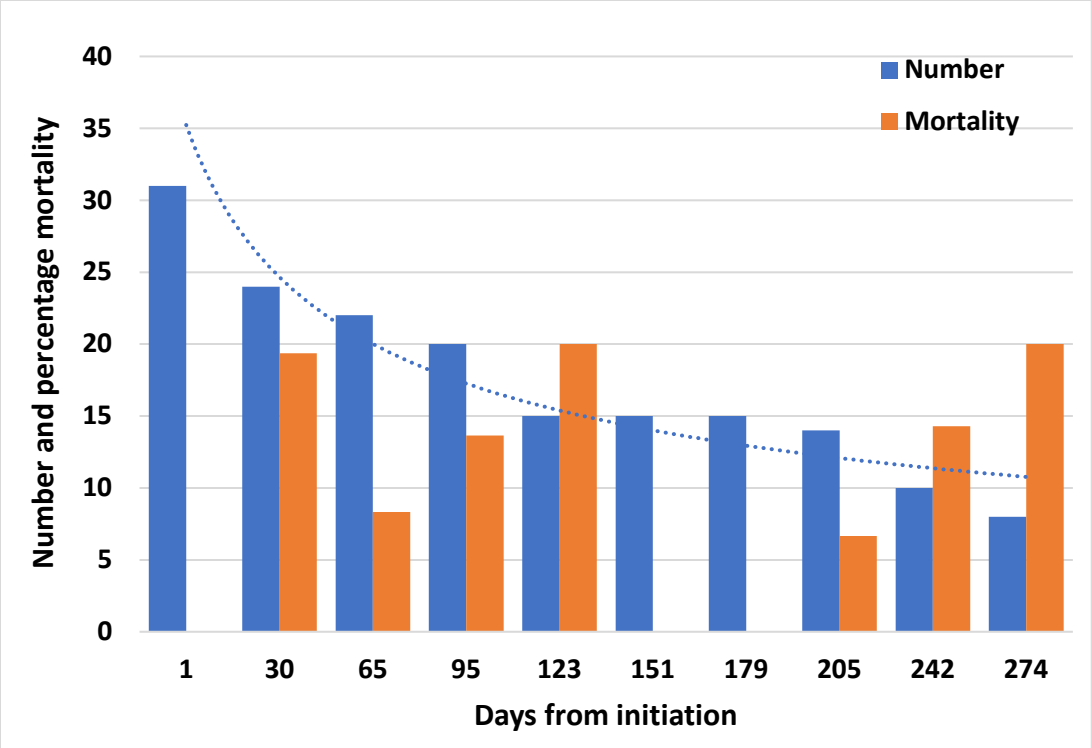

Figure 3. Number of individuals counted during each census, shown as number of days from the initiation of the common garden on February 12, 2018. The percentage mortality between censuses (accounting for individuals removed or added) is also given. The trendline shows the trajectory of surviving individuals based on a power function.

Egg incubation and immature growth

We observed 26 individual brooding female *Stygobromus* that produced 139 eggs. The average brood size across all *Stygobromus* females was approximately 5.3 eggs per brood with 27% released from the marsupium as free-swimming neonates. Twenty-one of these brooding females were *S. pecki*, producing 120 eggs. Of the *S. pecki* eggs that were observed to conclusion, (several mothers are still brooding) 24% developed into free-swimming juveniles. We observed four *S. russelli* mothers with a combined total of six eggs, two developing to free-swimming juveniles and we recorded one *S. flagellatus* adult female with nine eggs of which six developed into free-swimming juveniles. From our common garden culture we observed five females (out of a starting number of 19) to brood a total of 28 eggs. The average egg incubation time observed among eight brooding females (seven *S. pecki* and one *S. flagellatus*) beginning with fresh eggs was 49.5 ± 11.5 days (49.7 ± 12.4 days for only *S. pecki*).

Stygobromus pecki hatched in the laboratory from fresh eggs were never observed to molt before dying; the longest-lived individuals that hatched in captivity were not recorded to last longer than 32 days. This indicates that individuals probably take greater than 32 days before their first molt after leaving the marsupium, at least under the conditions they were exposed to at the SMARC. The average length of these instar 1 *S. pecki* was 2.87 ± 0.16 mm (n = 26). This was lower compared to instar 1 of *S. flagellatus* that were reared under the same protocol (3.32 ± 0.17 mm; n = 6), but higher than *S. russelli* also reared under the same protocol (2.23 ± 0.02 mm; n = 2).

Of the 72 wild-caught *Stygobromus* subjects reared in the laboratory, 30 survived for at least 60 days and were determined to undergo at least one molt. Number of days observed between molts and starting instar was used to determine the number of days spent in successive instars for each individual (**Appendix F**). Individuals were estimated to stay in instar 2 for 48.1 ± 24.3 days, instar 3 for 50.7 ± 19.2 days, and instar 4 for 52.3 ± 22.7 days. Considering the high variation in our estimates, we chose to take the rounded average (50 days) to represent the amount of time between molts and used this number to estimate the number of days to reach sexual maturity (see section below).

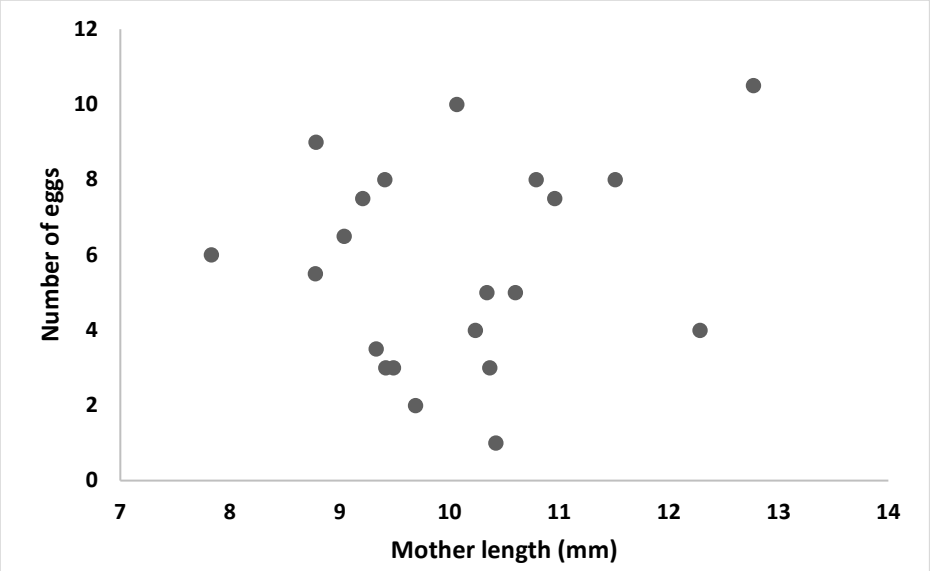

Figure 4. Scatter plot of number of eggs per brooding female of *Stygobromus pecki*.

There was no relationship between the number of eggs per brooding female and female size of *S. pecki* (F-value = 0.67; p-value = 0.42) (**Fig. 4**). Our expectation was that the number of eggs would be would be correlated with female size as have been shown for various epigean species of amphipods (Bollache and Cézilly 2004b). It is possible that these results represent natural conditions, but cannot be compared to wild populations, since brooding females of *S. pecki* have never been collected in the wild. It is also likely that our numbers are underestimated considering the stress evidentially endured during captivity. We discovered that brooding females can drop eggs occasionally during routine handling while preforming condition checks. On one occasion an *S. pecki* mother was observed eating a juvenile just after releasing it from her marsupium, and on at least two occasions the adult female was observed eating an egg that had been released from the marsupium, prematurely. Several linear developed eggs that were dropped prematurely during handling were placed in a K-cup to observe survivorship; all eggs were rotten within a week.

Because of the mother's tendency to eat her own young, neonates were sometimes coxed out of the mother's marsupium. A pipette was used to gently push water over the marsupium, often resulting in the adult female to release developed neonates. This method was found to be preferable because brooding chambers were not effective; neonates observed in the marsupium during one check were often gone during the next condition check. We surmise that developed neonates left the marsupium but did not make it to the side of the chamber where they would be safe from parental predation.

A modified chamber used in conjunction with the method of removing juveniles by hand when they become fully developed, may decrease the mortality of the juveniles. Alternatively, placing females within a small flow-through aquarium "nursery" with a great amount of heterogenous habitat (perhaps several layers of aquarium stones) and plenty of food may provide the mother with a less stressful

environment and could allow her off spring the opportunity to find hiding places. Once the marsupium has been emptied, the mother can be returned to general holding and the nursery could serve as the first-stage habitat for rearing young.

Number of instars until maturity

Fifty-eight wild-caught individuals were graded into size classes ranging from 2.5 mm to 9 mm. Only one individual was found at size class 2.5 mm, indicating that it represented an earlier instar than size class 3 mm or was *S. russelli*. *S. russelli* are presumed to be smaller than *S. pecki* for equivalent stages of development (see egg incubation and immature growth section above). Also, it was increasingly difficult to find specimens > size class 7.0 mm that had all of their body parts.

Only one male was sexed at size class 6 and its distal process on the peduncle of uropod 1 was not completely developed. At size class 6.5 mm, half of the specimens were males with well-developed apical processes, indicating that most individuals in this size class were sexually mature (at least one female had brooding plates indicating that she recently reproduced). Therefore, individuals that reached size class 6.5 mm and above were considered to be mature; specimens with an apical process were considered male and those without a process were considered female. Individuals at size class 6 mm and below were considered to be immature. From this, 16 individuals were identified as sexually mature.

PCA indicated that there was a strong association of individuals to a size class at early instars, but there was increasing variation among size classes at later instars (**Fig. 5**). Principal components 1 and 2 explained 88.6 and 2.4 percent of the variation, respectively. The character states with the highest loading on component 1 were the number of uropod 1 peduncle spines (0.576), the number of uropod 1 outer ramus spines (0.343), the number of uropod 2 peduncle spines (0.311), the number of uropod 2 inner ramus spines (0.304), the number of uropod 1 inner ramus spines (0.228), and the number of uropod 2 outer ramus spines (0.210).

At size class 6 mm there was a noticeable amount of variability between individuals within this size class and those beyond. It was also noted that there were a few individuals that appeared to cluster within the previous classes. Also, there appeared to be a considerable amount of overlap among successive size classes that may indicate a combining of instars, for instance size class 4.5 mm and 5.5 mm appeared to be distinct instars based on the ordination; however, size class 5 mm overlapped with both of these classes, indicating that individuals of this size class represented more than one instar. Loadings for all characters on principal components 1 and 2 are given in **Appendix G**.

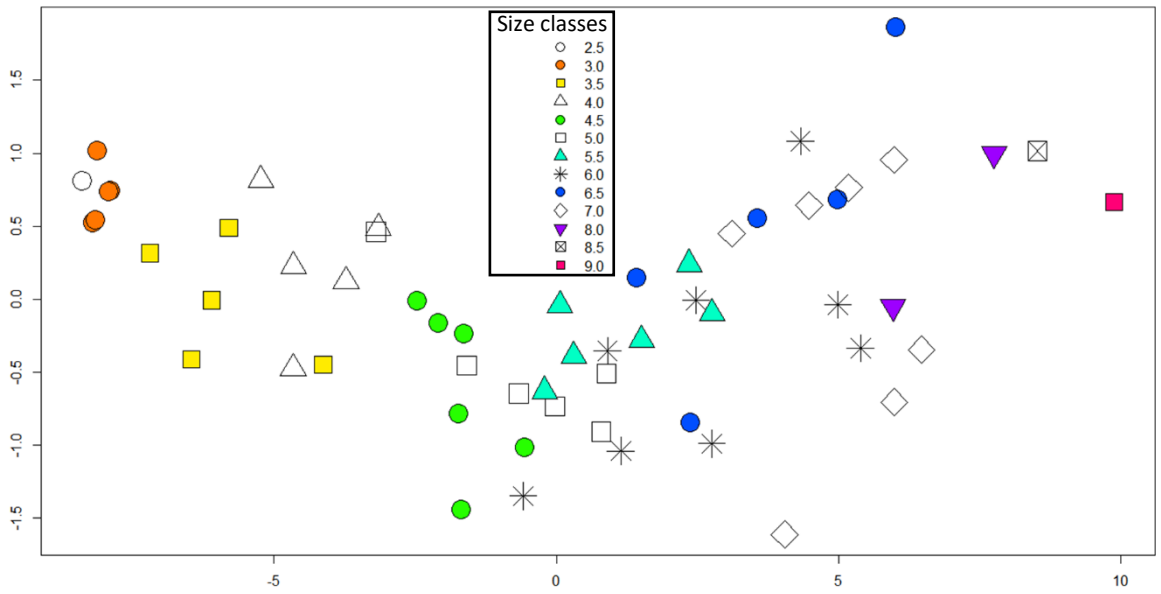

Figure 5. Ordination of size classes, given by the set of symbols, over principal components 1 and 2 of character states.

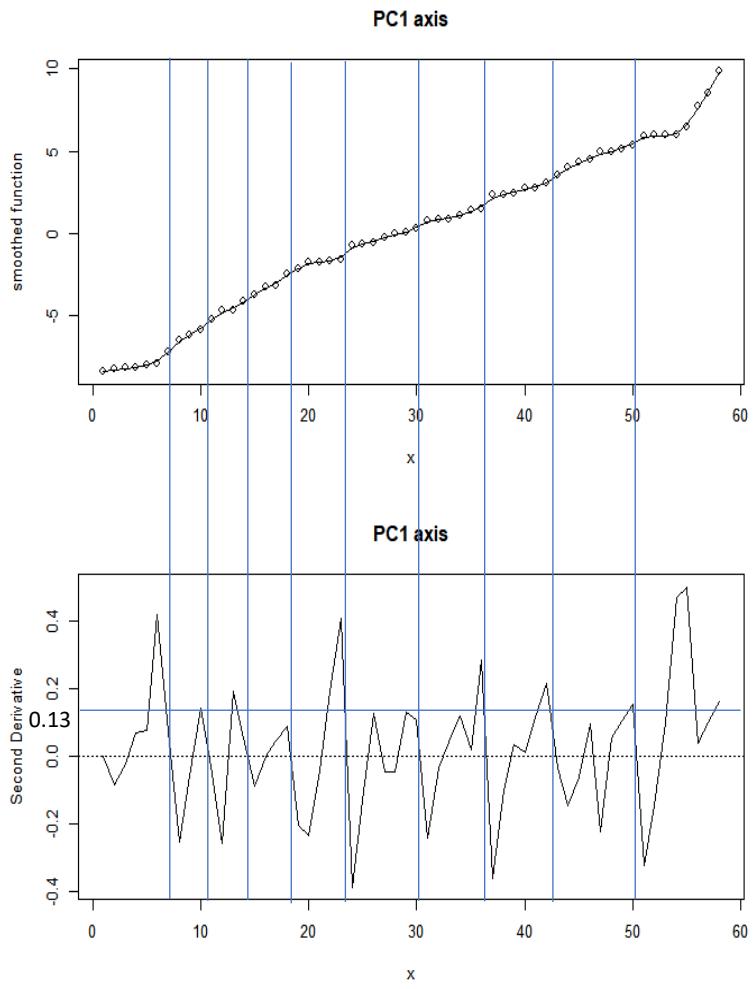

Figure 6. Instars determined by the second derivative of a smooth spline of PCA axis 1. A minimum change in rate of 0.13 to zero was interpreted as a break between instars.

Each individual was ranked by their coresponding scores for principal component 1 and a smooth spline was fit over an even distribution of the points. The second derivative found 13 inflection points going from high to low; however, some of the rate changes were not great and therefore we set a threshold of 0.13 as a minimum change of the second derivative to zero required for an inflection to be included as an estimated break between instars. Ten instar breaks were delineated in this way; however, because there were few representatives of larger size classes, and to stay consistent with the other methods, 8 instars were delineated where individuals allocated to the eighth instar were considered greater than 7 (**Fig. 6**).

The gap statistic of the hierarchical cluster analysis delineated 8 clusters among classes (**Fig. 7**). However, individuals falling into cluster 8 were considered to represent instars 8 and higher; it was presumed that if more representatives of size classes greater than 7 mm had been available the analysis would have delineated more clusters and it was likely that cluster 7 was already a mix of several instars (**Fig. 8**).

Measurements of the antenna 2 peduncle correlated strong with body length (r^2 = 0.979; *p-value* < 0.001, *n* = 58) and therefore was considered a good surrogate of growth (**Fig. 9**). Inflections points estimated 12 potential instars. However, considering that the representation of individuals beyond size class 7 mm was limited, the number of instars were capped at 8 where individuals falling into instar 8 were considered to be in instar 8 or higher (**Fig. 10**).

Instar estimates of wild-caught specimens by all three methods, the average of these, and instars delineated for laboratory-reared subjects by BPJ ("immature growth dataset") are given in **Table 1** and **Fig. 11.** In general, all methods were in agreement towards grading individuals into early instars, but showed increasing disagreement for later instars. Formal statistics were not performed, but BPJ and PCA-delineated instars were in close agreement based on body length, whereas length of antenna 2 peduncle tended to grade larger sizes into earlier instars and cluster analysis tended to grade larger individuals into later instars (**Fig. 11**).

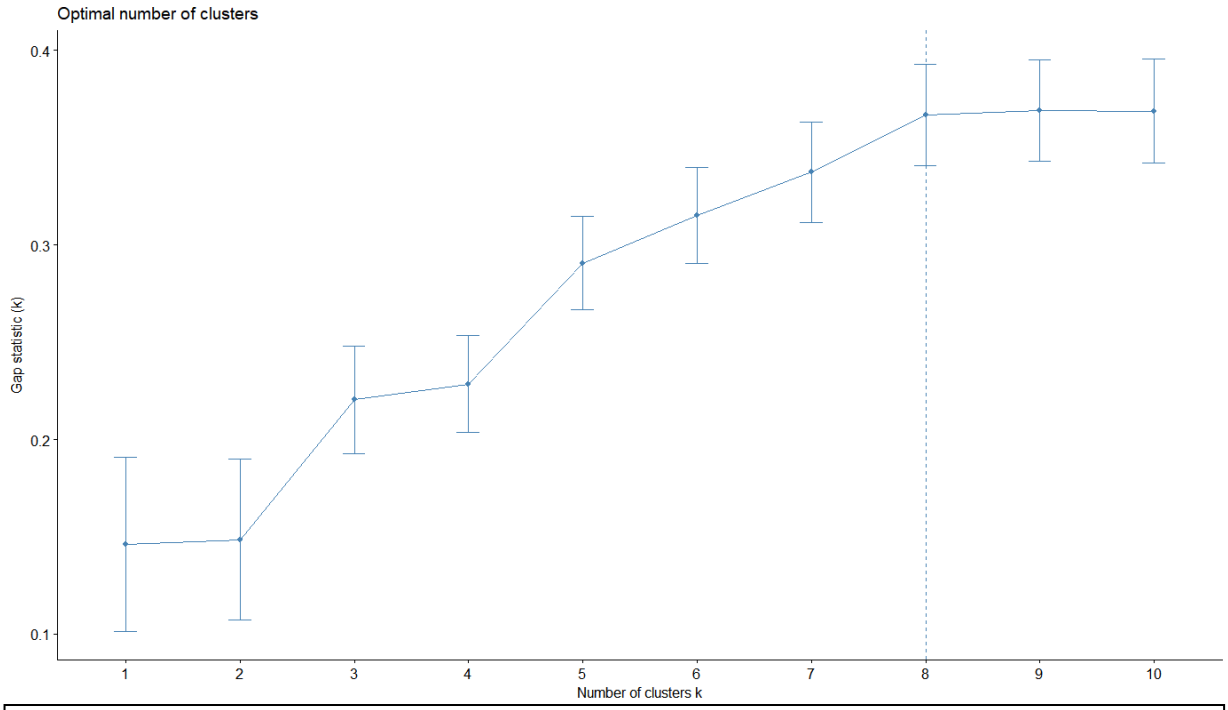

Figure 7. Number of clusters determined by the gap statistic of a hierarchical cluster analysis.

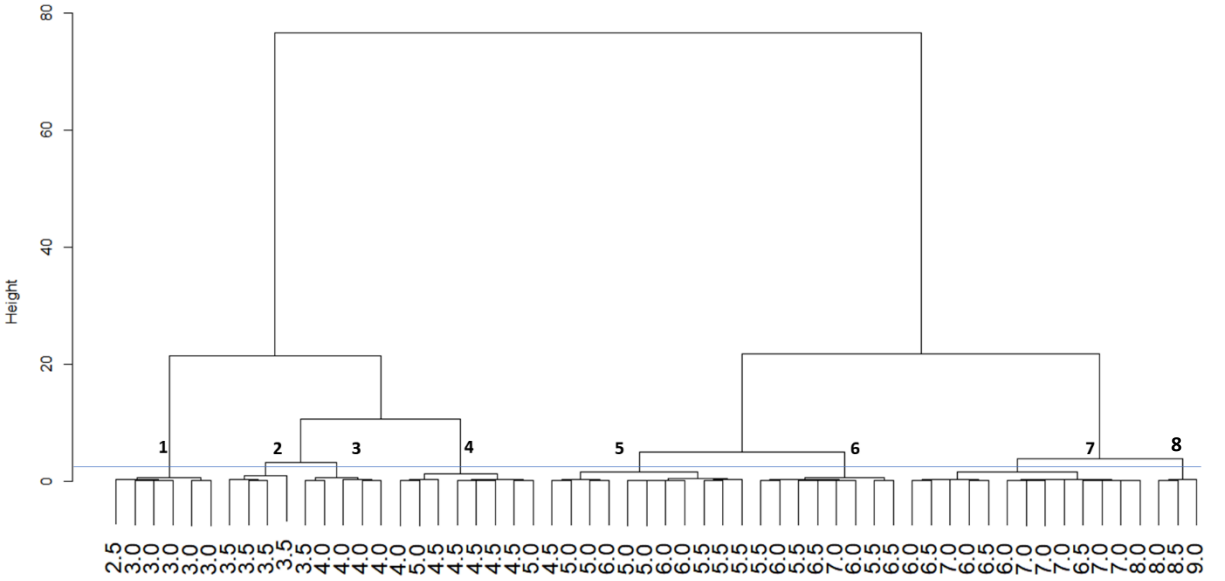

Figure 8. Hierarchical cluster analysis of size classes delineated from Bray-Curtis distance of 34 morphological characters. Estimated instars given by the gap statistic are numbered.

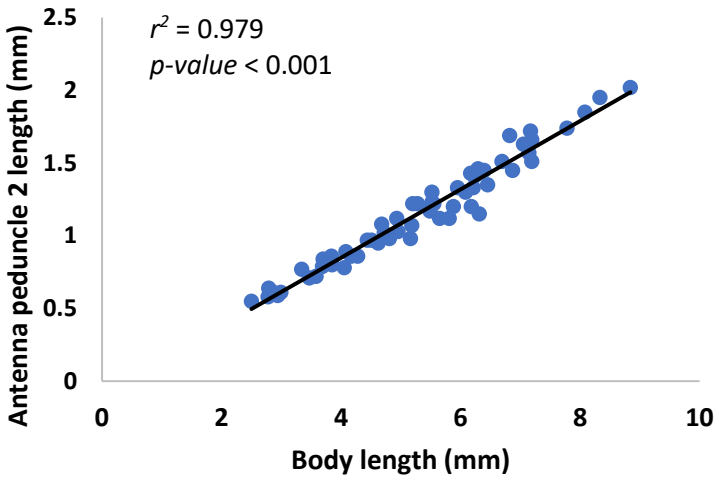

Figure 9. Relation between body length and antenna 2 peduncle.

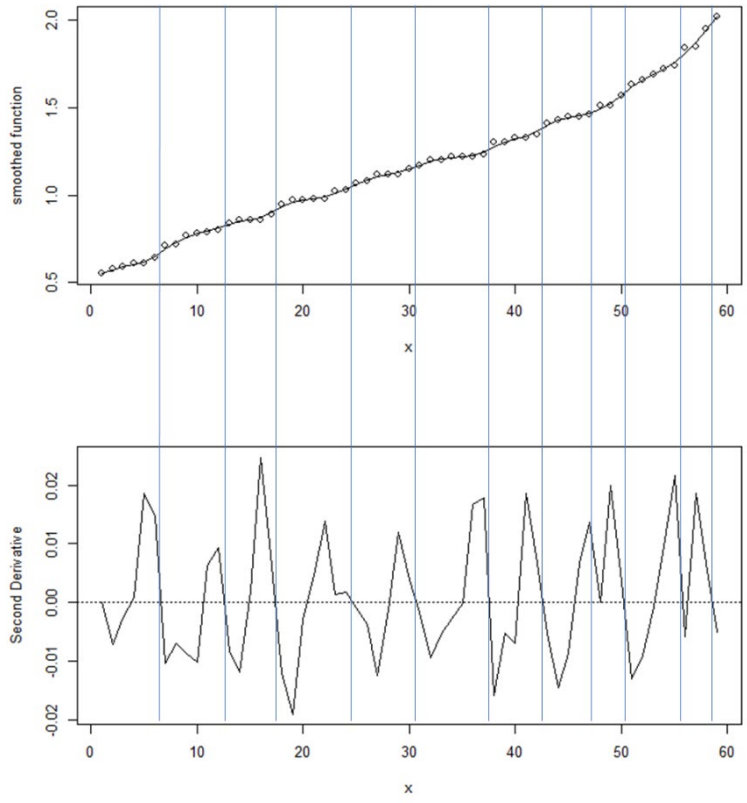

Figure 10. Instars determined by the second derivative of a smooth spline of ranked antenna 2 peduncle lengths. A change in rate from the high peak to zero was interpreted as a break between instars.

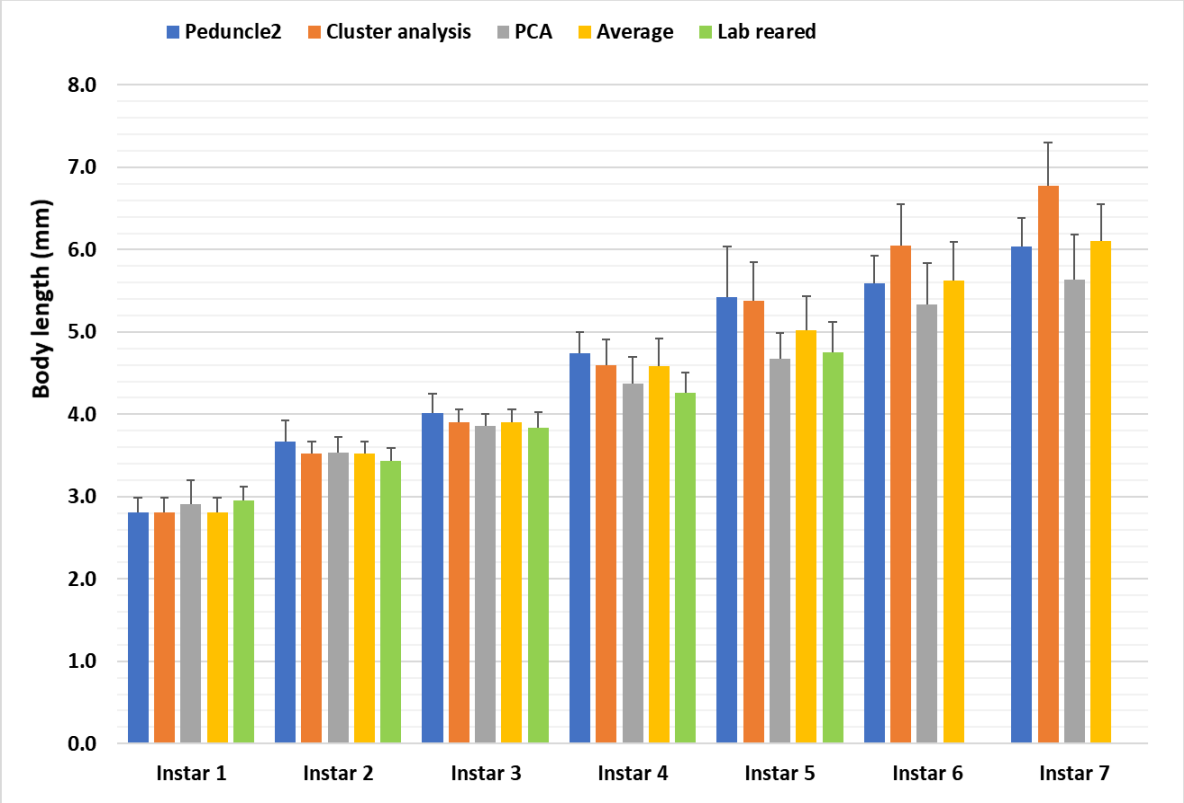

Fig. 11. Average body length of estimated instars determined by quantitative methods utilizing character states, the average of those three methods (antenna 2 peduncle length, cluster analysis, principal component 1), and estimates based on best professional judgment of individuals reared in the lab.

Table 1. Mean body size of estimated instars determined by the antenna 2 peduncle, cluster analysis, and PCA for wild-caught specimens. Instar estimates of laboratory reared subjects by best professional judgment ("immature growth dataset") and the estimated length of time spent in instars are also given.

		Instar estimate							Instar 8
Method		**Instar 1**	**Instar 2**	**Instar 3**	**Instar 4**	**Instar 5**	**Instar 6**	**Instar 7**	**& greater**
Peduncle2	Mean	2.81	3.66	4.01	4.74	5.43	5.59	6.04	-
	SD	0.17	0.26	0.24	0.25	0.61	0.34	0.35	-
	n	6	6	5	7	6	7	5	16
Cluster analysis	Mean	2.81	3.52	3.90	4.60	5.38	6.05	6.78	-
	SD	0.17	0.15	0.16	0.32	0.47	0.50	0.52	-
	n	6	4	5	8	11	8	13	3
PCA	Mean	2.91	3.54	3.86	4.37	4.67	5.33	5.64	-
	SD	0.29	0.18	0.14	0.33	0.32	0.50	0.54	-
	n	7	3	4	4	5	7	6	22
Average: "final instar"	Mean	2.81	3.52	3.90	4.58	5.02	5.63	6.10	-
	SD	0.17	0.15	0.16	0.34	0.42	0.46	0.45	-
	n	6	4	5	7	5	9	8	14
"Immature growth dataset"	Mean	2.96	3.44	3.84	4.27	4.75			
	SD	0.16	0.15	0.18	0.24	0.37			
	n	13	27	29	22	6			
	Mean no. days	-	48.1	50.7	52.3	-			
	SD		24.3	19.2	22.7				

The "final instar" number provided by the average of the three estimation methods found one mature individual in instar 6, two mature individuals in instar 7, and 13 mature individuals in instars 8 and above. The "final instar" number of individuals from size class 6.5 mm (considered the minimum size to reach maturity) one individual was found in instar 6, two in instar 7, and 3 in instar 8. Based on the limited information gathered by these studies we estimate that individuals will go through at least 6 instars before reaching maturity, but many may not reach sexual maturity until they enter instar 8 or higher. Using the rounded average of 50 days between molts provided from the "immature growth dataset" (**Table 1**), we provide an estimate based on the "final instar" number of size class 6.5 individuals of 370 ± 40 days (range = 300 – 400 days) before reaching maturity at given ambient conditions at the SMARC refuge. However, we expect this estimate to vary considerably between individuals based on access to resources and stress. Caution should be used if applying this number to efforts related to propagation or husbandry.

Degree days were recorded for the majority of the length of our studies. However, no amphipods were tracked for an extensive amount of time to make a direct relation to degree days. If we consider the average number of degree days to be 21.8 ± 0.6°C (BIO-WEST, unpublished), individuals will require ca. 8066 degree days (range = 6540 – 8720 degree days) to reach sexual maturity, assuming temperature is a driving factor.

Our estimates on number of instars to reach maturity are arguably greater than what occurs naturally. Considering that their natural habitat temperatures are probably higher, it is likely that they grow faster in the wild, given the same food resources. However, captive individuals are fed a high protein diet that is probably richer than what they encounter in the wild and in this respect, they may grow faster compared to wild populations. Furthermore, food resource differences may contribute to differences in timings of molts. For instance, captive individuals may undergo seven molts in a little over a year before reaching sexual maturity; however, wild populations may undergo fewer molts over a longer period of time before reaching sexual maturity. It should also be noted that the average number of days

estimated between molts in the laboratory may be overestimated. Individual measures were taken ca. once every four weeks for most of the specimens. Molts of reared individuals were "observed" on an average every 50 days; however, individuals were surely molting within the four-week period of time between photographs. Thus, it is likely that they number of days between molts is less.

Our estimates of time between molts is also incomplete. Considering none of our captive reared neonates were observed to molt over a maximum period of 32 days, we do not know how much time is spent in the first instar. Our estimates of number of days between molting from instar 2 to 3, instar 3 to 4, and instar 4 to 5 were 48, 51, and 52 days, respectively. Though subtle, this implies that older individuals may take longer to molt and therefore our estimates are likely incomplete for many life stages.

Mating trials

We did not observe any behavior suggestive of mating. Interactions were seldom and typically brief; subjects mostly avoided each other and often remained inactive for hours. After the trials, females were inspected every few weeks to a month and as of November 13, 2018, two of the three females remain alive, but no eggs or brood plates have been observed. It is likely that no mating took place during any of the trials and a longer trial duration would be needed to observe mating behavior. It is still unknown if females are only receptive towards mating only after a molt, and if so, trails may need to be conducted for several months. Although it would not be feasible for direct viewing or video recording, it is recommended that similar mating chambers containing nylon mesh or other heterogenous habitat with plenty of food is used during mating experiments.

Feeding studies

HIGH-feeding treatment

This experiment was conducted from March 20, 2018 through July 13, 2018. At the time this study was discontinued, two out of 10 females and males, respectively, remained in the community tank. In May, one female was found brooding with two eggs. Another female was found in June with fully developed brood plates, but no eggs. Although this experiment indicated that 20% of the females had produced a brood with elevated resources within a three-month period of time, it also suggested that the conditions were not suitable for long-term survival as 80% of the subjects died during this time.

Compared to the other *S. russeli* common gardens, it appears that increases in resources are likely to increase female brooding; however, more cleaning will likely be necessary for maintaining a healthy habitat. More work is needed to gain a better understanding of the feeding requirements for producing healthy broods.

Food preference

Fish flakes were found to be more preferable to conditioned leaf (*t-value* = 5.88; *p-value* = 0.004), the control plastic strip (*t-value* = 2.45; *p-value* = 0.07), and to restrained *Hyalella* sp. (*t-value* = 3.13; *p-value* = 0.035). All other paired treatments were not significant. With these comparisons the preferred food of *S. flagellatus* and probably most species of *Stygobromus* is the fish flake which is a high protein resource.

The response of *S. flagellatus* to the initial encounter with free swimming *Hyalella* sp. was indifference or it appeared disturbed, jetting away after contact. However, on two occasions the subject was left with *Hyalella* sp. overnight and these prey items were found to be partially or fully consumed the following morning. Predatory responses were observed in two out of five trials with free-swimming

Lirceolus sp. (**Fig. 12**). These responses suggest that *Stygobromus* will eventually identify and subdue living prey items but from these observations, it appears that they do not have any sensory detection for these prey items other than mechanoreception by direct contact during random encounters. On the other hand, it appeared that *S. flagellatus* was able to sense fresh fish flakes and was observed to feed on these within the 20 min trial duration 80% of the time. It was noted that the diluted fish flake with no other food item was eaten in three out of five trials (60%). Some experiments have suggested that amphipod species may be able to detect amino acids (Ide et al. 2006) which may explain why flake food was most preferred. This suggests that predator responses do occur; however, longer acclimation times may be necessary before subjects are ready to subdue prey.

Figure 12. Example of predation behavior of *Stygobromus flagellatus* feeding on *Lirceolus* sp.

Conclusions and recommendations

Many questions remain about the life histories of *S. pecki* that cannot be answered within a one-year study. New strategies for accommodating brooding females and rearing immature subjects need to be considered. Our estimates on the development time for individuals to reach sexual maturity suggest ca. a year which is in line with previous work (Fries el al. 2004). It should also be noted that the number of instars estimated to reach maturity and the time it takes to proceed from one instar to another were determined with wild-caught captive individuals. There may be differences in growth, time between molts, size of each instar, and number of instars compared to F1 reared subjects. Additionally, we can conclude the following with respect to captive holding of *S. pecki:*

- Fewer individuals should be housed within multiple mesocosms, when possible.
- Mesocosms should contain plenty of heterogenous habitat with plentiful surface area.
- Flow-through aquariums are suitable mesocosms; however, other types of mesocosms can be considered.
- Commercial fish flakes are a suitable food for their diet; however, living prey items may provide an added supplement.
- Conditioned leaf material may or may not provide a useful food resource, but it probably does provide additional habitat, which is important.
- Brooding mothers should be provided with the most stress-free habitat possible with plenty of heterogenous habitat to protect newly released neonates and sufficient amounts of food material (but not too much to ruin the water quality!).

- Eggs should be fully developed and released within ca. 50 days but may require more time and if necessary, removal of neonates should be considered at this period of time to ensure maximum survival.
- Hatchlings will require close to a year or more before reaching sexual maturity and it is probably best to keep them apart from adults until they are ready to mate. It may also be worth separating early instars as they develop, at least into smaller groups to avoid cannibalism. (If you ever observed a praying mantis egg sac to hatch in an aquarium you would remember that hundreds of little mantids would kill each other off until there were just a few).

Due to the natural aggressive behavior and apparent susceptibility to stress, maintaining a self-propagating refuge of 500 animals should include multiple mesocosms. Even more important is the amount of habitable surface area provided within the mesocosm itself. The combination of nylon mesh, leaves, and rocks appears to provide suitable cover; however, the implementation of other substrates, such as the PVC shavings is worth exploring. We recommend that an adequate refuge should also include separate mesocosms to ensure sustainable offspring propagation.

Acknowledgments

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Literature Cited

Adams, J., and P. J. Greenwood. 1983. Why Are Males Bigger than Females in Pre-Copula Pairs of *Gammarus pulex*. Behavioral Ecology and Sociobiology 13:239-241.

Bollache, L., and F. Cezilly. 2004a. State-dependent pairing behaviour in male *Gammarus pulex* (L.) (Crustacea, Amphipoda): effects of time left to moult and prior pairing status. Behavioural Processes 66:131-137.

Bollache, L., and F. Cézilly. 2004b. Sexual selection on male body size and assortative pairing in *Gammarus pulex* (Crustacea: Amphipoda): field surveys and laboratory experiments. Journal of Zoology 264:135-141.

Bulnheim, H. P. 1978. Interaction between genetic, external and parasitic factors in sex determination of the crustacean amphipod *Gammarus duebeni.* Helgolander Wissenschaffliche Meeresuntersuchungen 31:1-33.

Bulnheim, H. P., and J. Vávra. 1968. Infection by the microsporidian *Octosporea effeminans* sp. n., and its sex determining influence in the amphipod *Gammarus duebeni* . The Journal of Parasitology 54:241-248.

Crawford, D. M., and D. C. Tarter. 1979. Observations on the life history of the freshwater amphipod, *Crangonyx forbesi* (Hubricht and Mackin), in a spring-fed cistern in West Virginia. American Midland Naturalist 2:320-325.

Ethridge J. Z., J. R. Gibson, and C. C. Nice. 2013. Cryptic diversity within and amongst spring-associated *Stygobromus* amphipods (Amphipoda: Crangonyctidae). Zoological Journal of the Linnean Society 167:227-242.

Franceschi, N., J. Lemaitre, F. Cezilly, and L. Bollache. 2010. Size-assortative pairing in *Gammarus pulex* (Crustacea: Amphipoda): a test of the prudent choice hypothesis. Animal Behaviour 79:911-916.

Fries, J. N., J. R. Gibson, and T. L. Arsuffi. 2004. Edwards Aquifer spring invertebrate survey and captive maintenance of two species. Report for U. S. Fish and Wildlife Service. Austin Ecological Services Field Office, Austin, Texas.

Gibson J. R., S. J. Harden, and J. N. Fries. 2008. Survey and distribution of invertebrates from selected springs of the Edwards aquifer in Hays and Comal counties, Texas. The Southwestern Naturalist 53:74-84.

Gledhill, T., and M. Ladle. 1969. Observations on the life-history of the subterranean amphipod *Niphargus aquilex aquilex* Schiödte. Crustaceana 16:51-56.

Gross, M. Y., and D. S. Maycock, M. C. Thorndyke, D. Morritt, M. Crane. 2001. Abnormalities in sexual development of G. pulex below sewage treatment works. Environmental Toxicology and Chemistry 20:1792-1797.

Holsinger, J. R. 1967. Systematics, speciation, and distribution of the subterranean amphipod genus *Stygonectes* (Gammaridae). U.S. National Museum Bulletin 259:1-176.

Holsinger, J. R. 1994. Pattern and process in the biogeography of subterranean amphipods. Hydrobiologia 287:131-145.

Ide, K., Takahashi, K., Nakano, T., Sato, M. and Omori, M. (2006) Chemoreceptive foraging in a shallow-water scavenging lysianassid amphipod: role of amino acids in the location of carrion in Scopelocheirus onagawae. Marine Ecology Progress Series 317:193-202.

Kosnicki, E., E. P. Julius, and J. R. Gibson. 2019. Variation in the number of lateral telson spines of *Stygobromus flagellatus* (Benedict, 1896) (Amphipoda: Crangonyctidae), a subterranean species from Texas, USA. Journal of Crustacean Biology https://doi.org/10.1093/jcbiol/ruy106

Lucas L. K., Z. Gompert, J. R. Gibson, K. L. Bell, C. A. Buerkle, and C. C. Nice. 2016. Pervasive gene flow across critical habitat for four narrowly endemic, sympatric taxa. Freshwater Biology 61:933-946.

McCabe, J., and A. M. Dunn. 1997. Adaptive significance of environmental sex determination in an amphipod. Journal of evolutionary biology 10:515-527.

Nowlin, W.H., B. Schwartz, R. Gibson, and M.L.D. Worsham. 2015. Refugia research: development of husbandry and captive propagation techniques for invertebrates covered under the Edwards Aquifer Habitat Conservation Plan. Edwards Aquifer Authority study no. 138-14-HCP.

Plaistow, S. J., and L. Bollache, F. Ceuzilly. 2003. Energetically costly precopulatory mate guarding in the amphipod *Gammarus pulex*: causes and consequences. Animal Behviour 65:683-691.

Robinson, B. W., and R. W. Doyle. 1985. Trade-off between male reproduction (amplexus) and growth in the amphipod *Gammarus lawrencianus*. Biological Bulletin 168:482-488.

Sutcliffe, D. W. 1992.Reproduction in *Gammarus* (Crustacea , Amphipoda): basic processes. Freshwater Forum 2 102-128.

Tibshirani, R., G. Walther, and T. Hastie. 2001. Estimating the number of clusters in a data set via the gap statistic. Journal of the Royal Statistical Society: Series B, 63:411-423.

United States Fish and Wildlife Service. 1997. Endangered and threatened wildlife and plants; final rule to list three aquatic invertebrates in Comal and Hays counties, TX, as endangered. Federal Register 62 (18 December 1997):66295-66304.

Watt, P. J., and J. Adams. 1993. Adaptive sex determination and population dynamics in a brackish-water amphipod. Estuarine, Coastal and Shelf Science 37:237-250.

Worsham, M. L. D., E. P. Julius, C. C. Nice, P. H. Diaz, and D. G. Huffman. 2017. Geographic isolation facilitates the evolution of reproductive isolation and morphological divergence. Ecology and Evolution 00:1–11. https://doi.org/10.1002/ece3.3474

Appendix A

Brooding Mother Accession and Condition Datasheet

Circle one: Accession or Condition check

MotherID:_____ Date(mm/dd/yyyy):_____

Photo#:_____(Only necessary for original start date and when brood is gone)

Specimen Orientation during photo (circle one): Straight Curved Ball

Length of mother:_____(If one was taken)

Population of Origin:_____

Species:_____

Brood Size Estimated:_____

Egg Condition (circle one): Fresh Eggs Linear Embryo

Neonate Free Swimming

No Eggs

Mother Returned? Yes No
(If yes update Mother Accession Table)

Mother Dead? Yes No
(If yes update Mother Accession Table)

Other notes:

Immature *Stygobromus* Growth and Accession Datasheet

Date:________________________________

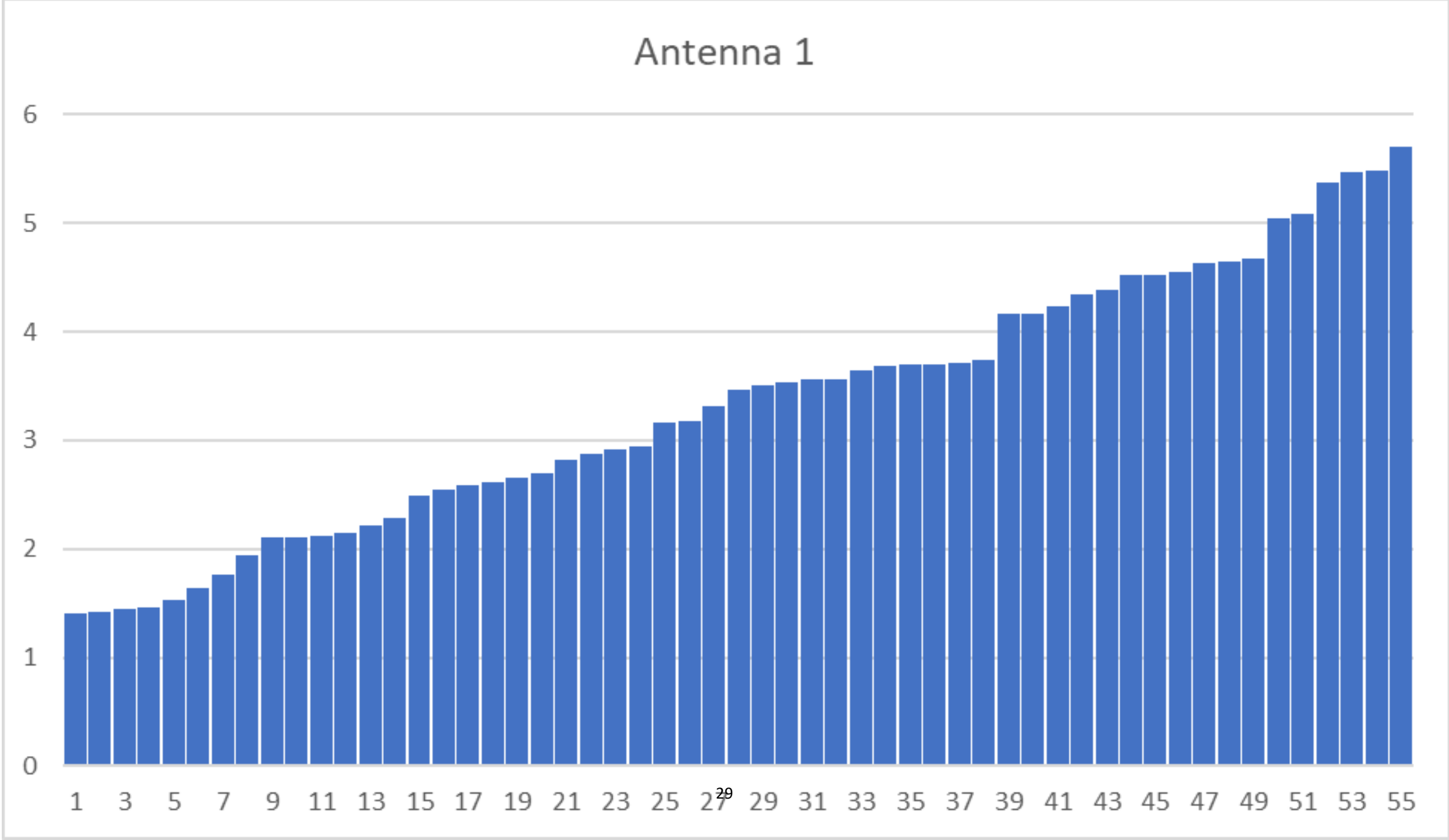
ImmatureID	**X** If First Measure	**X** if Dead	**X** if Returned	PhotoCode(s)	MotherID (If first measure)	Date Entered to db
Body Length (mm)		Antennae1 Length (mm)		Antennae2 Length (mm)	Notes	

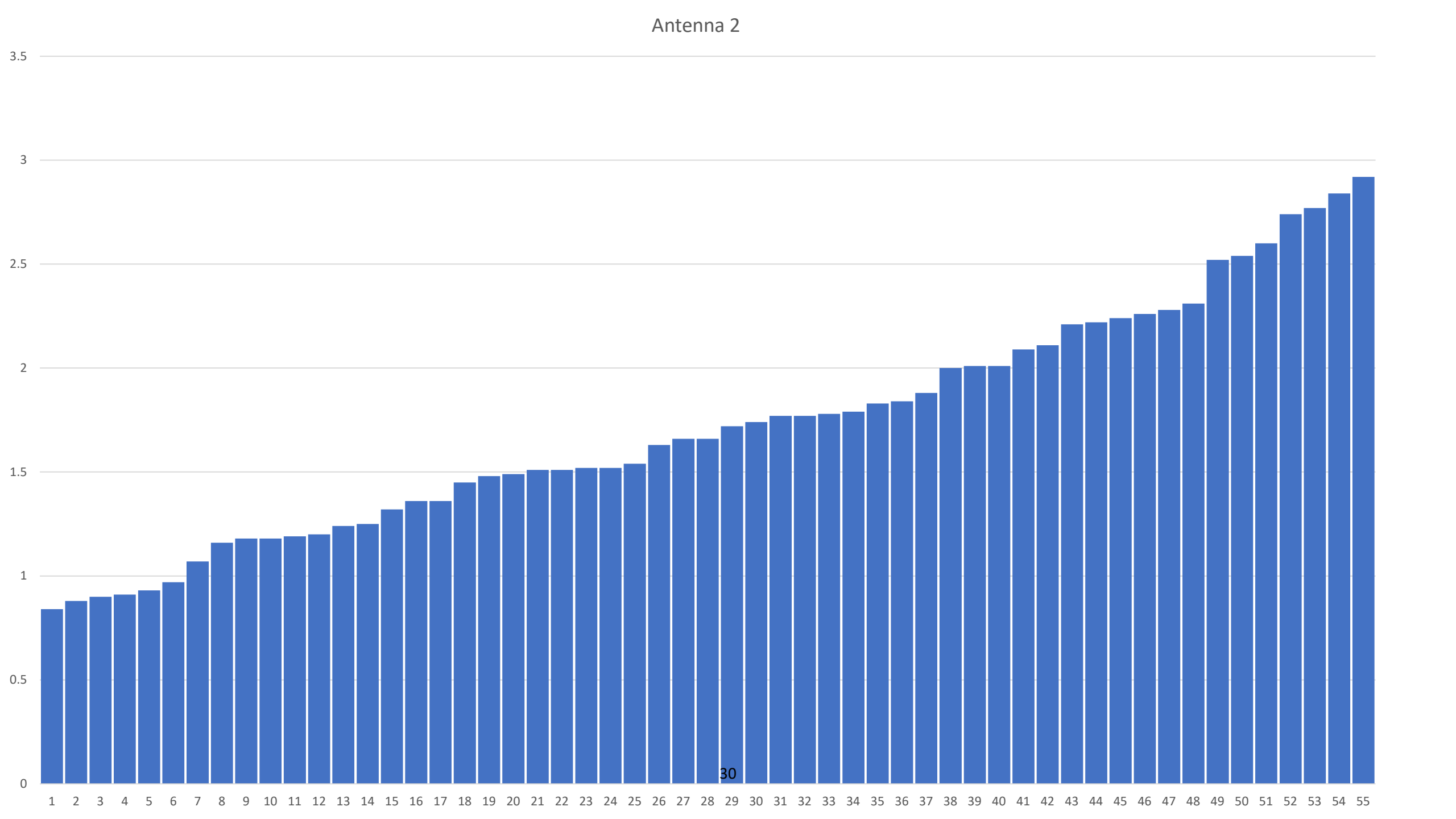
ImmatureID	**X** If First Measure	**X** if Dead	**X** if Returned	PhotoCode(s)	MotherID (If first measure)	Date Entered to db
Body Length (mm)		Antennae1 Length (mm)		Antennae2 Length (mm)	Notes	

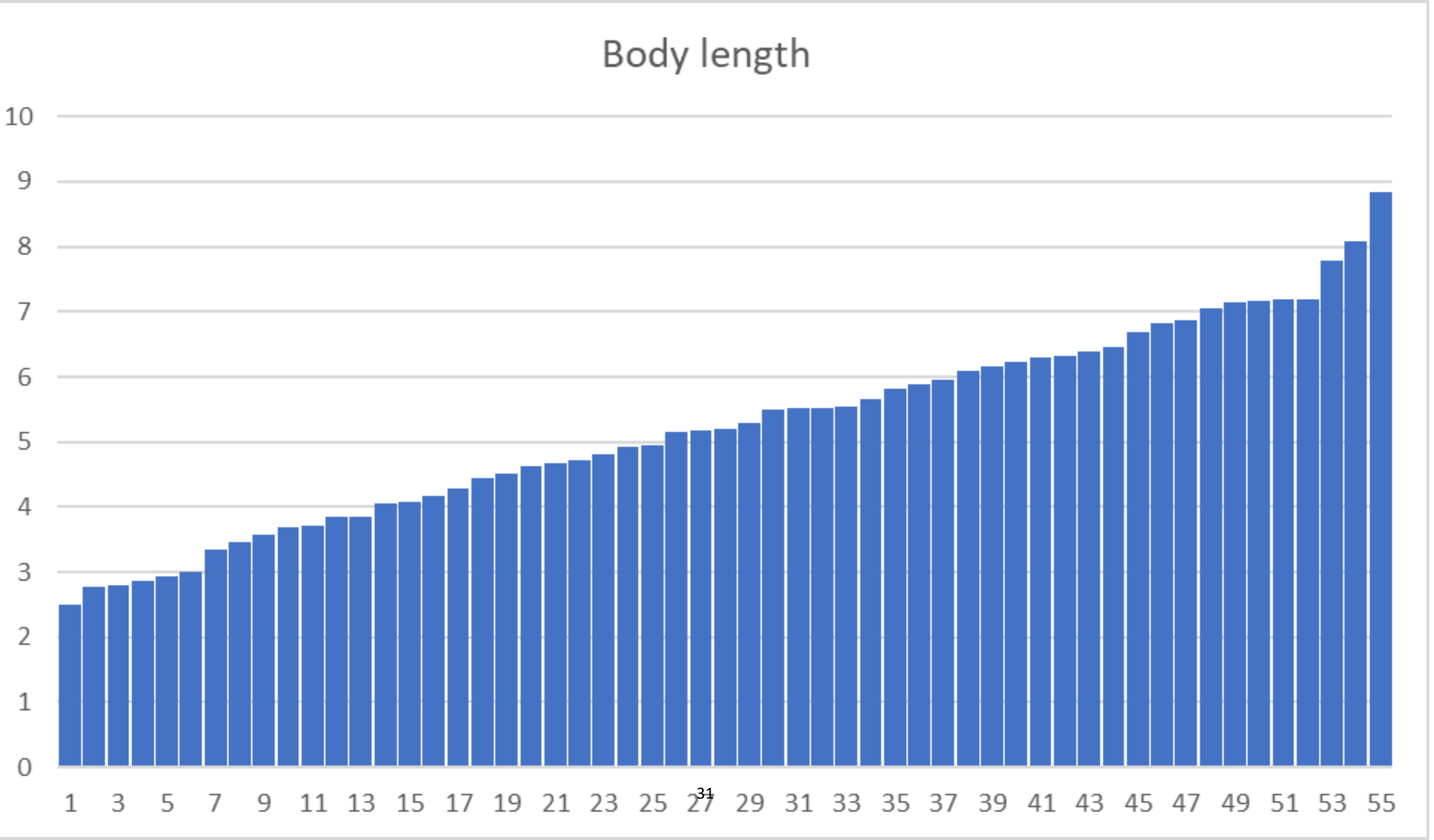
ImmatureID	**X** If First Measure	**X** if Dead	**X** if Returned	PhotoCode(s)	MotherID (If first measure)	Date Entered to db
Body Length (mm)		Antennae1 Length (mm)		Antennae2 Length (mm)	Notes	

ImmatureID	**X** If First Measure	**X** if Dead	**X** if Returned	PhotoCode(s)	MotherID (If first measure)	Date Entered to db
Body Length (mm)		Antennae1 Length (mm)		Antennae2 Length (mm)	Notes	

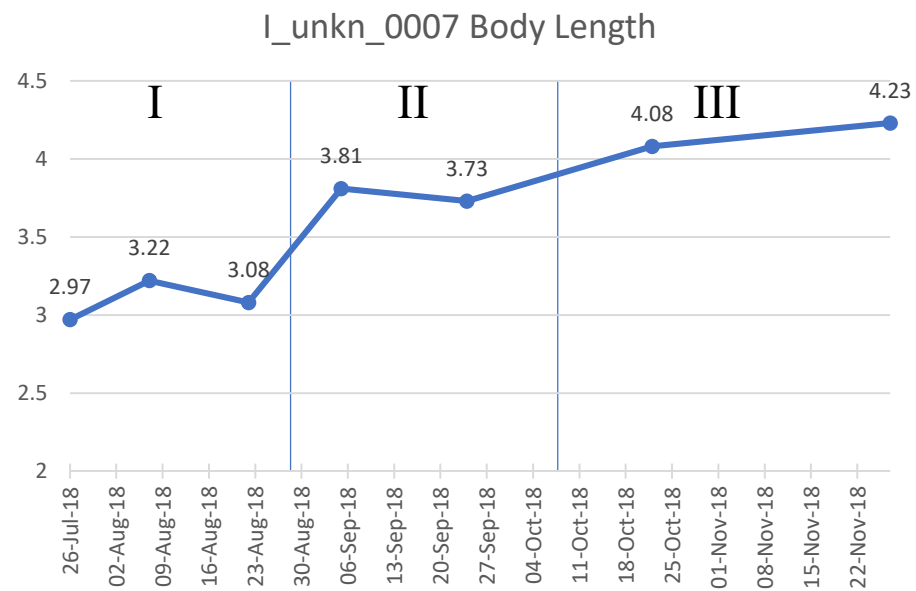
Appendix B

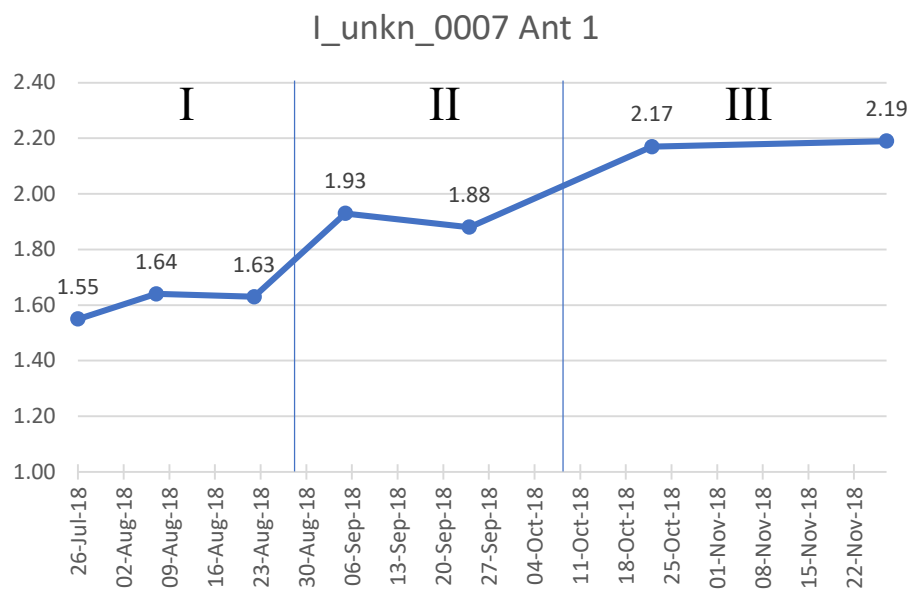

Antenna 1
6
5
4
3
2
1
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55

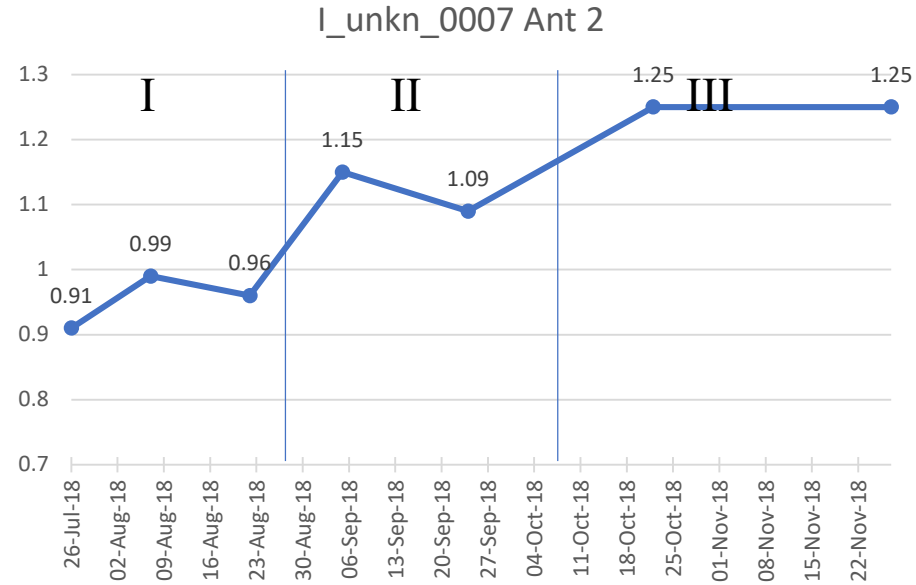

Antenna 2
3.5
3
2.5
2
1.5
1
0.5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55


Body length
10
9
8
7
6
5
4
3
2
1
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55

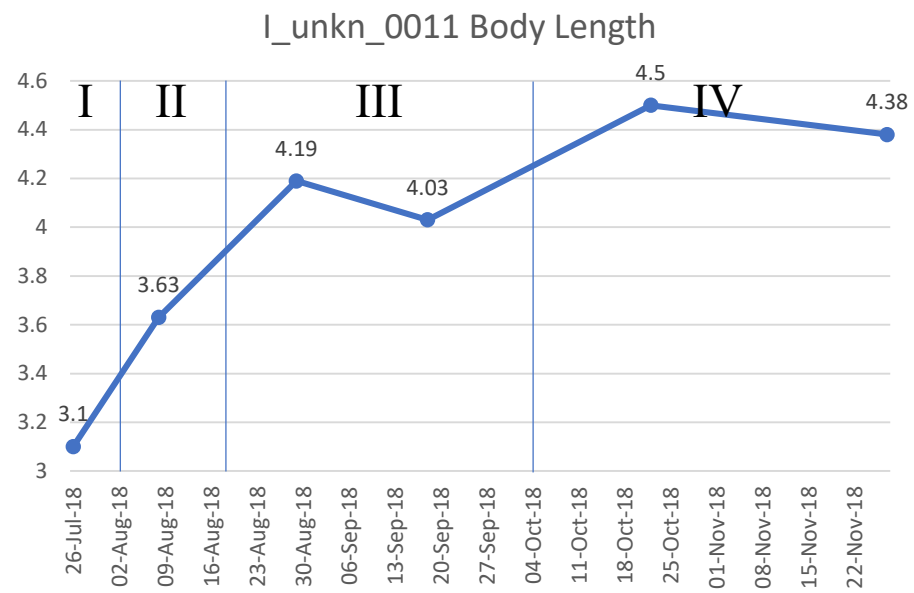
Appendix C

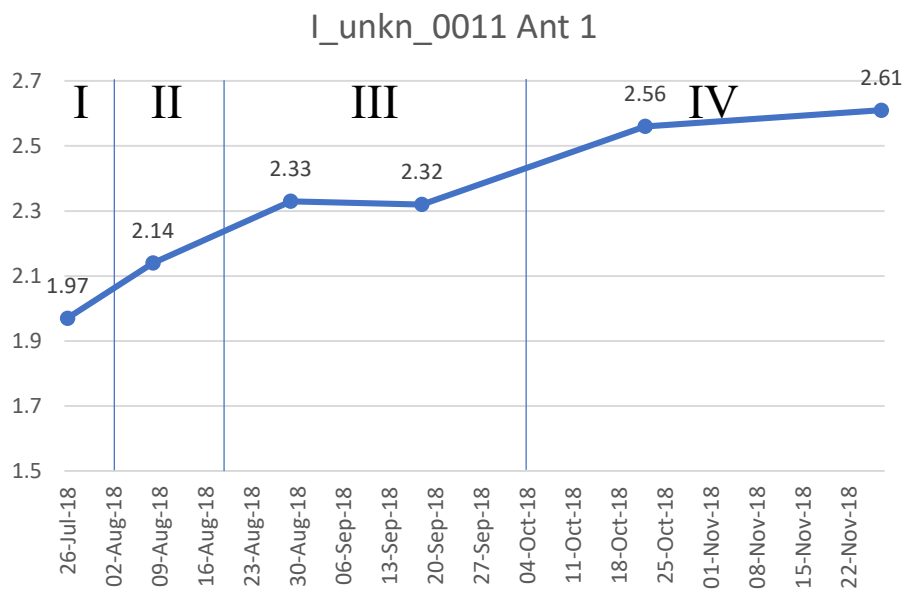


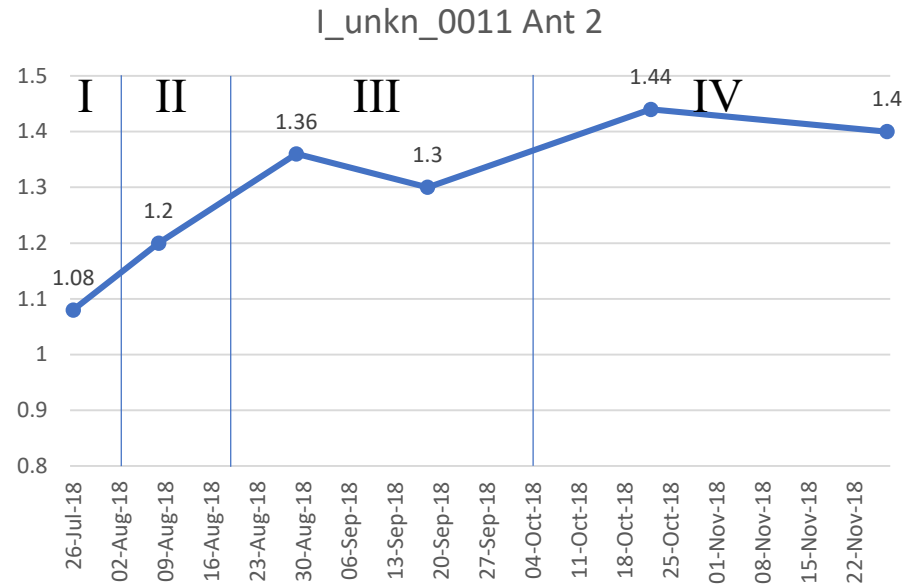




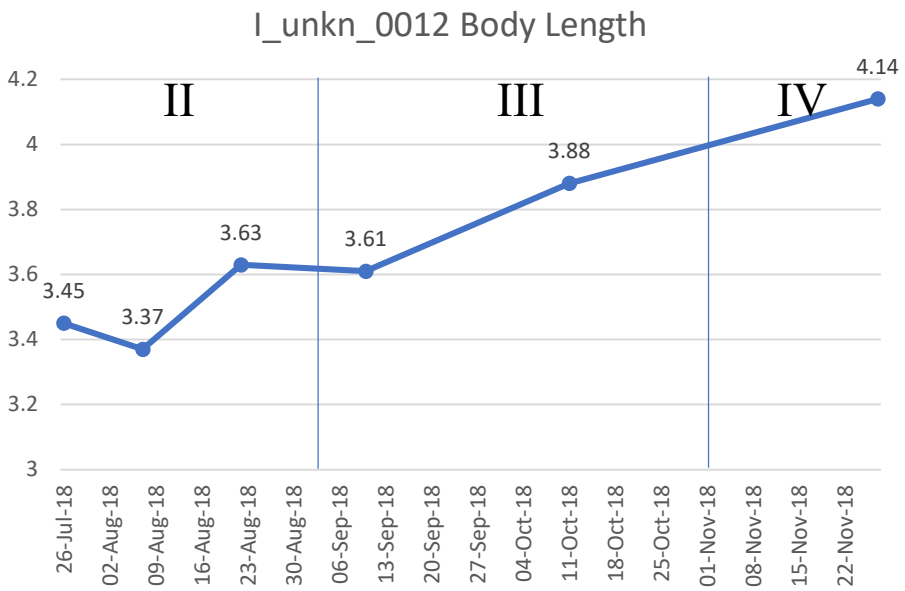
Instar determination of individual I_unkn_0007 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

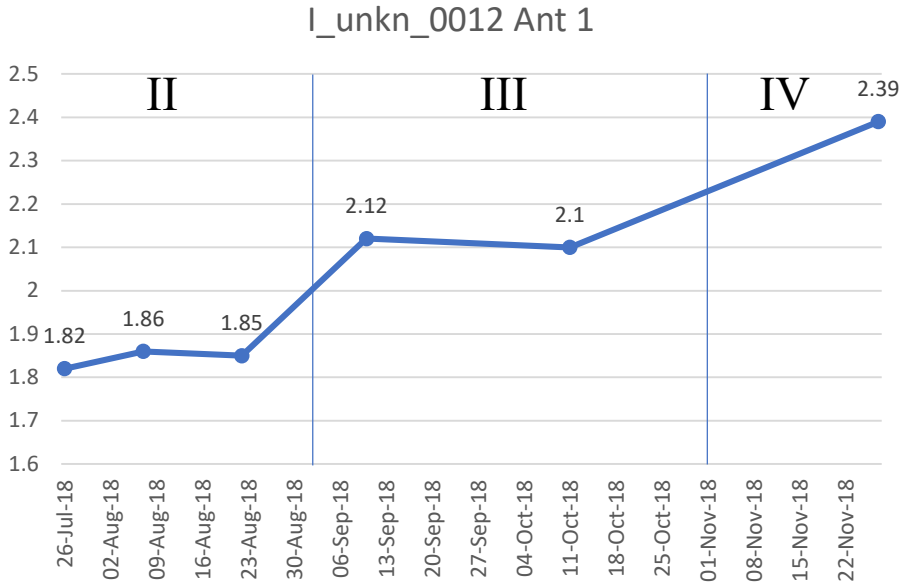


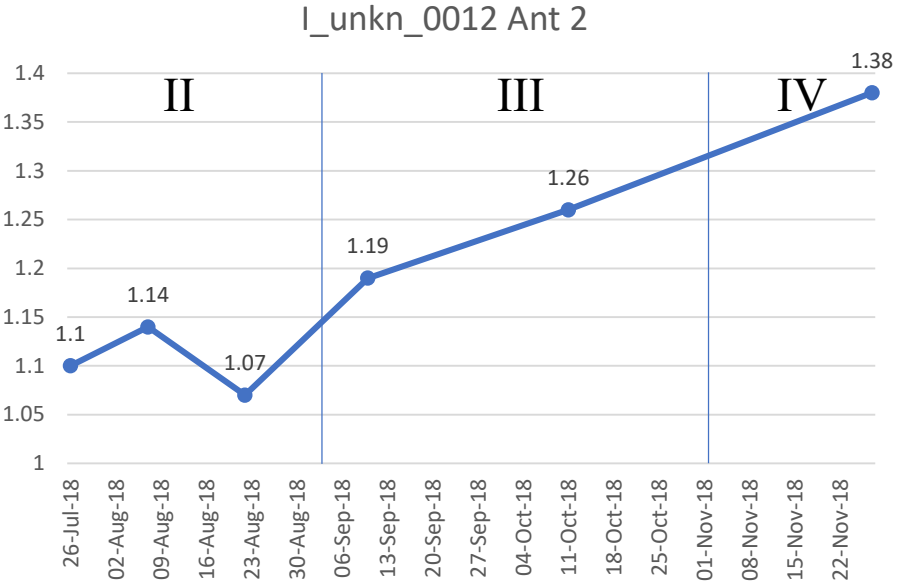




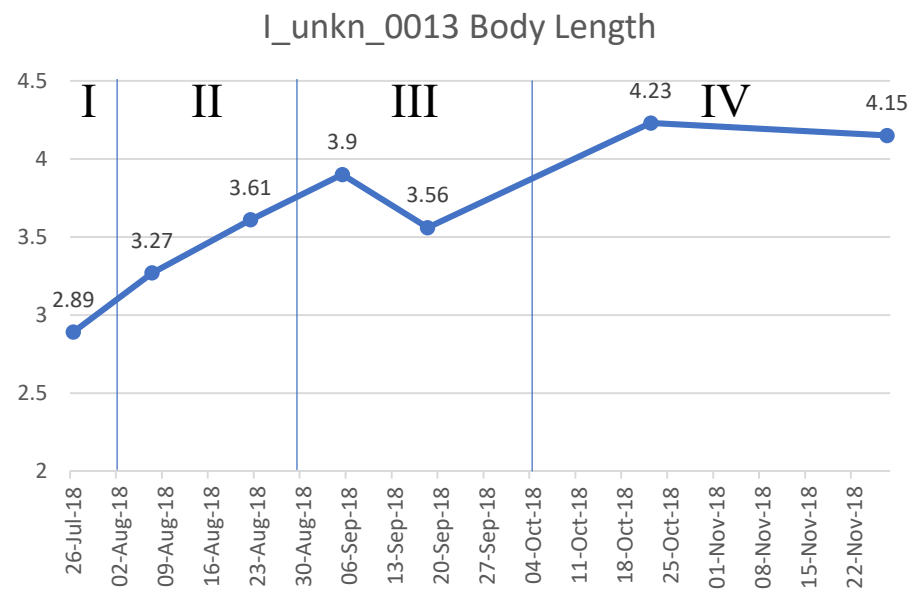
Instar determination of individual I_unkn_0011 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts. A molt was observed on 22-Oct by observing repaired antennae.

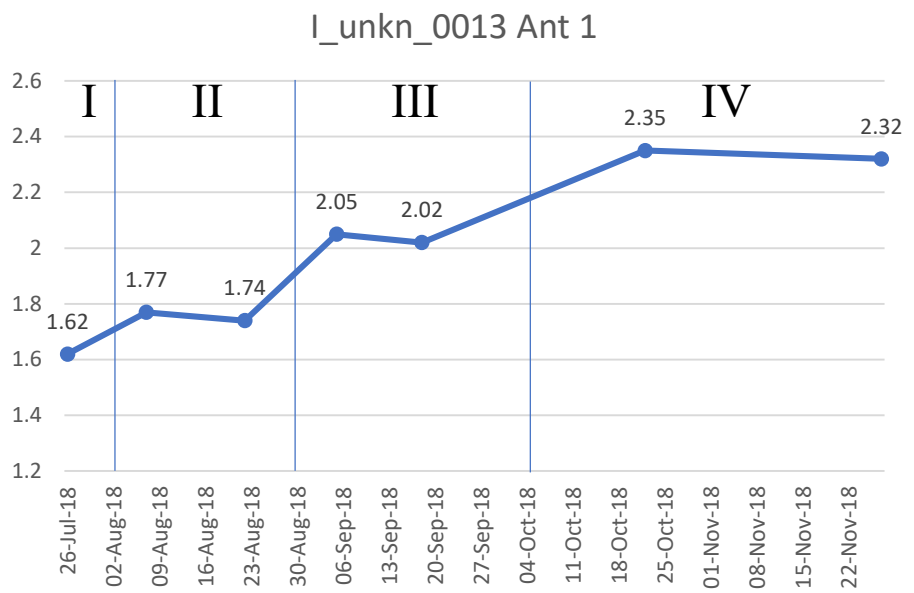


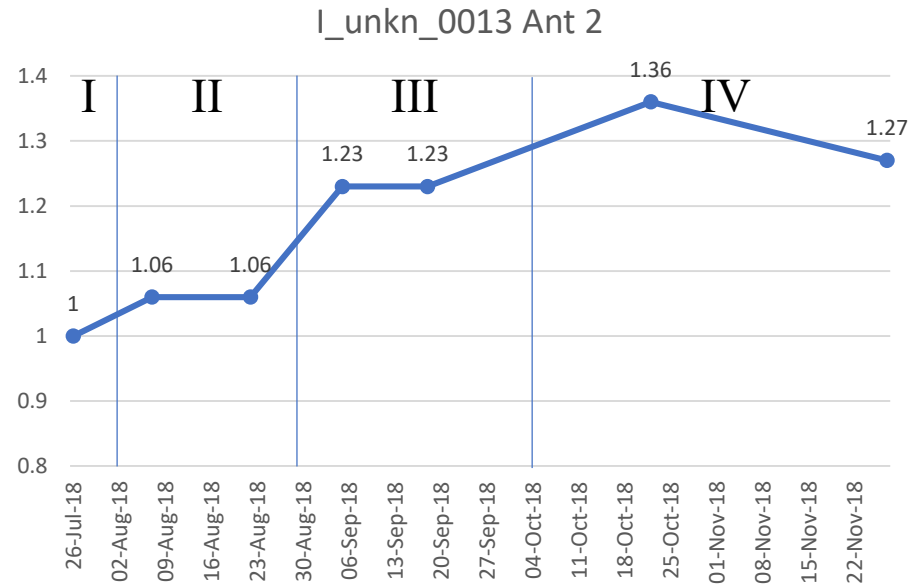




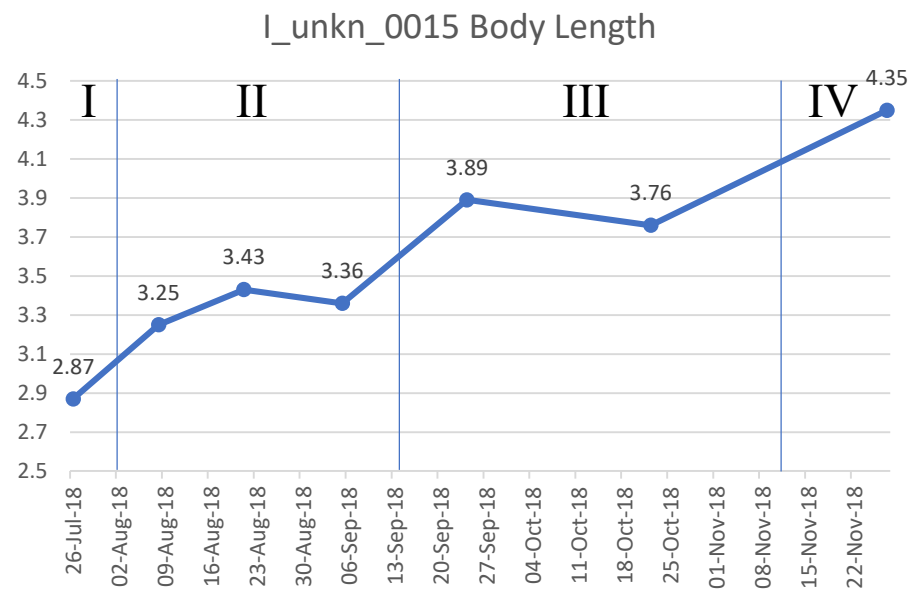
Instar determination of individual I_unkn_0012 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

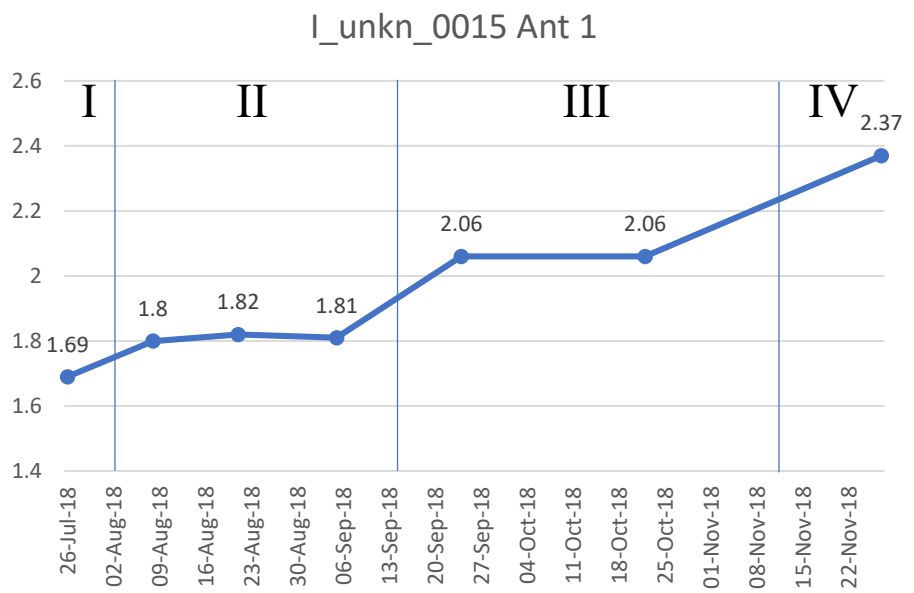


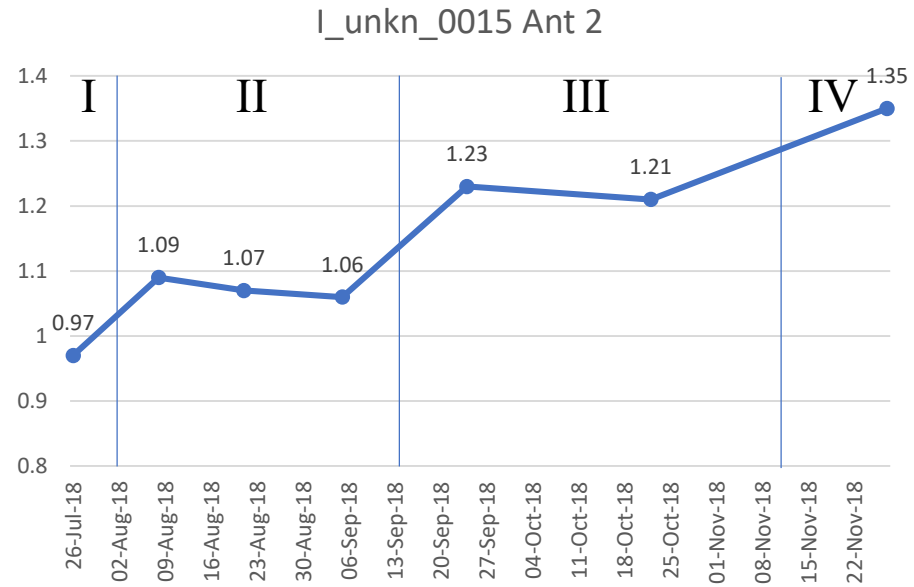




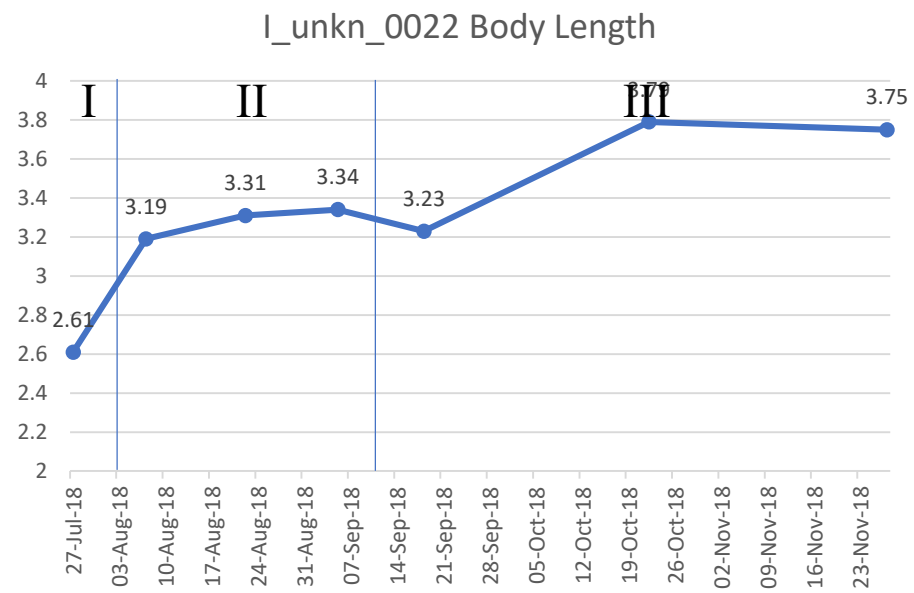
Instar determination of individual I_unkn_0013 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

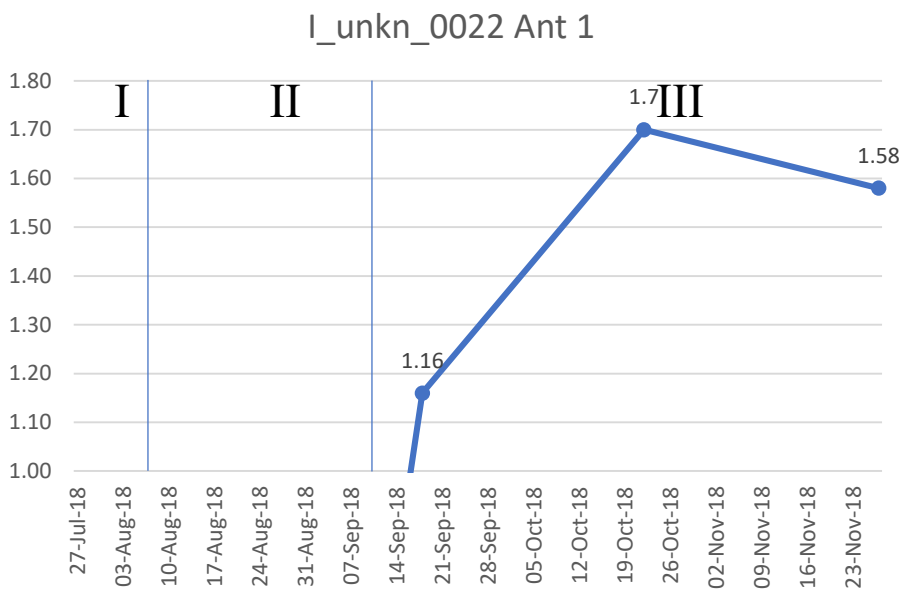


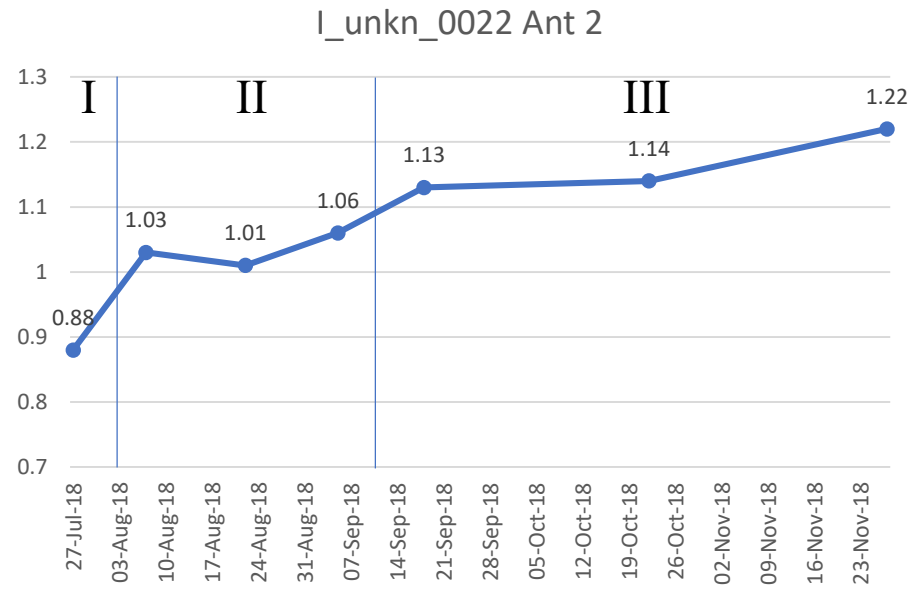




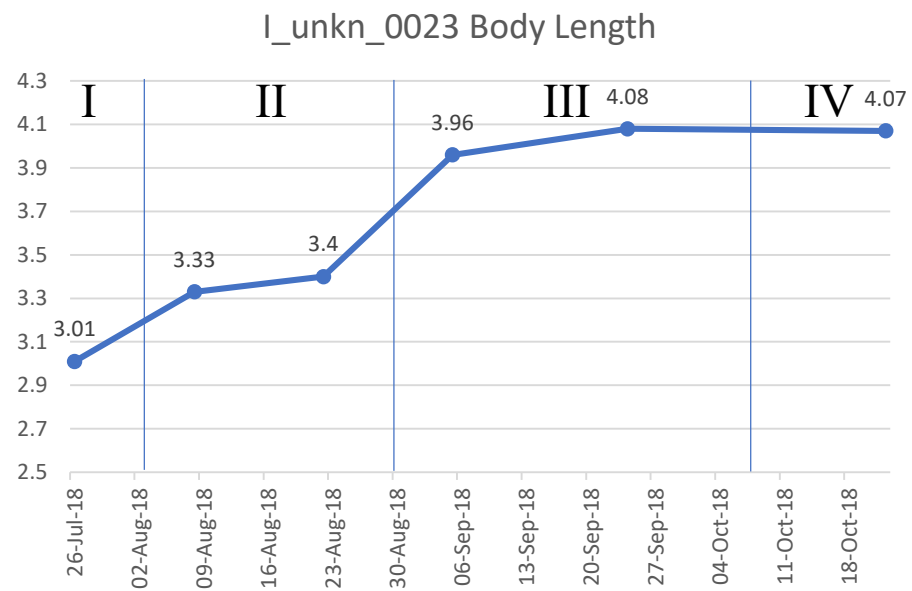
Instar determination of individual I_unkn_0015 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

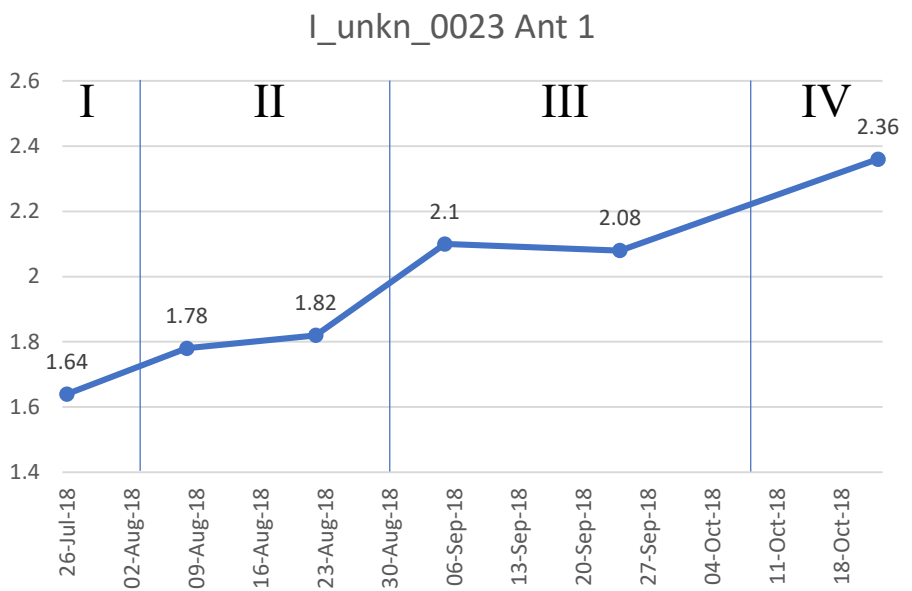


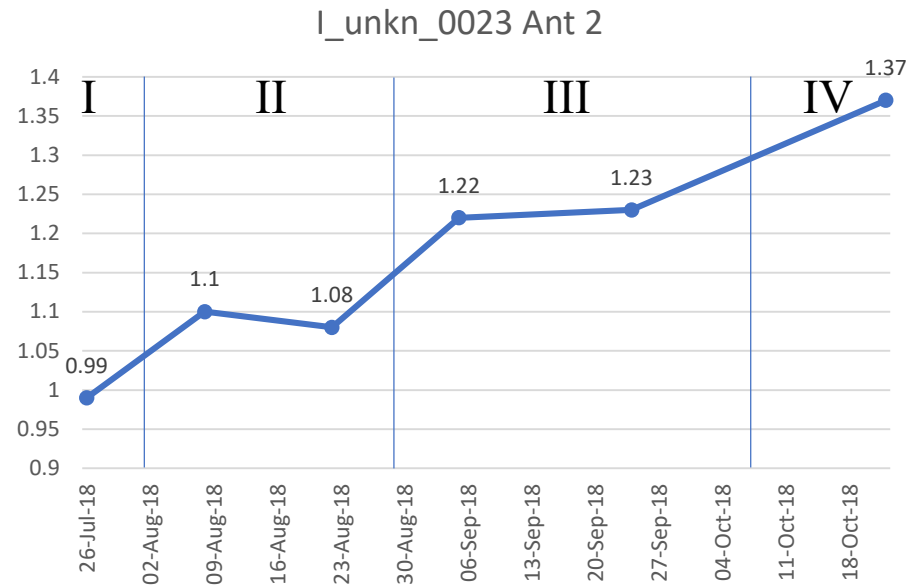




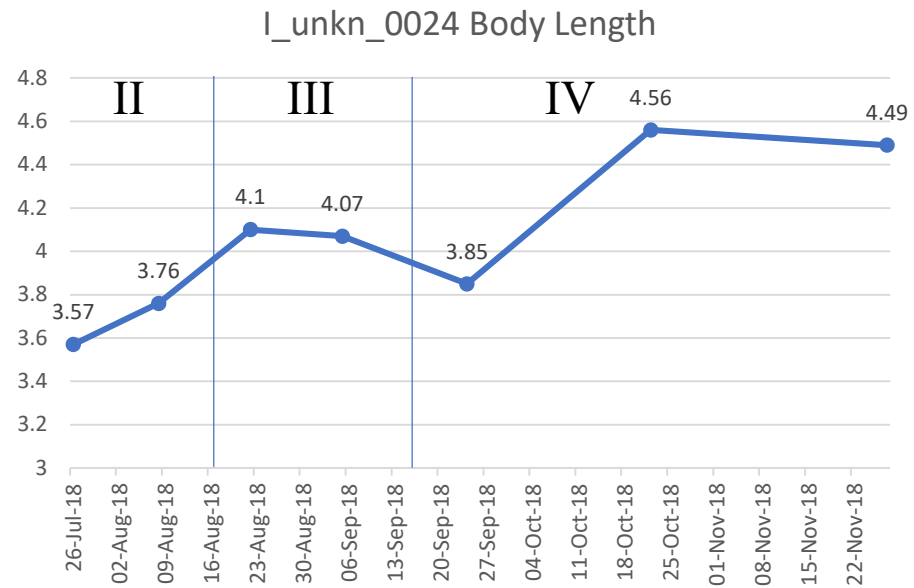
Instar determination of individual I_unkn_0022 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

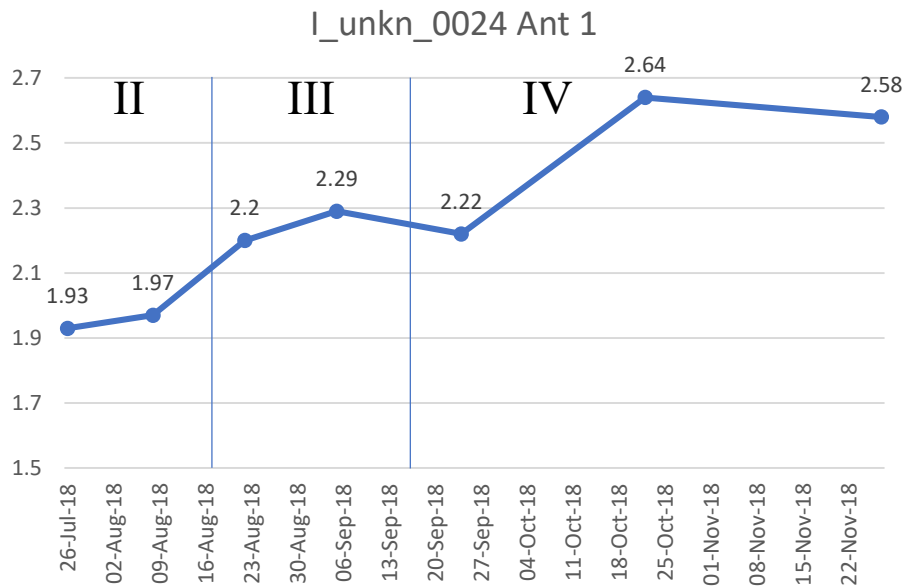


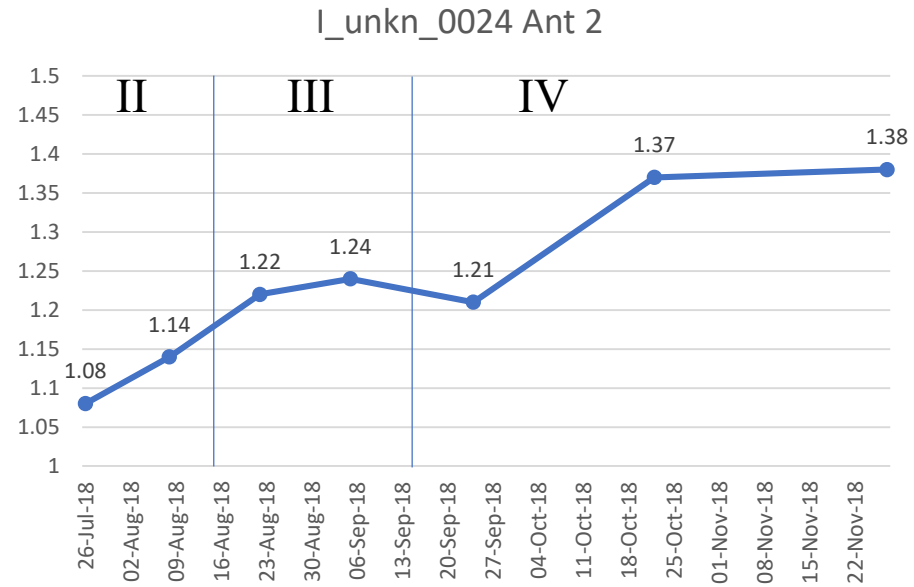




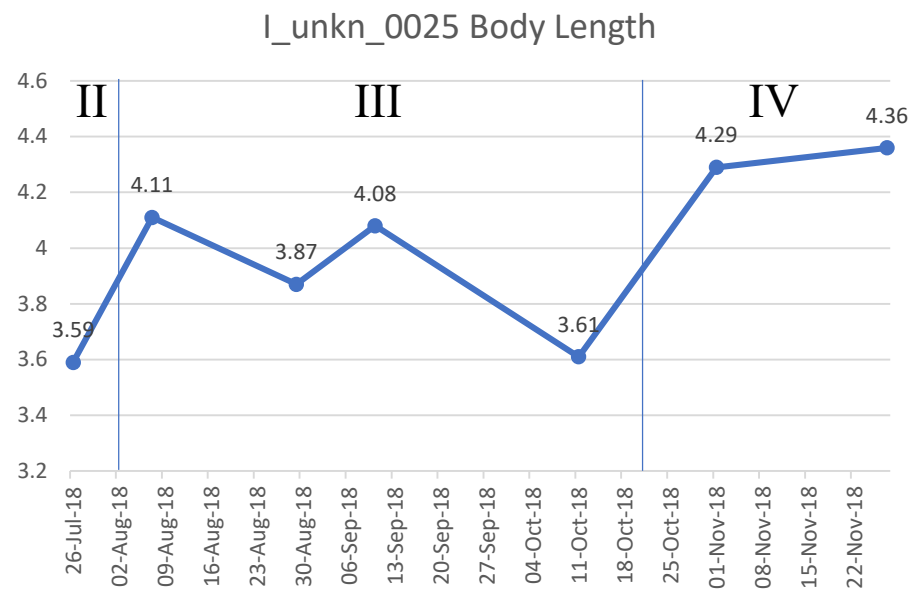
Instar determination of individual I_unkn_0023 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

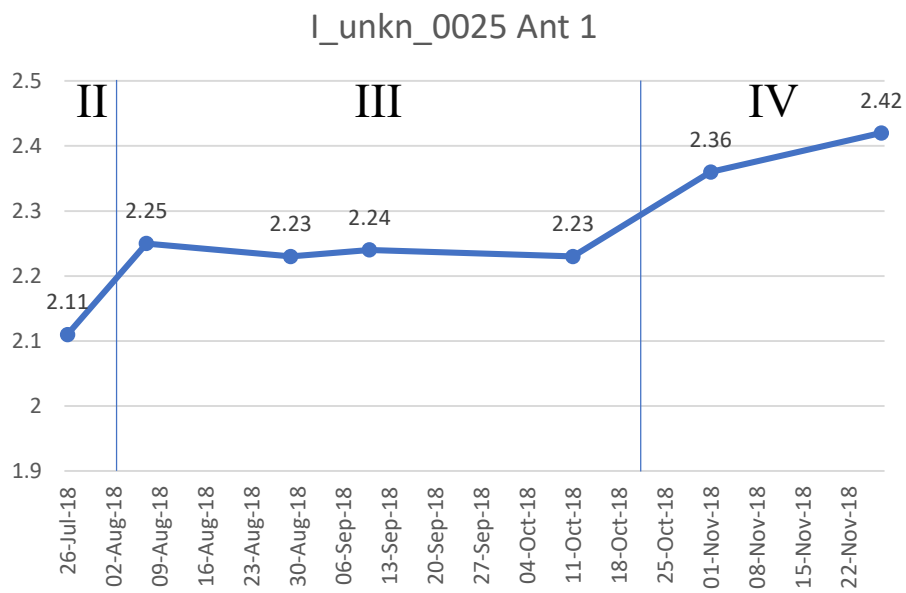


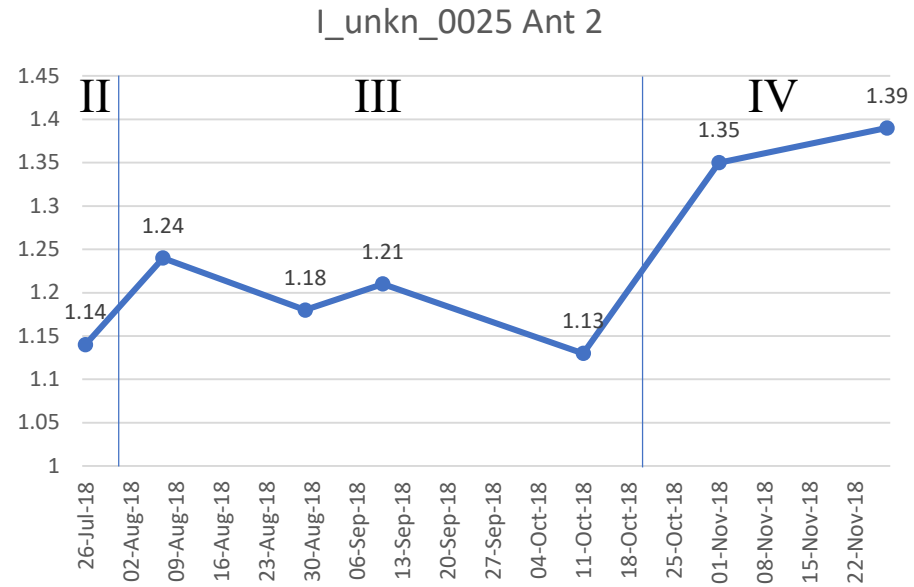




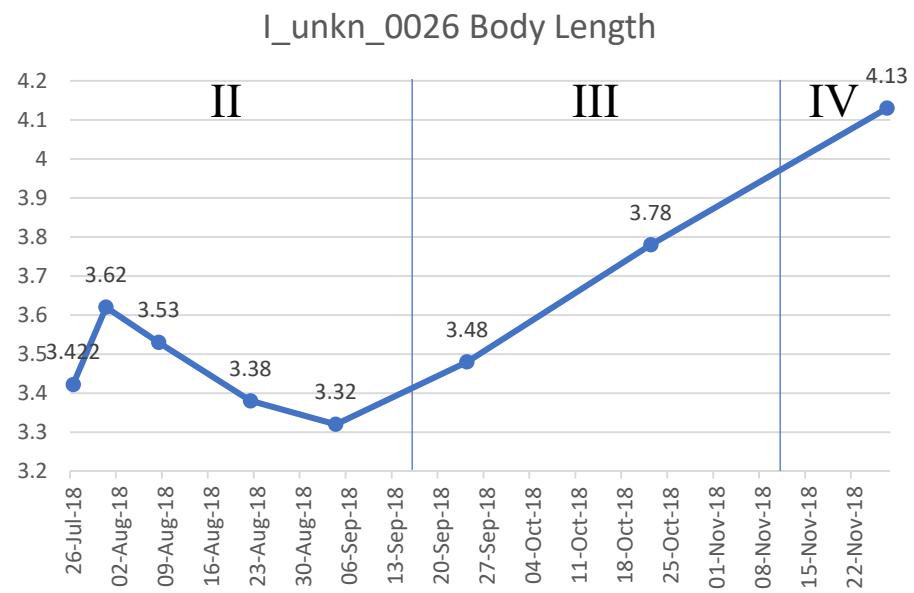
Instar determination of individual I_unkn_0024 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts. Right Antenna 1 was repaired between 5-Sep and 24-Sep indicating a molt occurred.

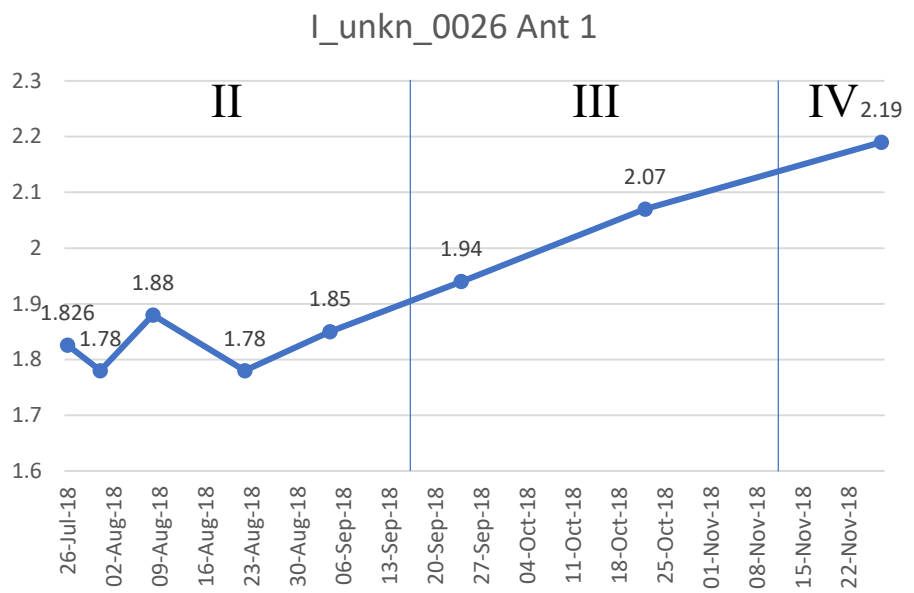


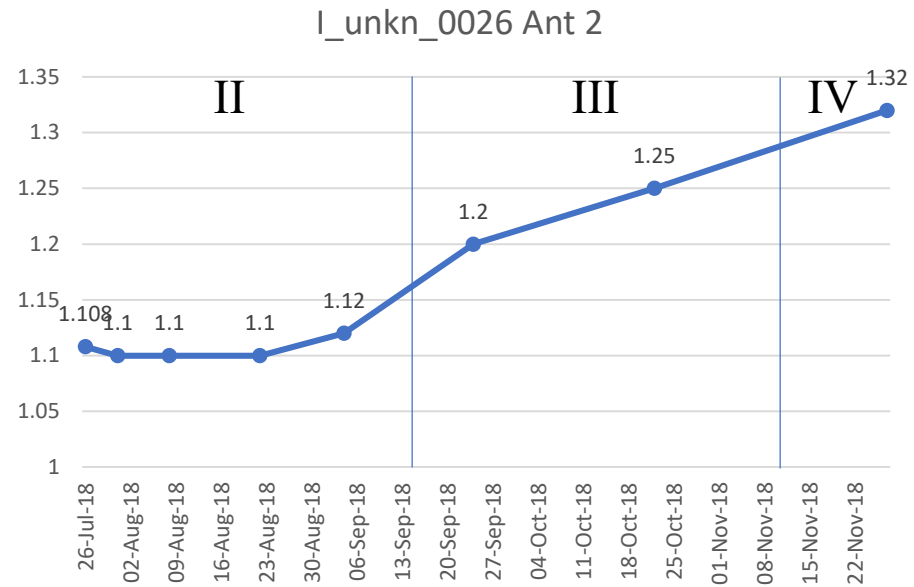




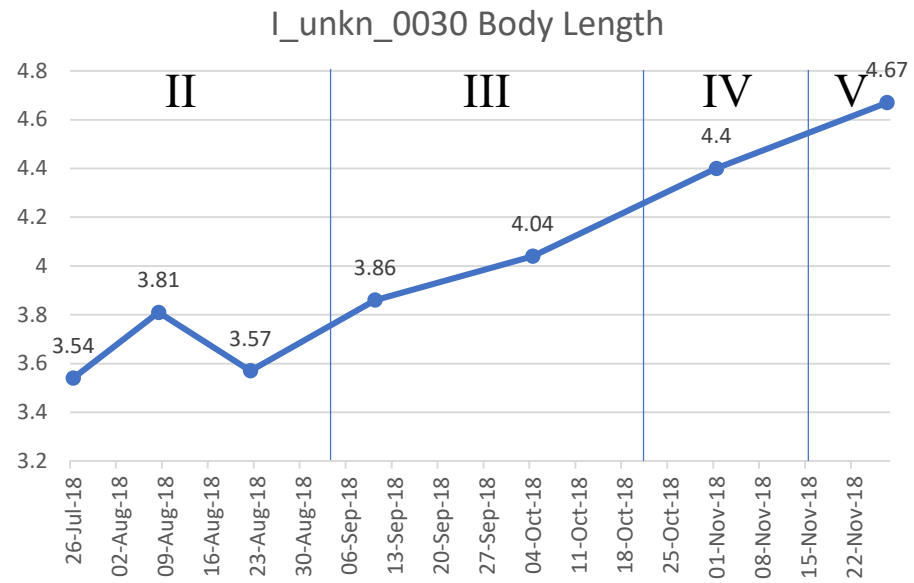
Instar determination of individual I_unkn_0025 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

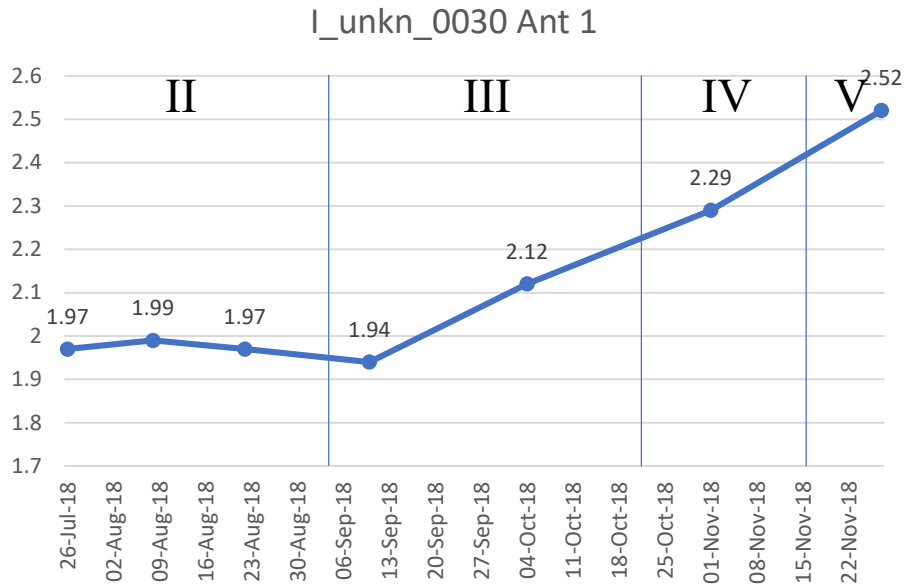


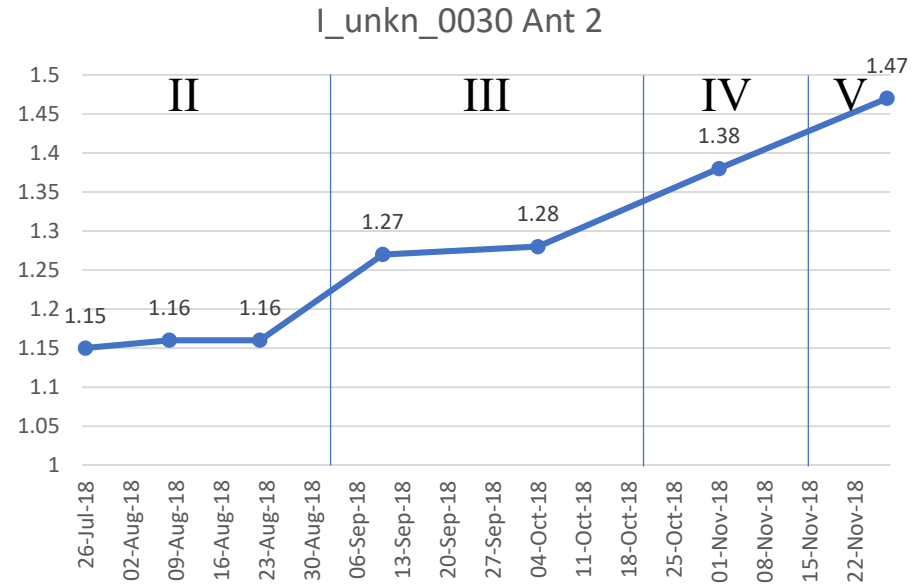




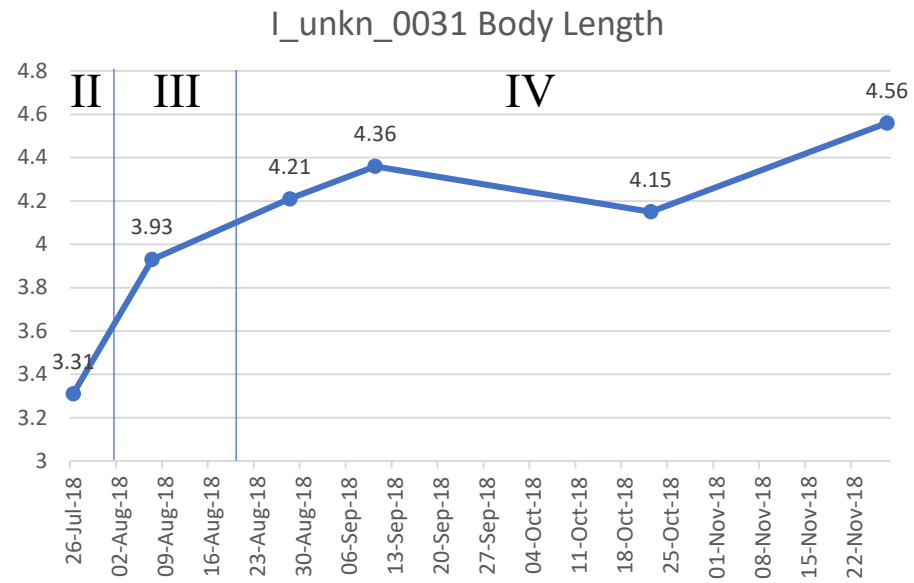
Instar determination of individual I_unkn_0026 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

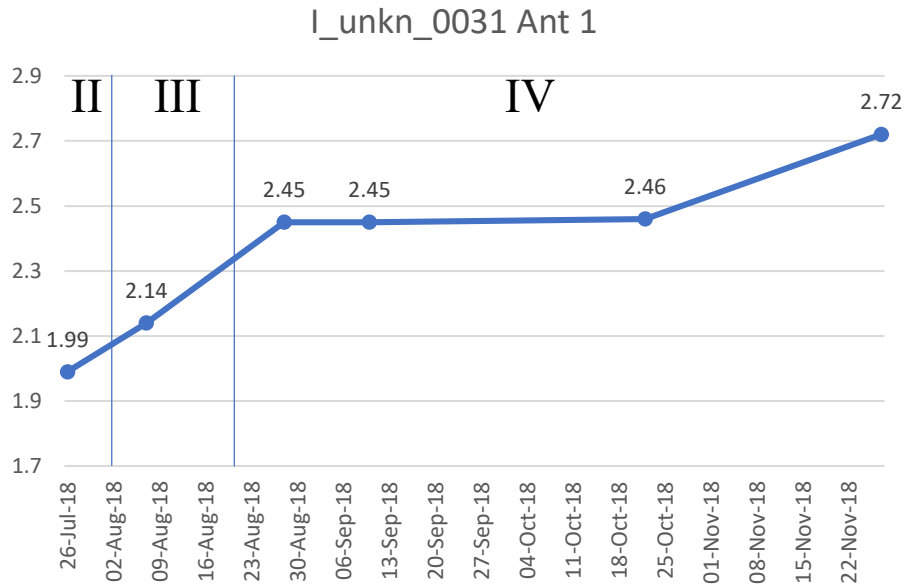


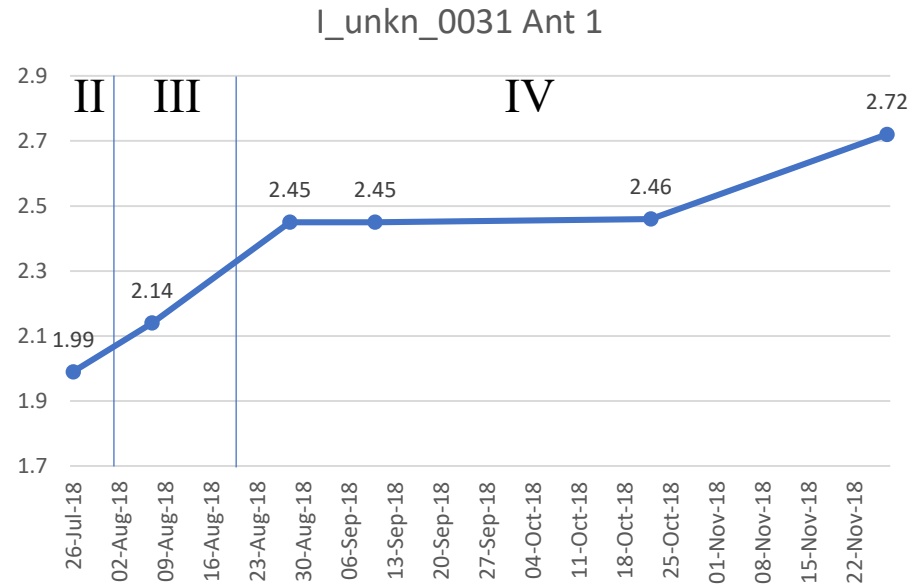




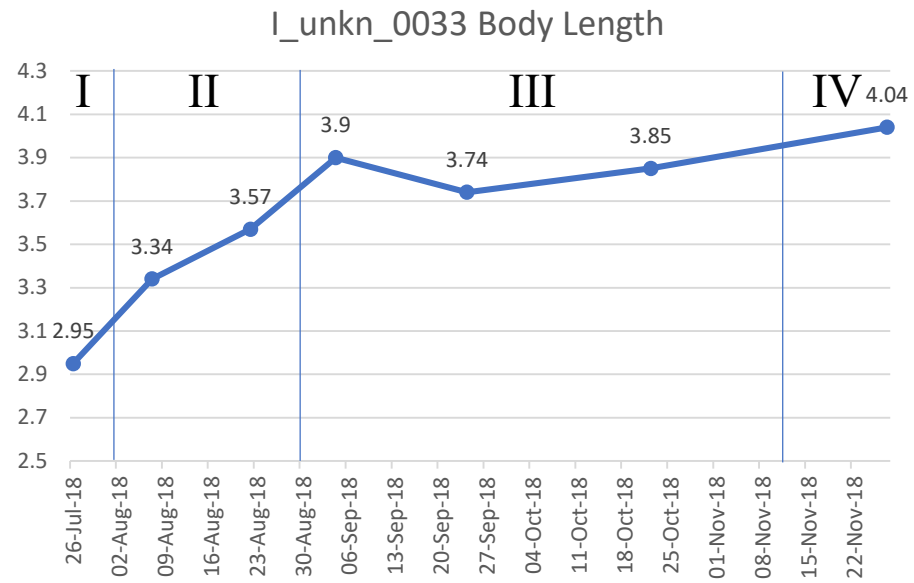
Instar determination of individual I_unkn_0030 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

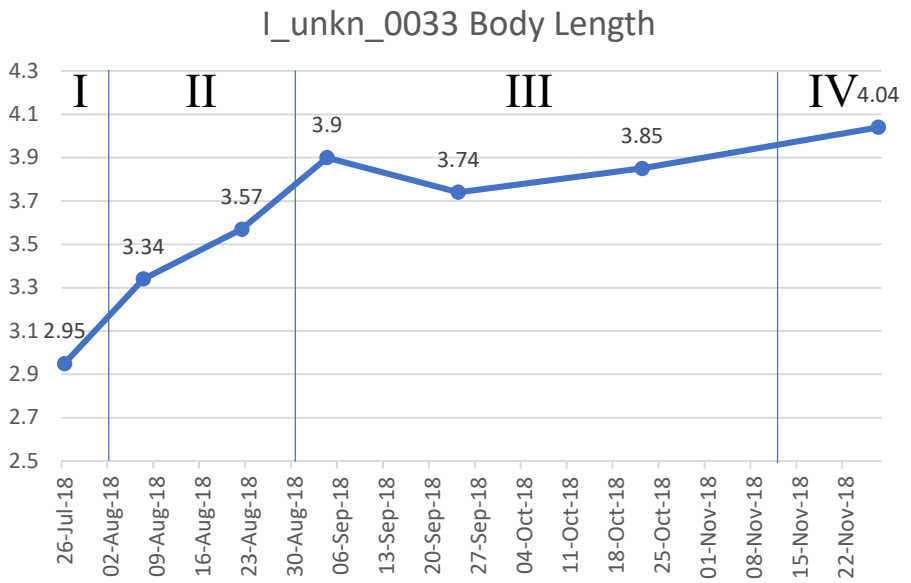


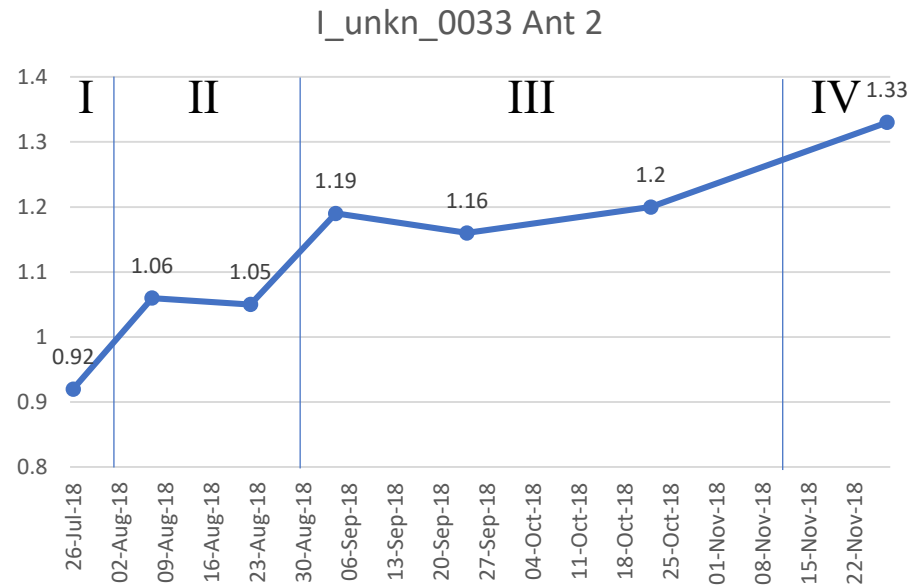




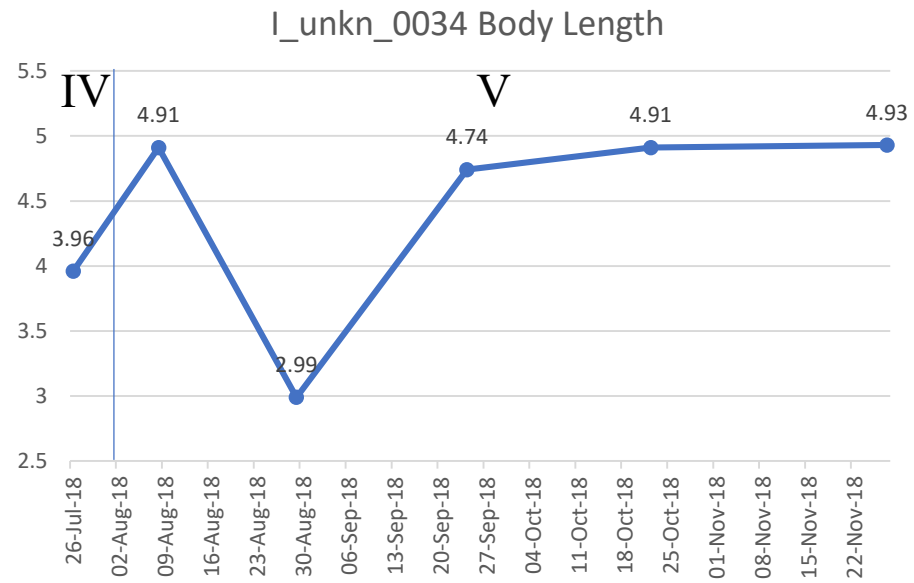
Instar determination of individual I_unkn_0031 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

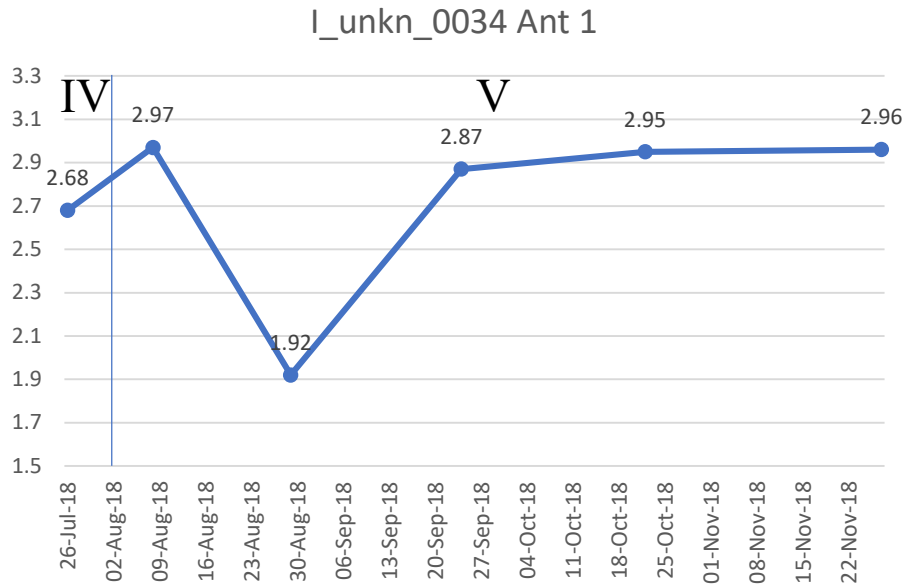


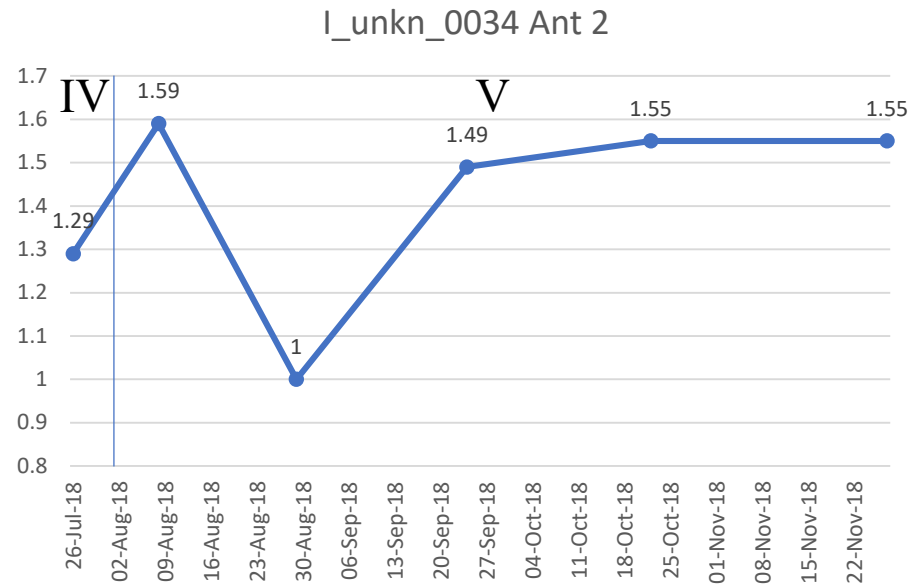




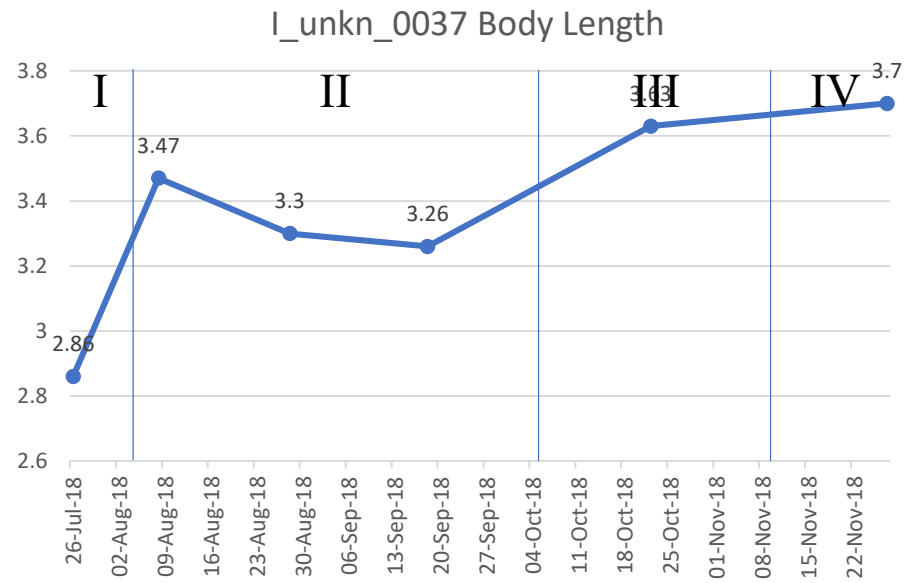
Instar determination of individual I_unkn_0033 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

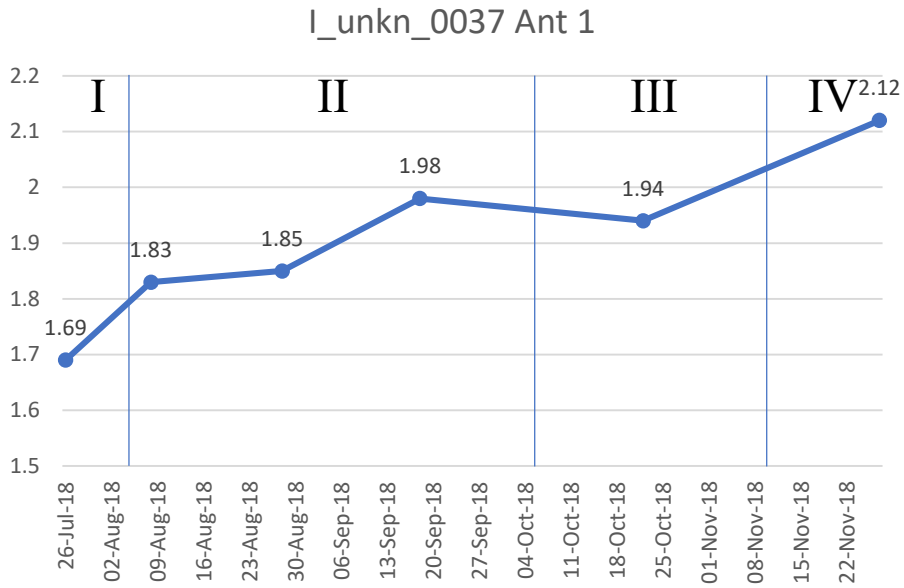


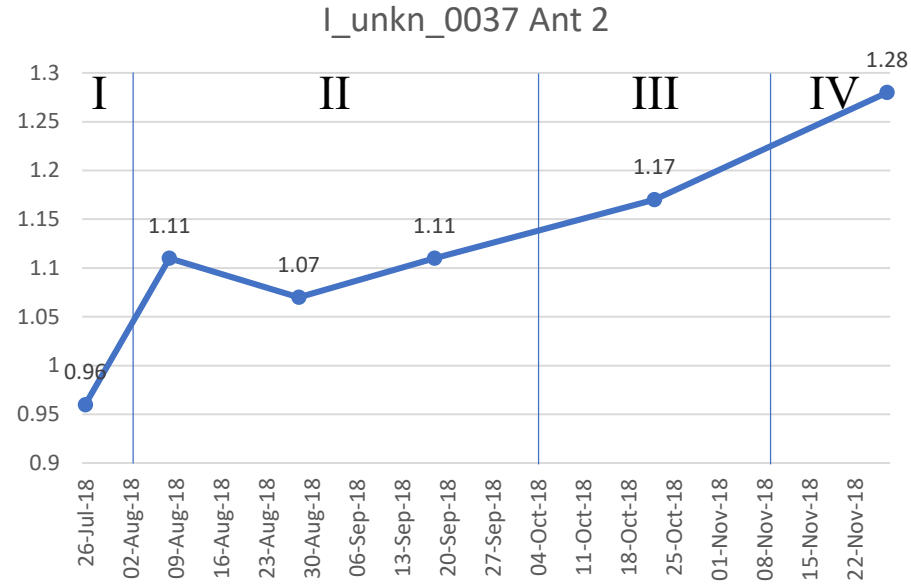




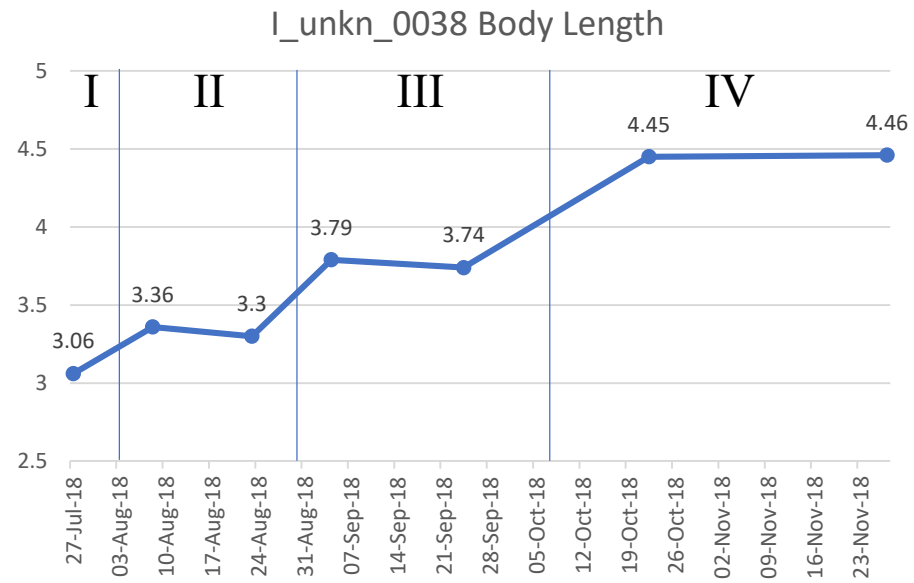
Instar determination of individual I_unkn_0034 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts. Bad measure on 29-Aug.

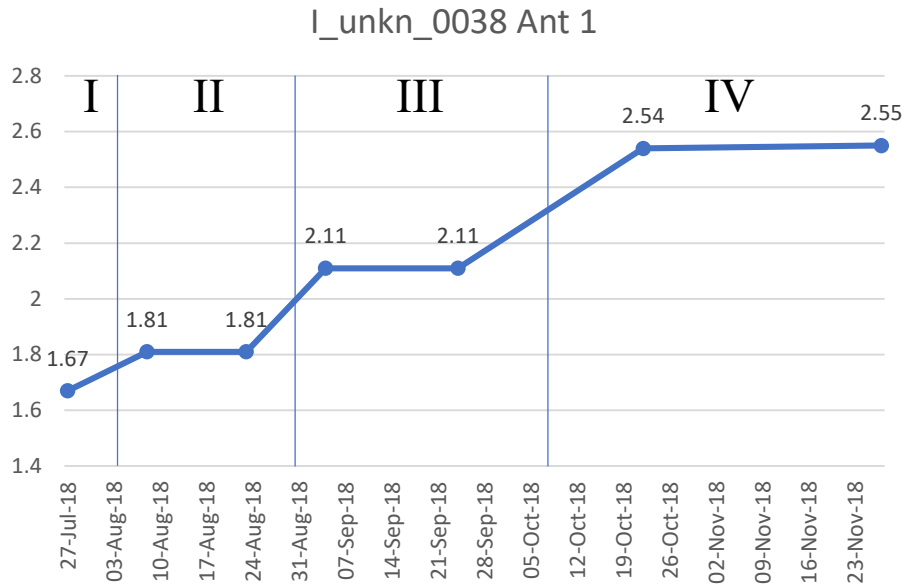


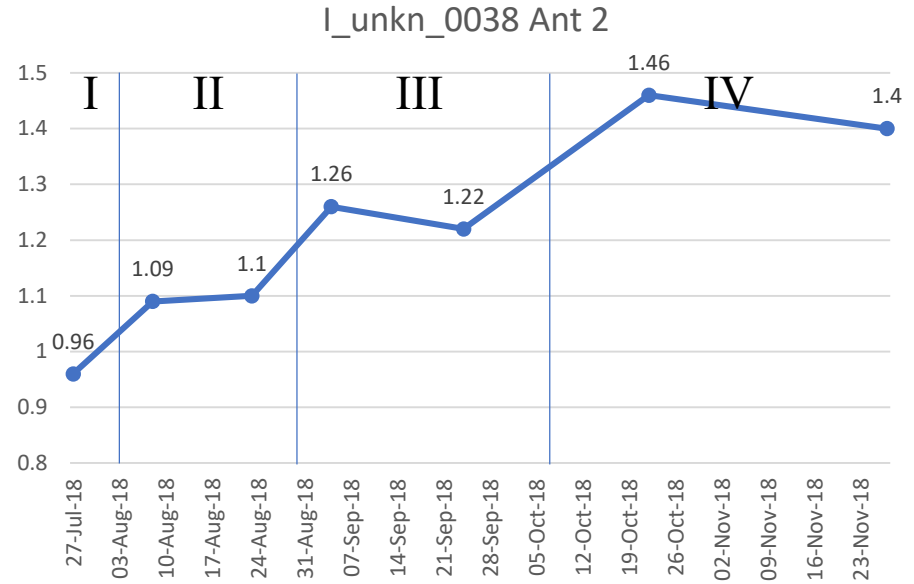




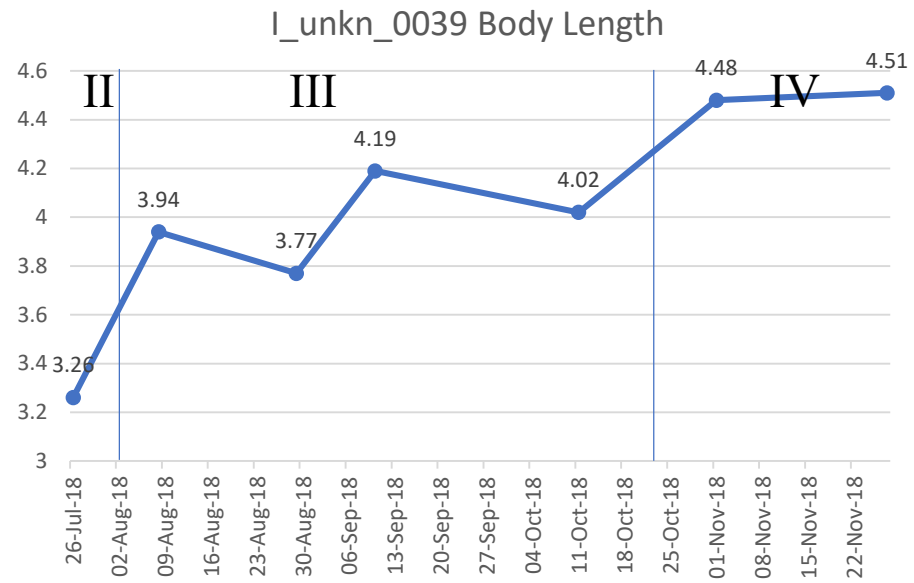
Instar determination of individual I_unkn_0037 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

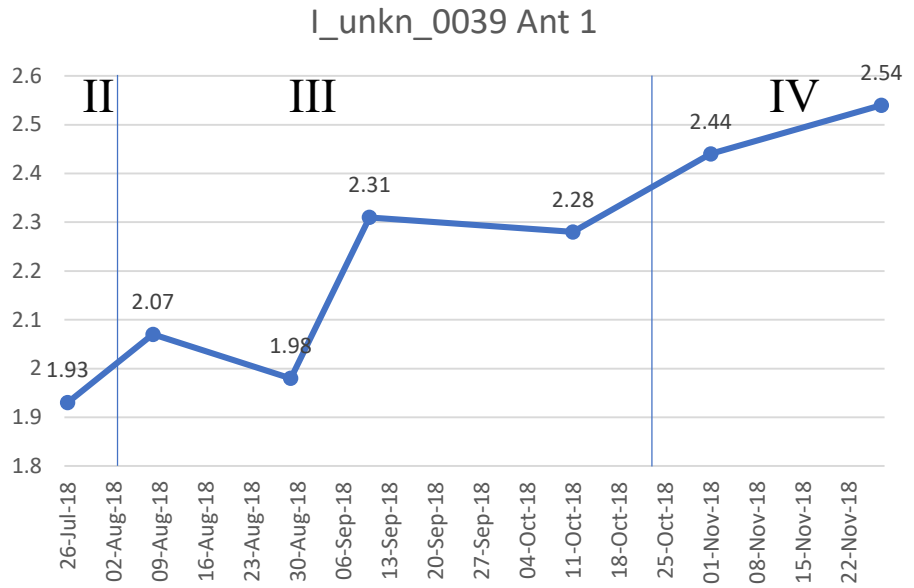


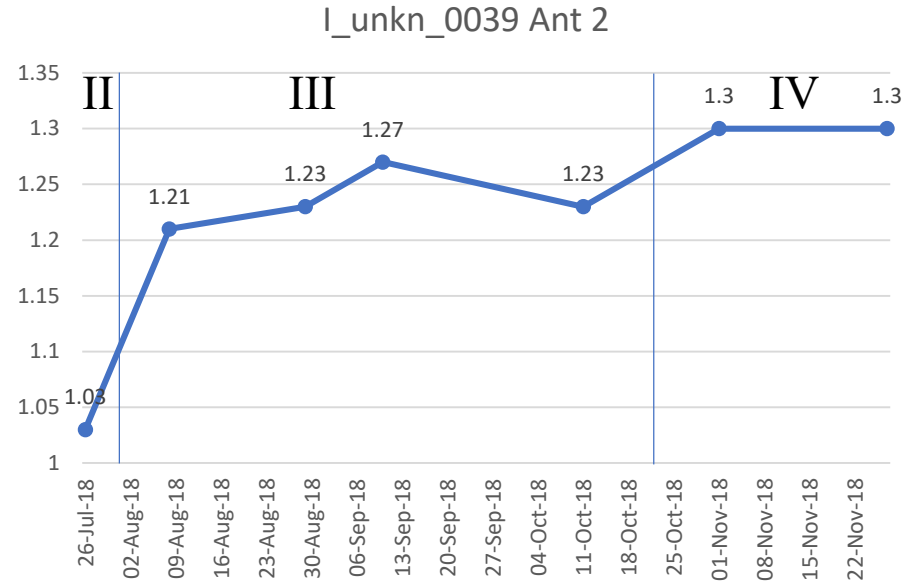




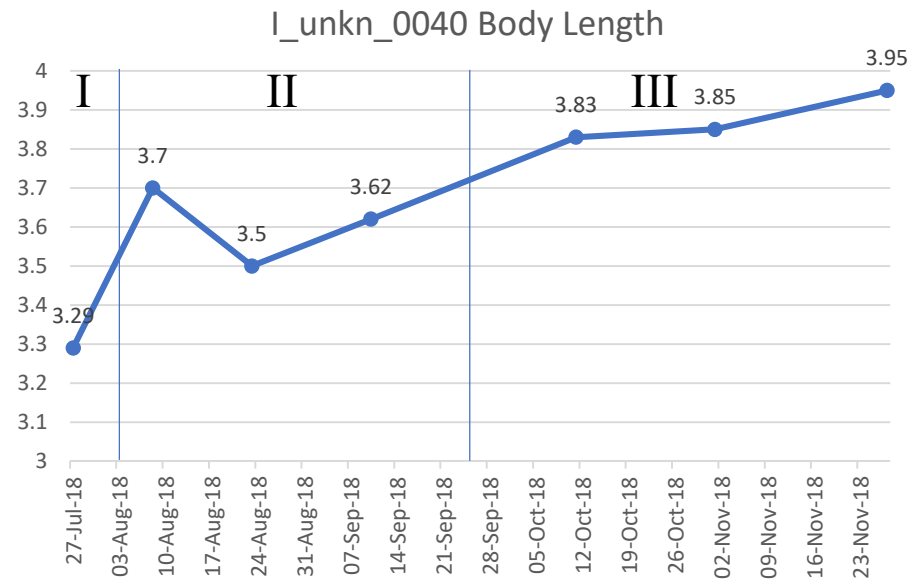
Instar determination of individual I_unkn_0038 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

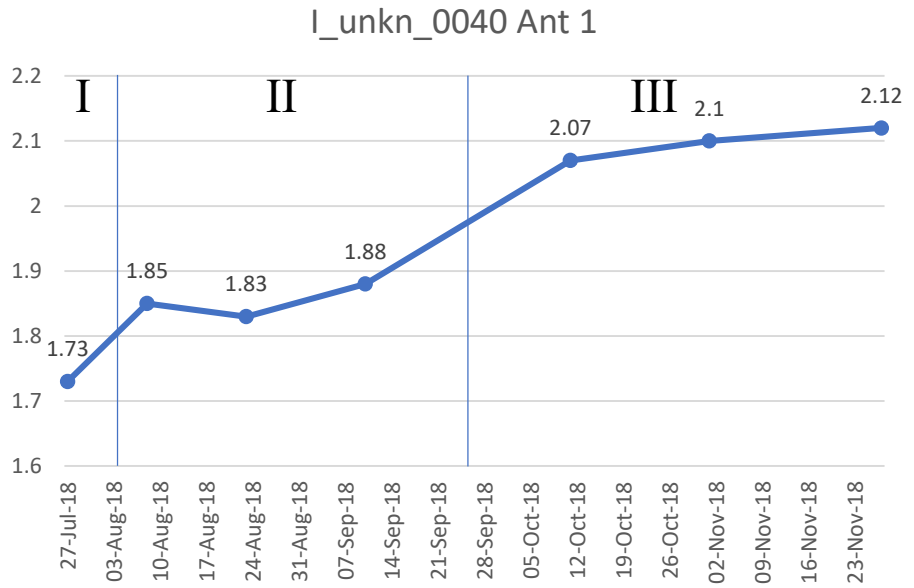


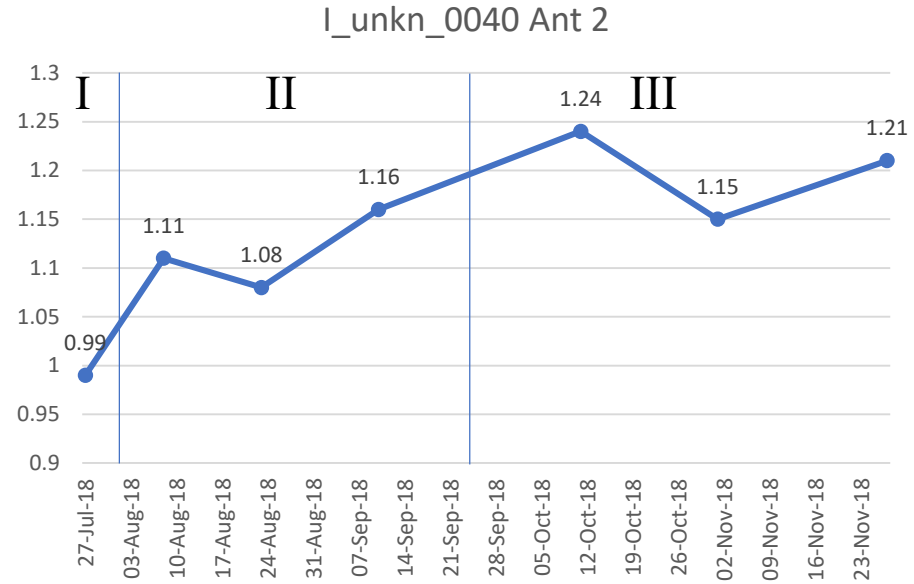




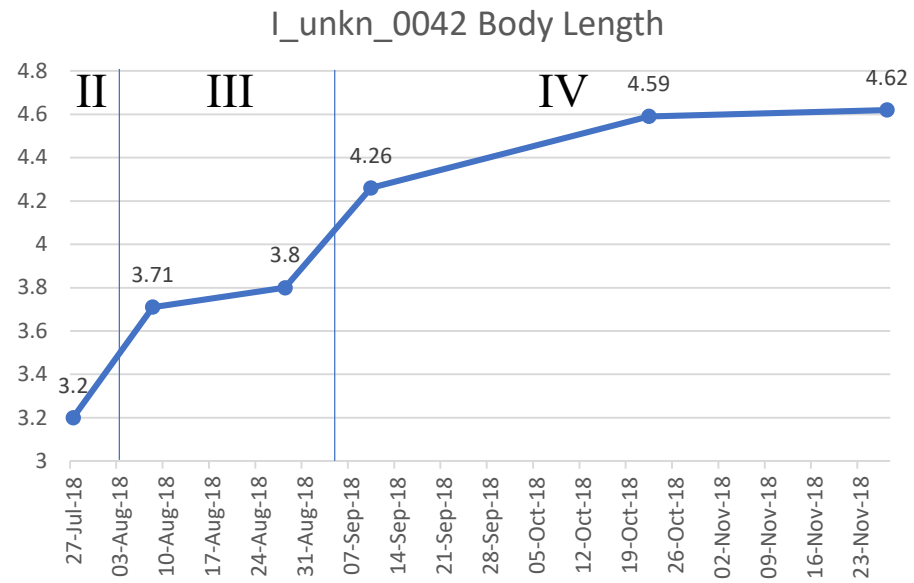
Instar determination of individual I_unkn_0039 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

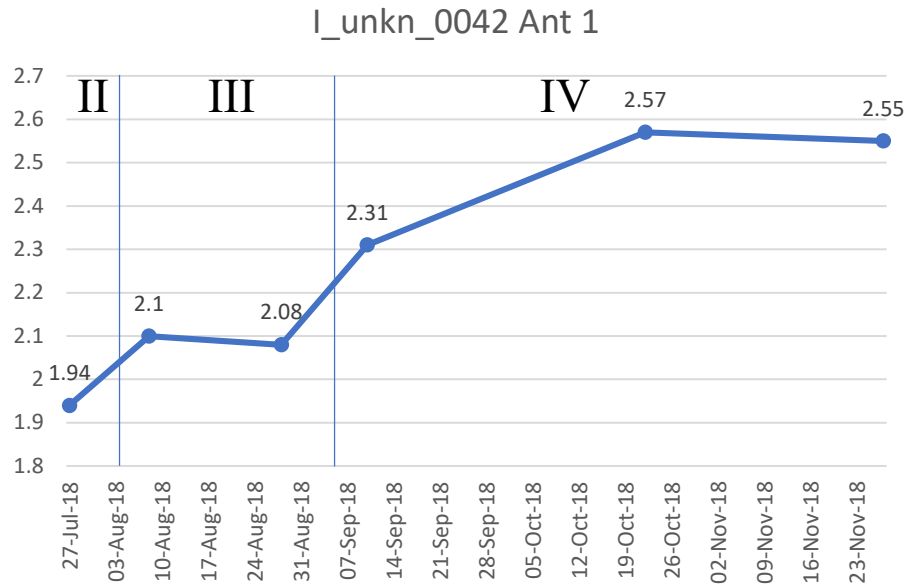


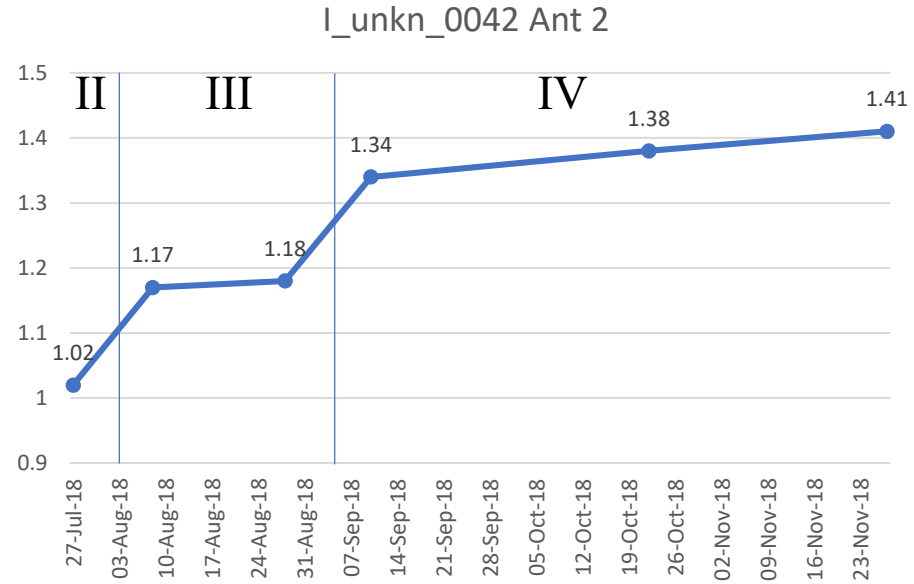




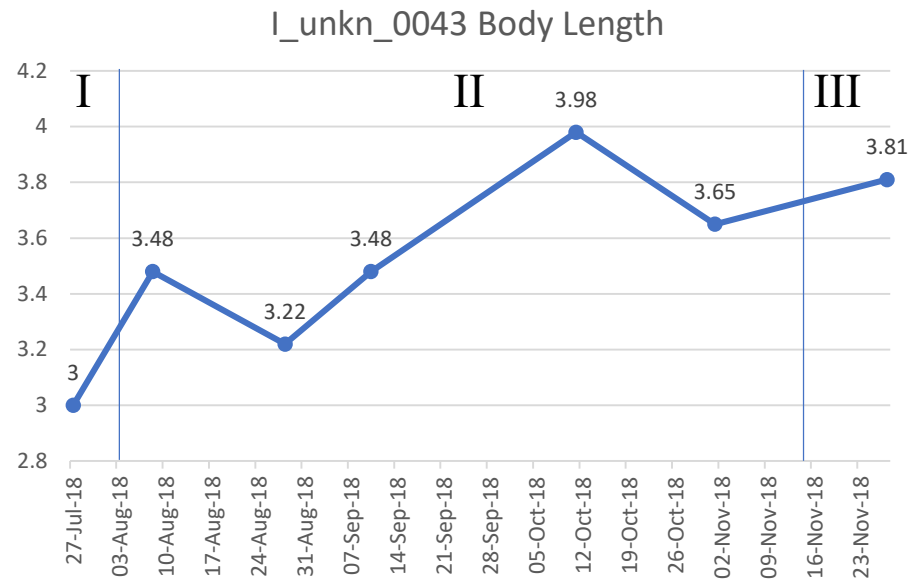
Instar determination of individual I_unkn_0040 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

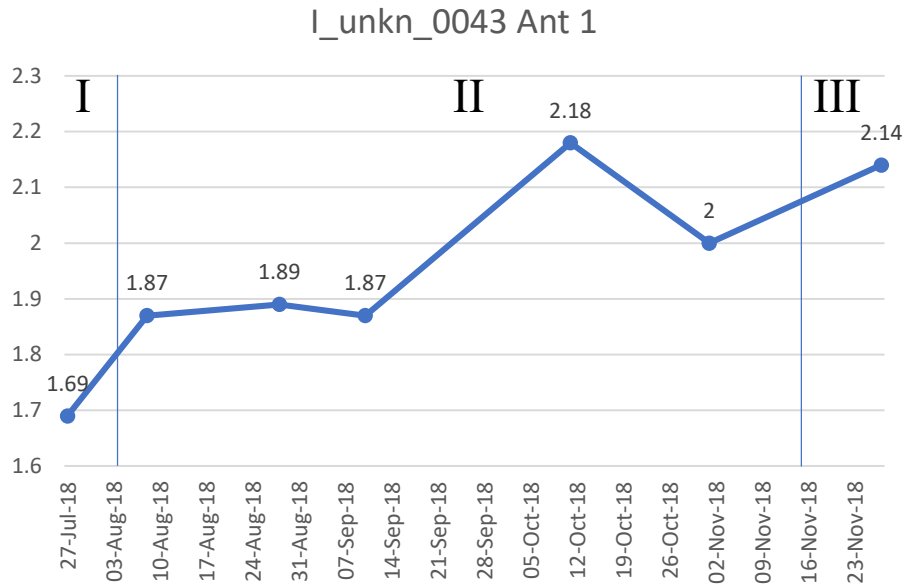


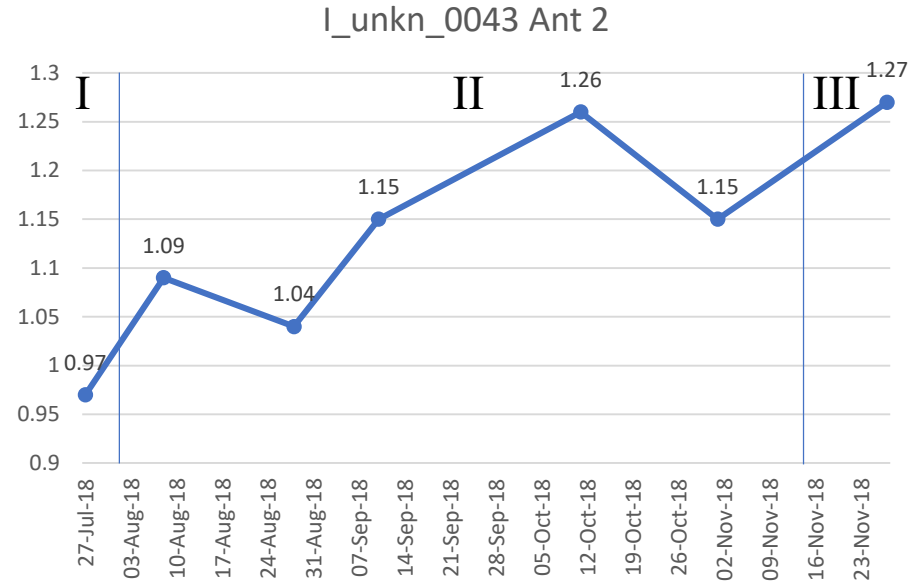




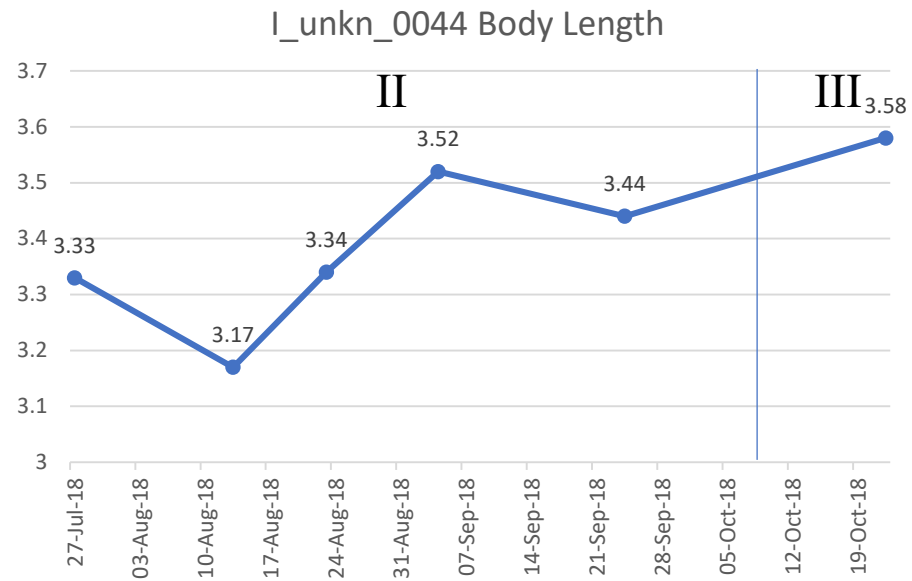
Instar determination of individual I_unkn_0042 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

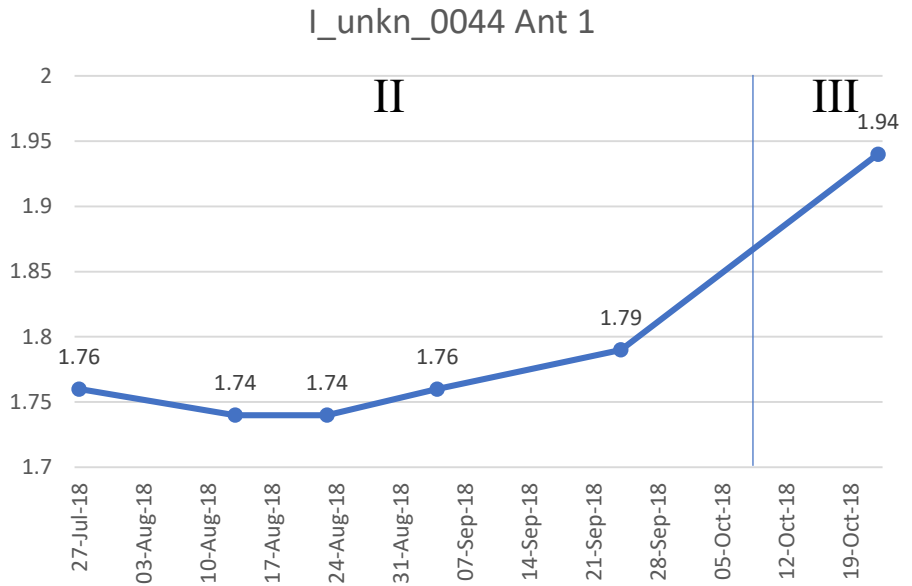


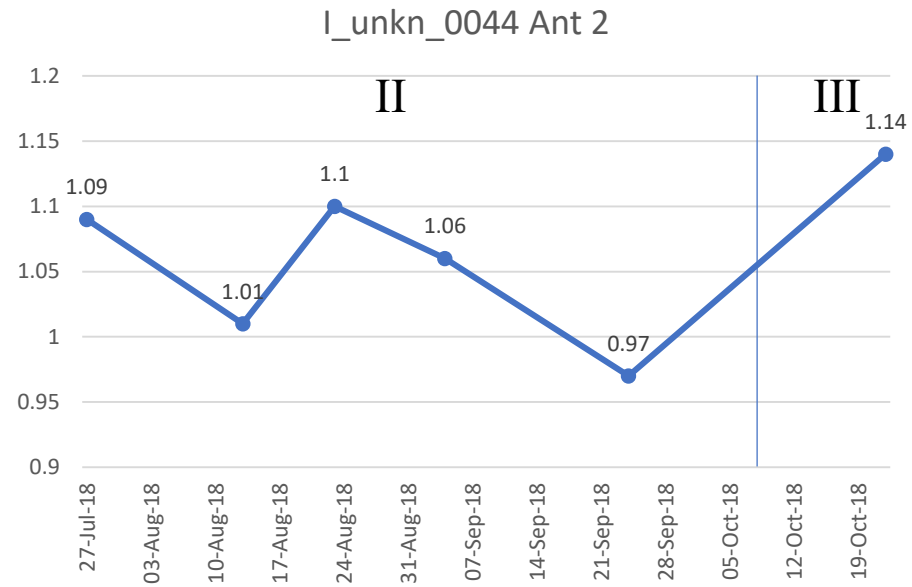




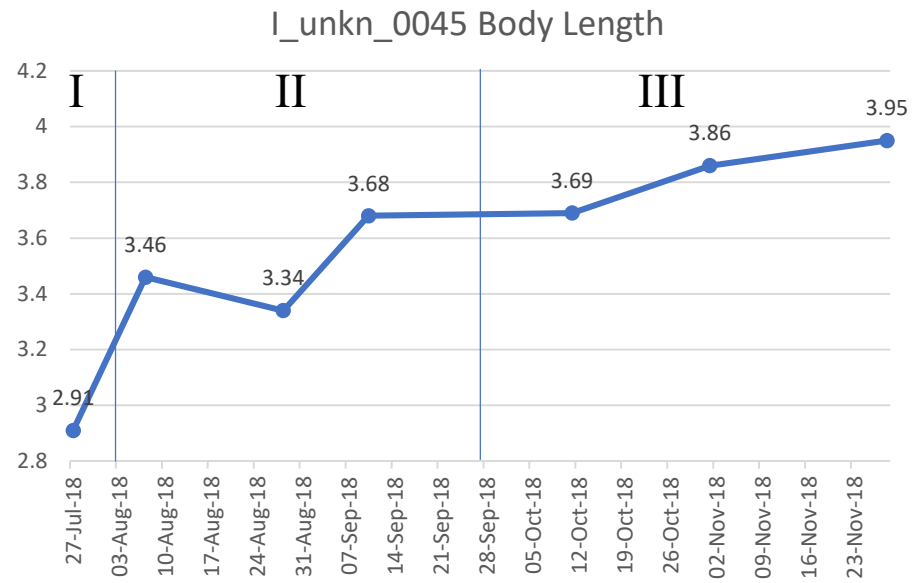
Instar determination of individual I_unkn_0043 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts. Left antenna 1 was repaired between 1-Nov and 27-Nov, indicating a molt occurred.

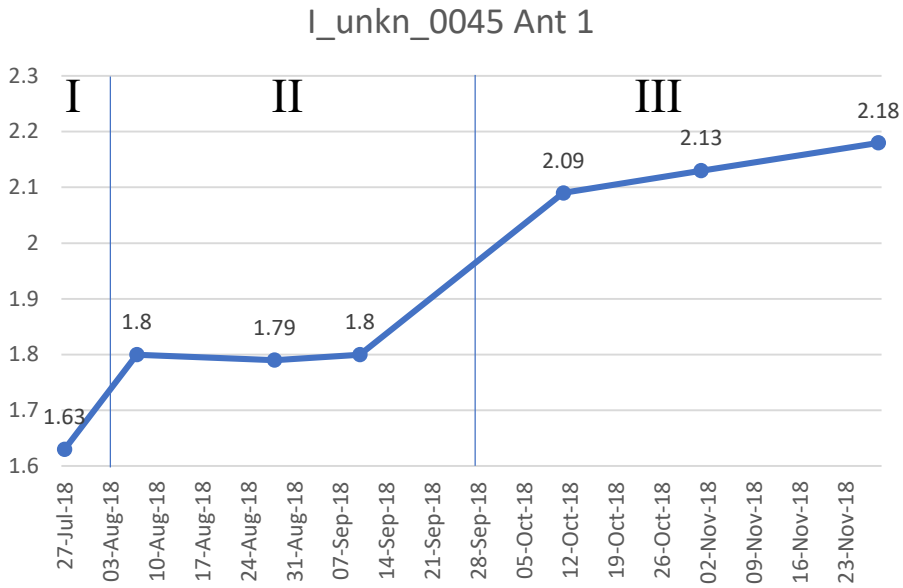


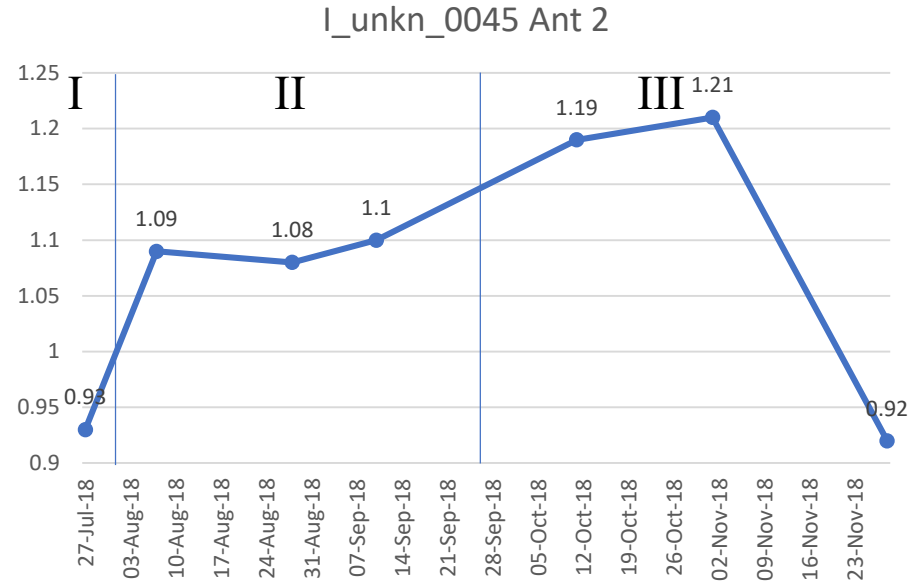




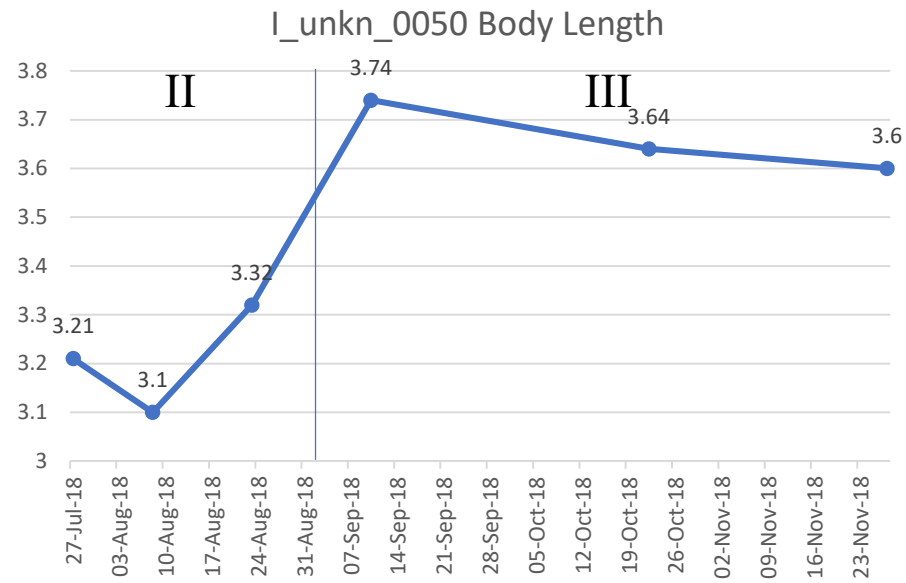
Instar determination of individual I_unkn_0044 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts. Right antenna 1 was repaired between 24-Sep and 22-Oct, indicating a molt occurred.

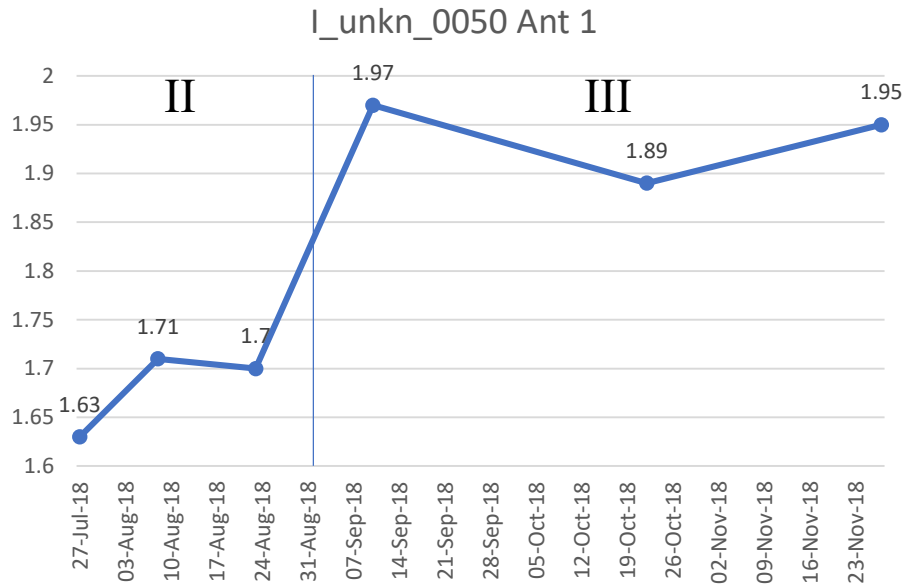


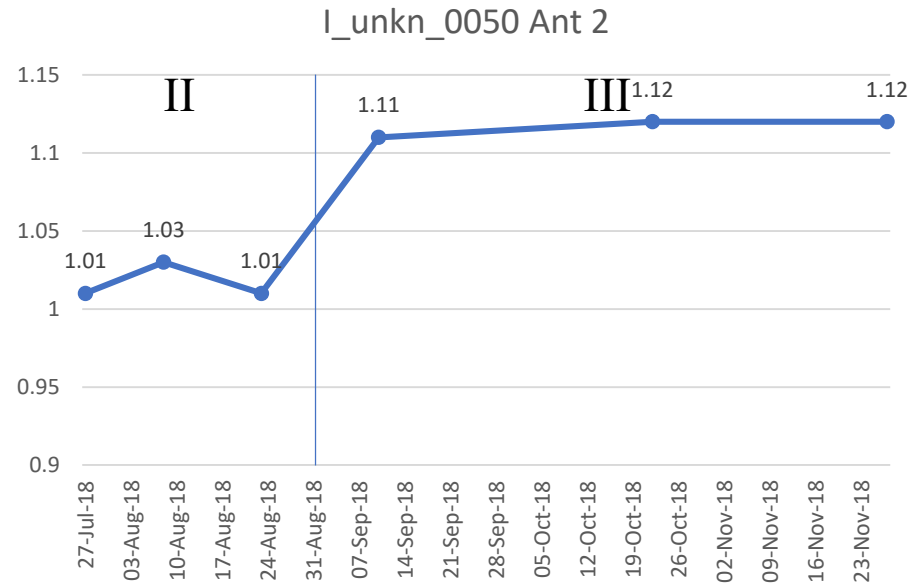




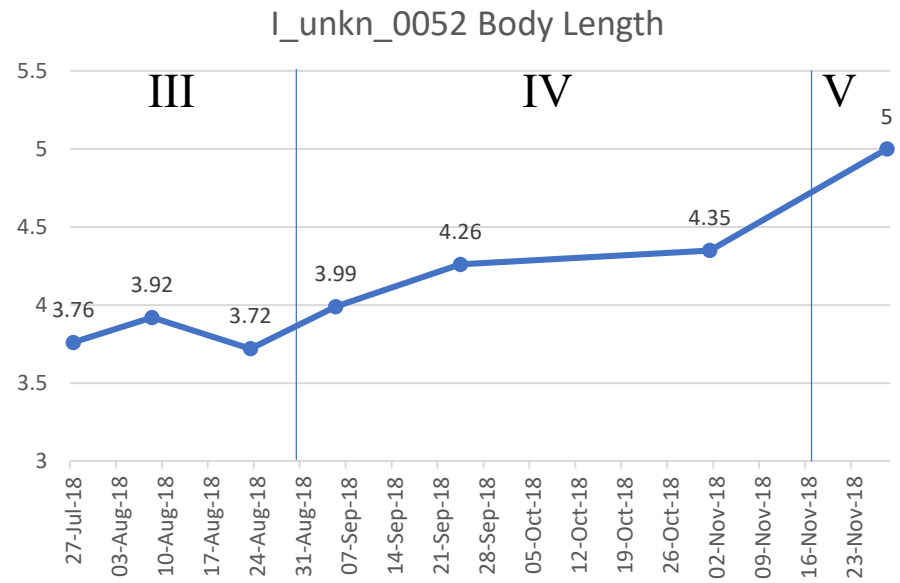
Instar determination of individual I_unkn_0045 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts. Right antenna 1 was repaired between 10-Sep and 11-Oct, indicating a molt occurred.

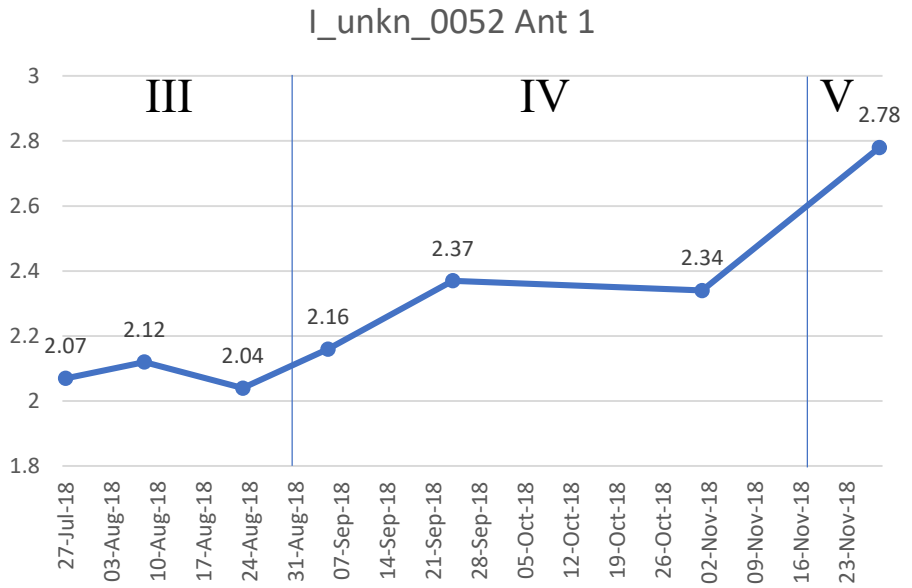


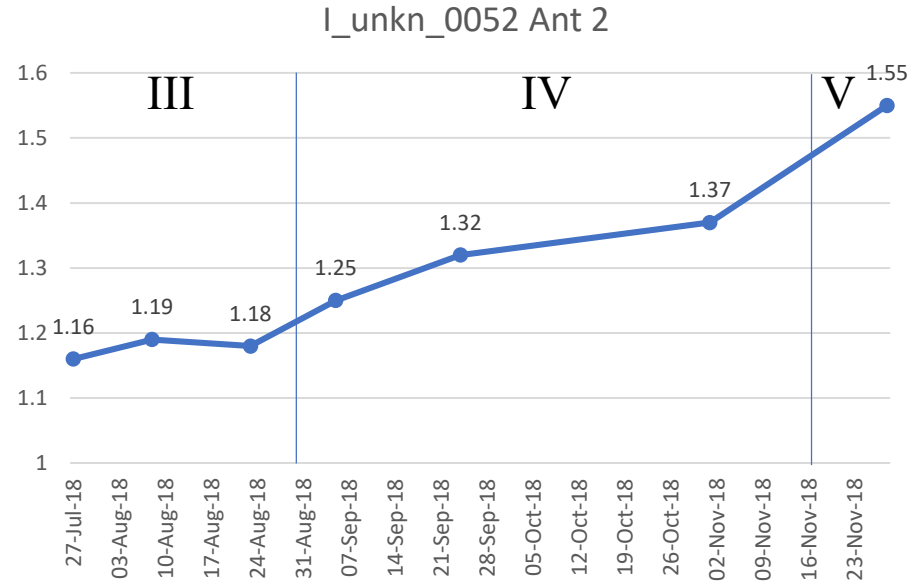




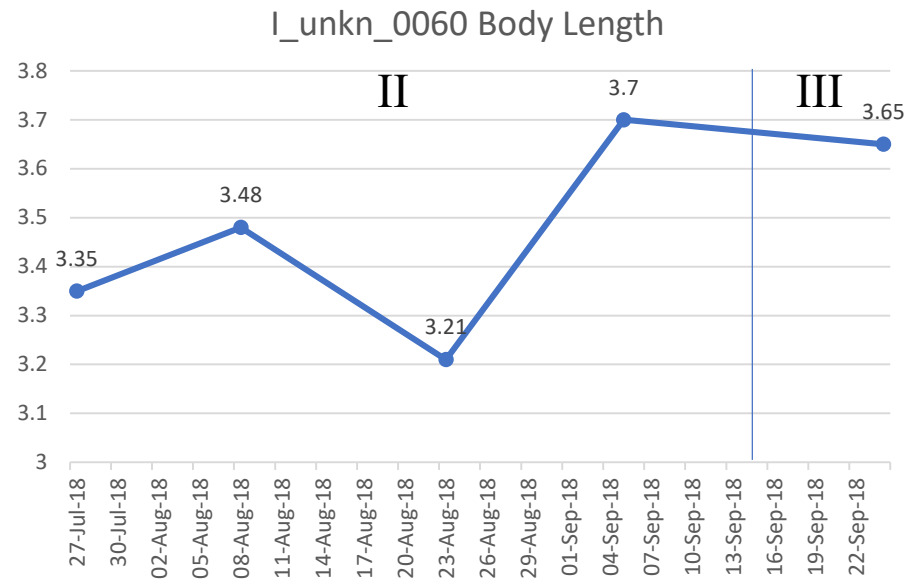
Instar determination of individual I_unkn_0050 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

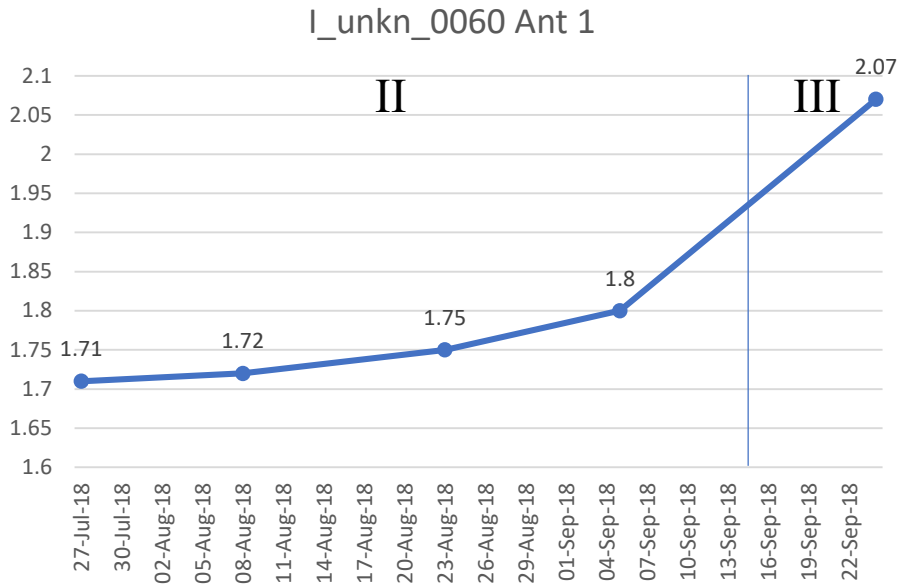


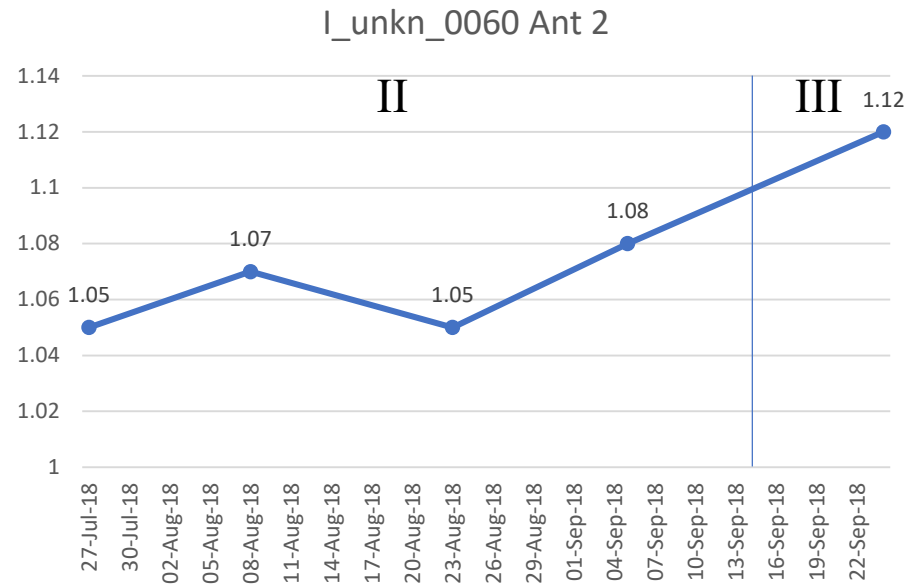




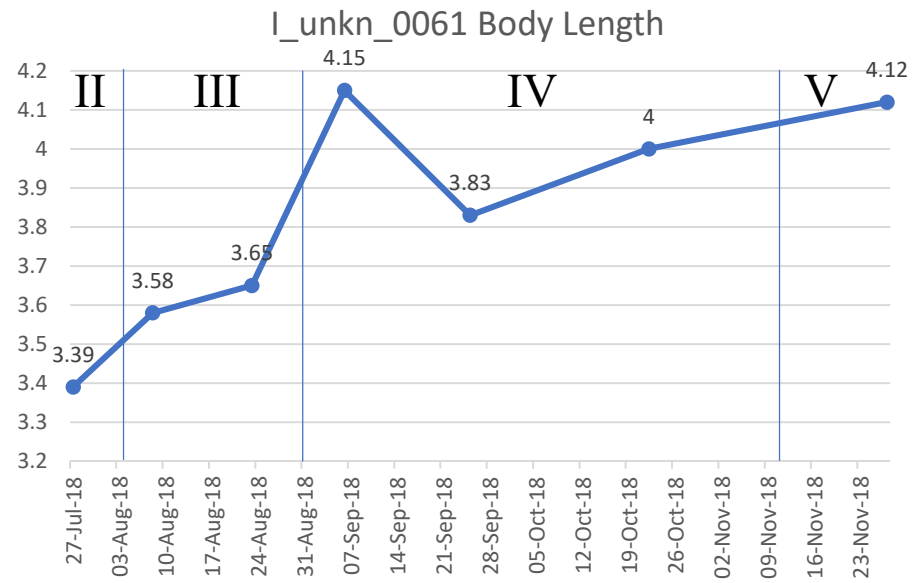
Instar determination of individual I_unkn_0052 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

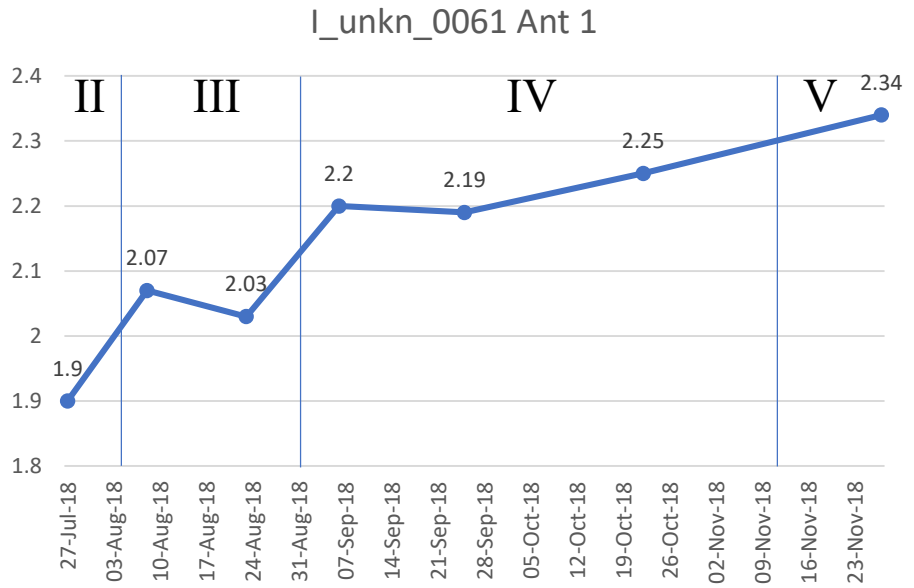


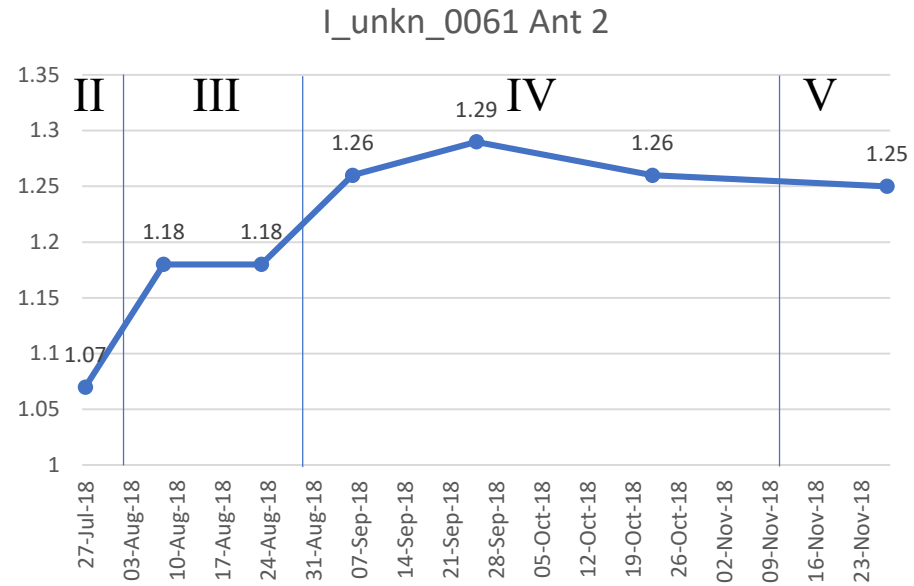




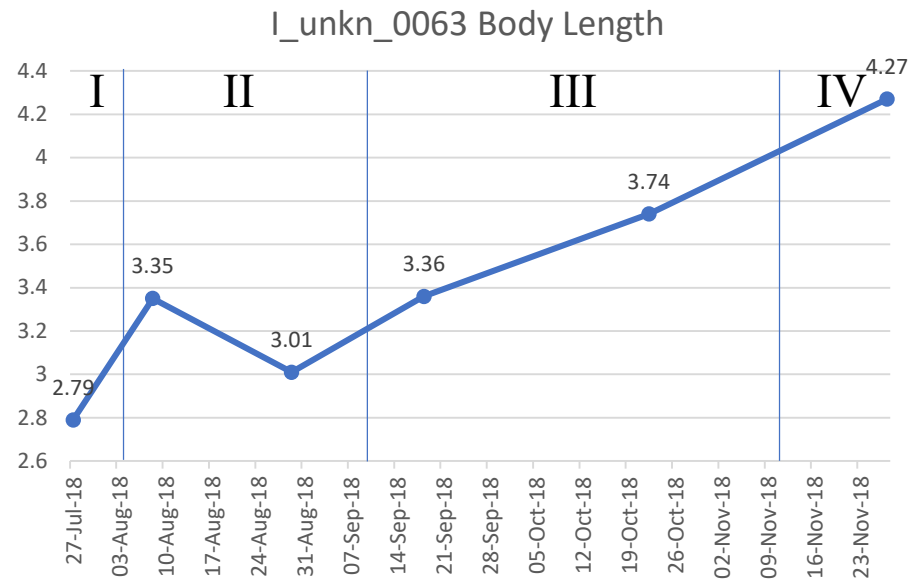
Instar determination of individual I_unkn_0060 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

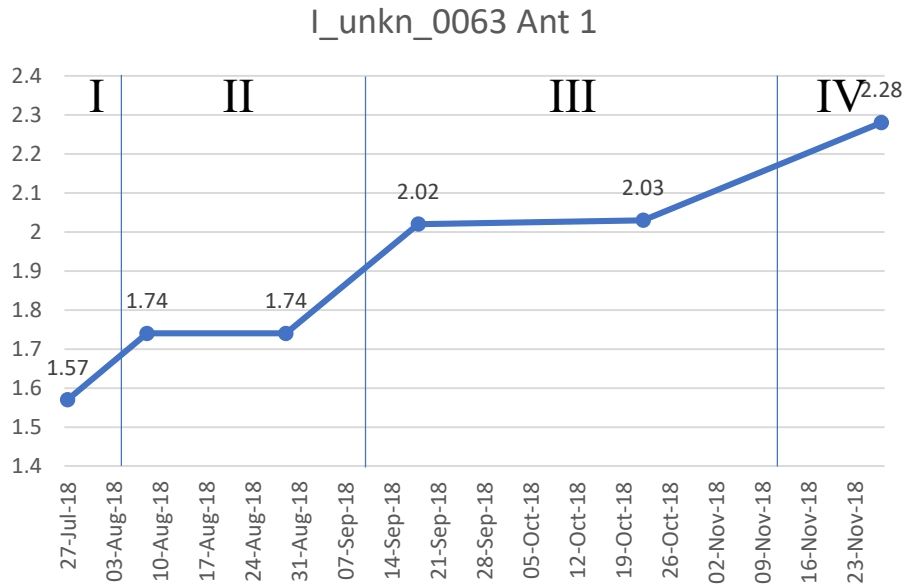


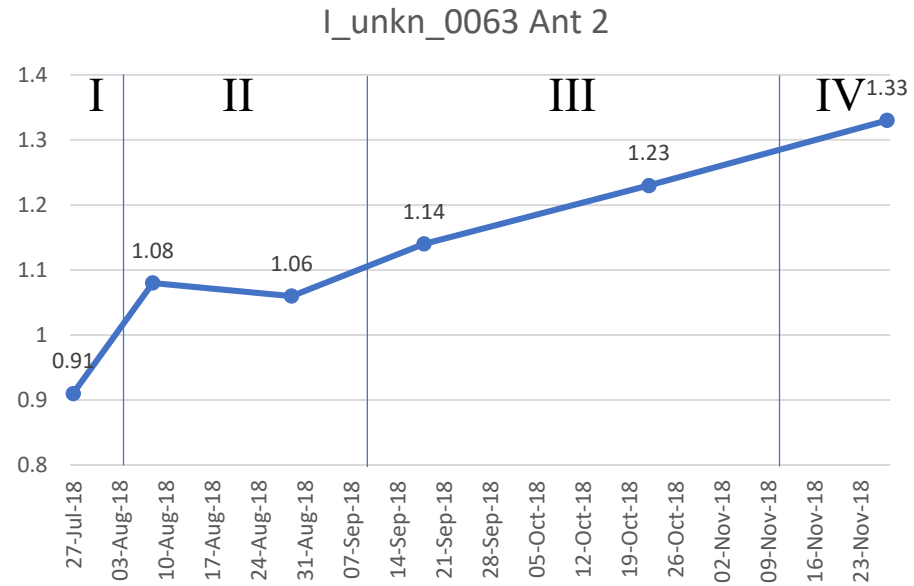




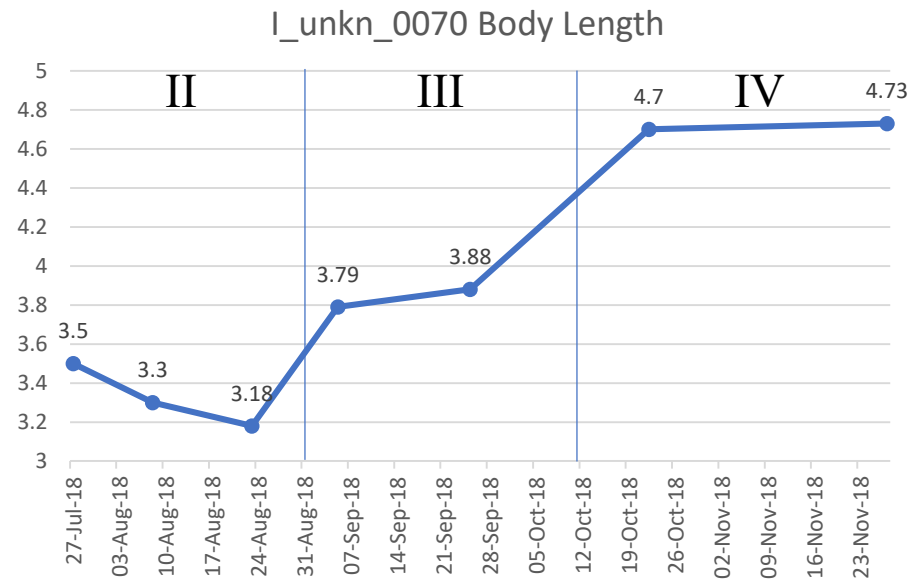
Instar determination of individual I_unkn_0061 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

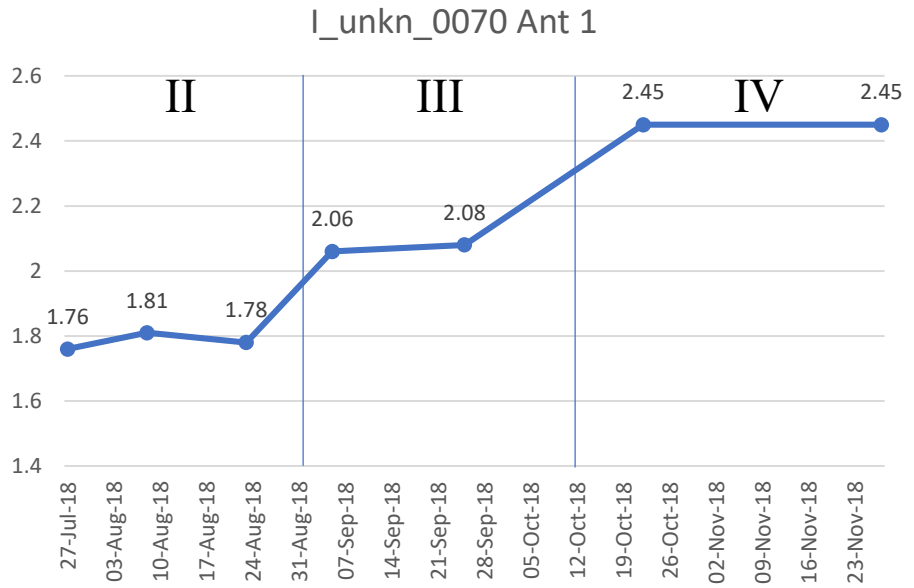


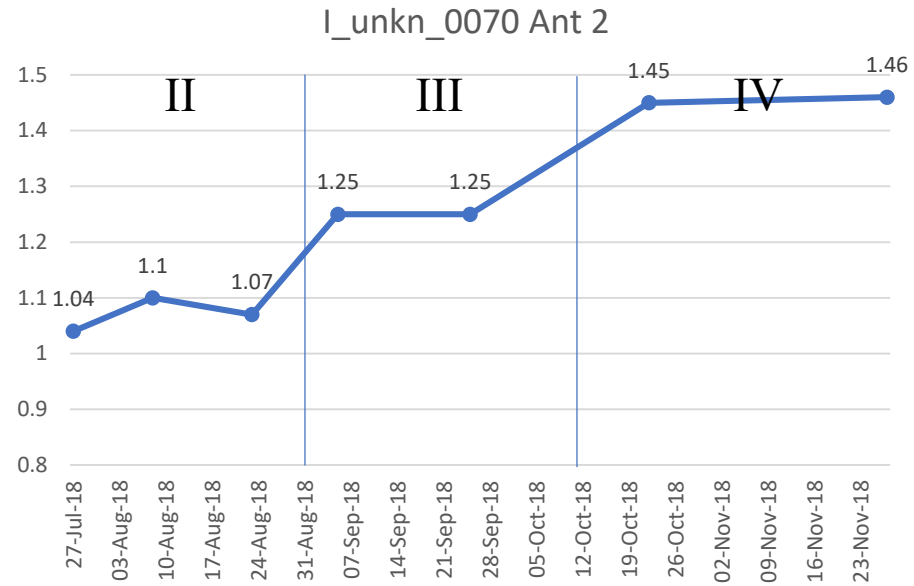




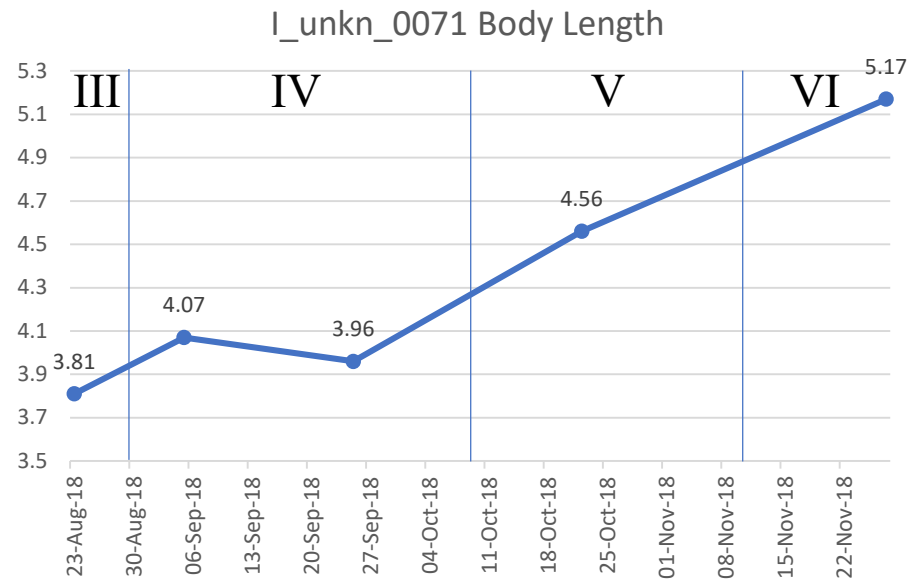
Instar determination of individual I_unkn_0063 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

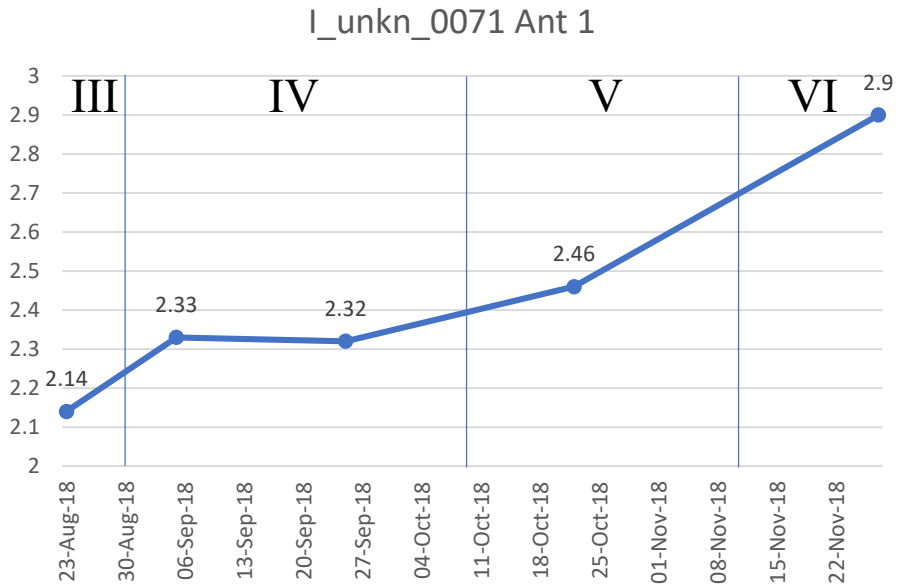


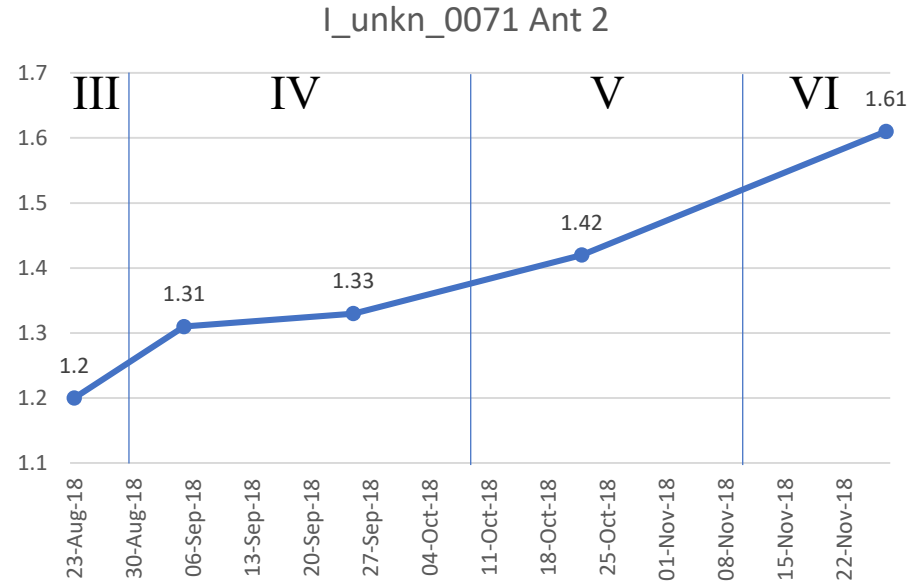




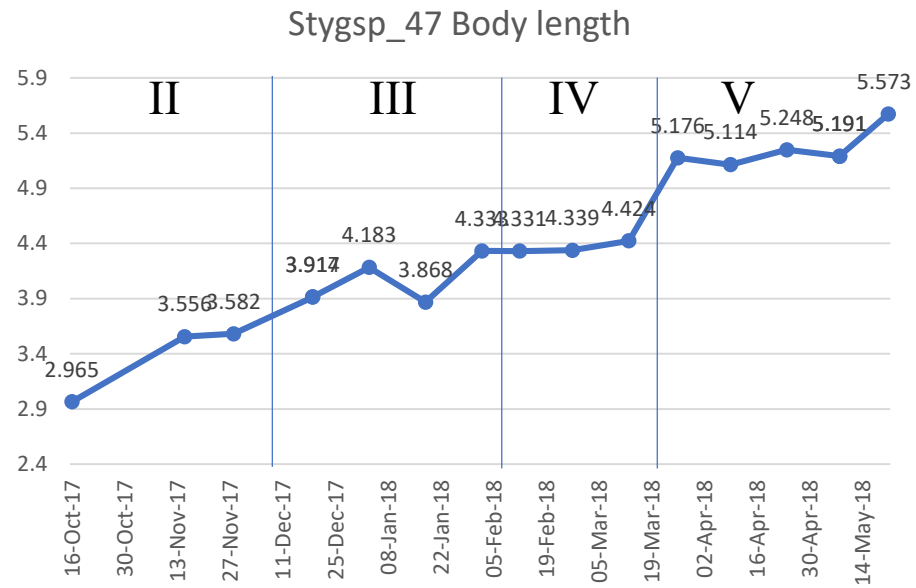
Instar determination of individual I_unkn_0070 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

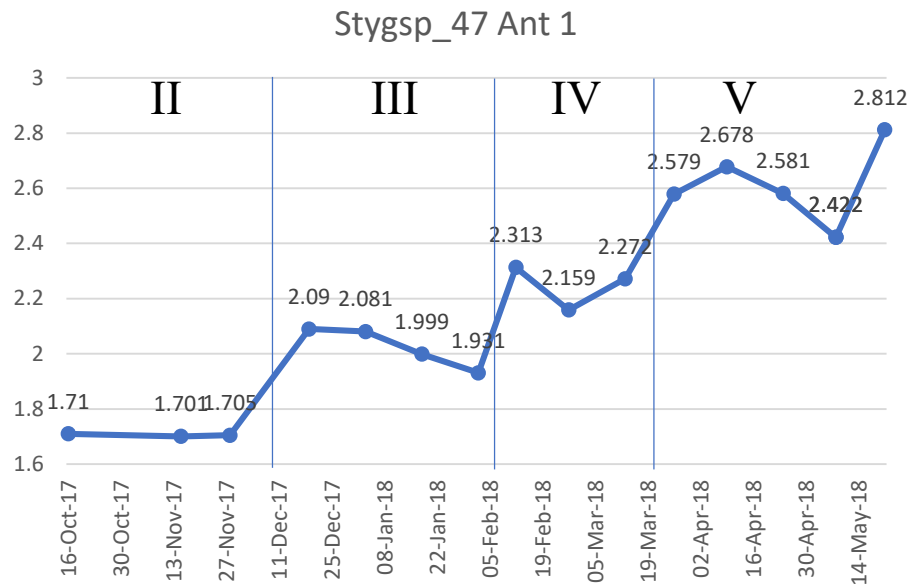


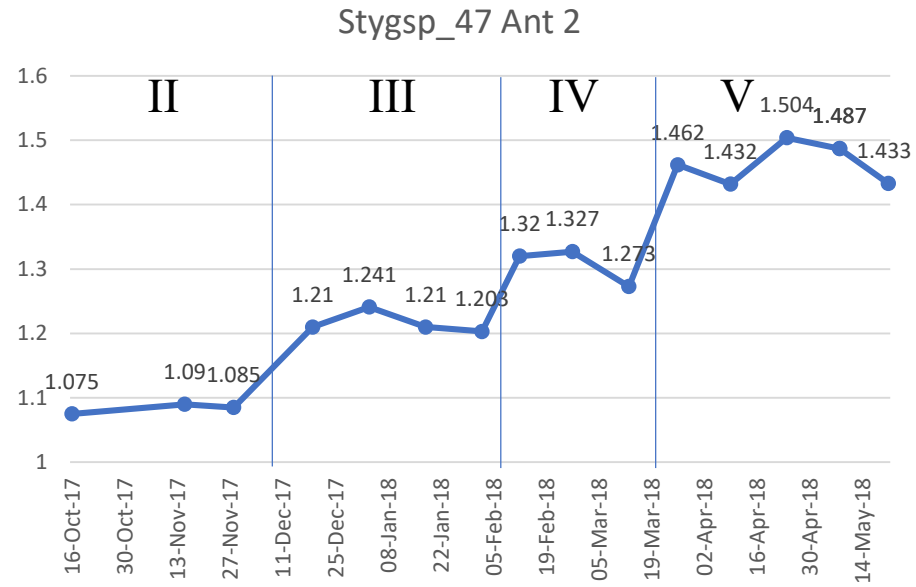




Instar determination of individual I_unkn_0071 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts. Right antenna 1 was repaired between 22-Oct and 27-Nov, indicating a molt.







Instar determination of individual Stygsp_47 based on visual inspection of body length, length of antenna segment 1 and 2. Breaks represent arbitrary delineation of molts.

Appendix D

Characters used for 58 individuals ranging is size classes from 3 mm (includes 2.5 mm) to 9 mm.

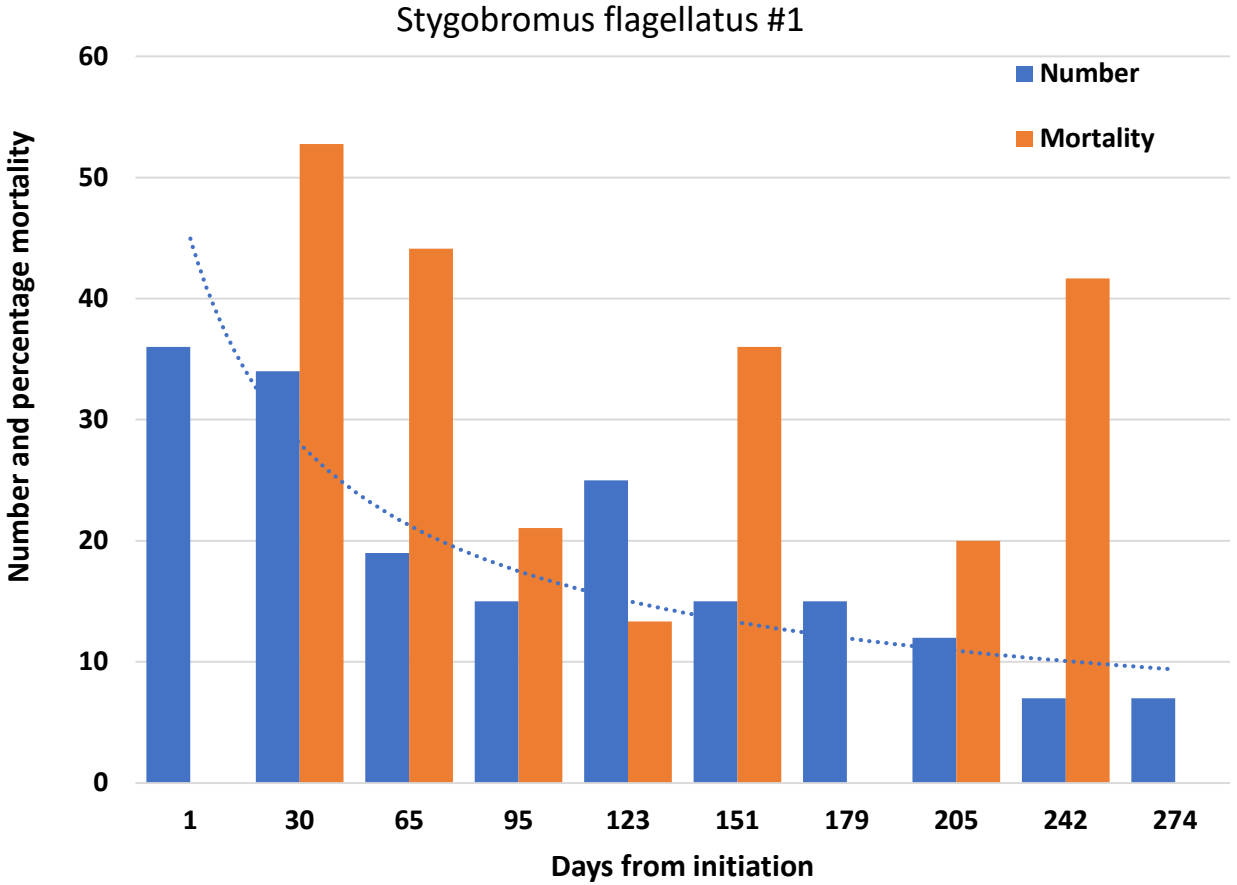
Character acronym	Definition	Type of state
Ind Id	Specimen code	Identifier
Body length	Total length of the body from the ocular lobe to the base of the telson, measured along the mid-laterad of the body.	Length
Size.class	Classification for individual based on the **Body length** measure.	Class
Ant Peduncle 1	Length of antenna peduncle 1.	Length
Ant Peduncle 2	Length of antenna peduncle 2.	Length
Gnath 1 CSP	Number of curved palm spines on gnathopod 1.	Count
Gnath 1 Prop leng	Length of propodus 1 measured from base of dactylus to base of propodus at maximum distance.	Length
Gnath 1 Prop wid	Width of propodus 1 measured from apical most curved spine perpendicular to **Gnath 1 Prop leng**.	Length
Gnath 1 PALM Length	Palmer length of propodus 1 measured from the basal most curved spine to corner of attachment to dactylus.	Length
Gnath 1 SC	Number of setae clusters along ventral margin of propodus 1.	Count
Gnath 2 CSP	Number of curved palm spines on gnathopod 2.	Count
Gnath 2 Prop leng	Length of propodus 2 measured from base of dactylus to base of propodus at maximum distance.	Length
Gnath 2 Prop wid	Width of propodus 2 measured from apical most curved spine perpendicular to **Gnath 2 Prop leng**.	Length
Gnath 2 PALM Length	Palmer length of propodus 2 measured from the basal most curved spine to corner of attachment to dactylus.	Length
Gnath 2 SC	Number of setae clusters along ventral margin of propodus 2.	Count
Corp 2 SC	Number of setae cluster along ventral margin of corpus 2.	Count
Peripod5 leng	Length of periopod 5 measured along central points of each segment from basal constriction of basis to tip of dactylus.	Length
basis5 leng	Length of basis 5 measured from basal constriction to center of apex.	Length
basis5 wid	Width of basis measured from anterior most spine perpendicular to **basis5 leng**.	Length
Peripod7 leng	Length of periopod 7 measured along central points of each segment from basal constriction of basis to tip of dactylus.	Length
basis7 leng	Length of basis 7 measured from basal constriction to center of apex.	Length
basis7 wid	Width of basis measured from anterior most spine perpendicular to **basis7 leng**.	Length
ur1 peduncle Length	Length of uropod peduncle 1 from apical connection of outer ramus to base parallel to outer margin.	Length
ur1 peduncle spines	Total number of spines occurring on the peduncle of uropod 1.	Count
ur1 INNER ramus Length	Length of the inner ramus of uropod 1 measured from base of apical-most spine to base.	Length
ur1 INNER ramus Spines	Total number of spines occurring on the inner ramus of uropod 1.	Count
ur1 OUTER ramus Length	Length of the outer ramus of uropod 1 measured from base of apical-most spine to base.	Length
ur1 OUTER ramus Spines	Total number of spines occurring on the outer ramus of uropod 1.	Count
ur2 peduncle Length	Length of uropod peduncle 2 from apical connection of outer ramus to base parallel to outer margin.	Length
ur2 peduncle spines	Total number of spines occurring on the peduncle of uropod 2.	Count
ur2 INNER ramus Length	Length of the inner ramus of uropod 2 measured from base of apical-most spine to base.	Length
ur2 INNER ramus Spines	Total number of spines occurring on the inner ramus of uropod 2.	Count
ur2 OUTER ramus Length	Length of the outer ramus of uropod 2 measured from base of apical-most spine to base.	Length
ur2 OUTER ramus Spines	Total number of spines occurring on the outer ramus of uropod 2.	Count
ur3 apical Spines	Number of apical spines on uropod 3.	Count
telson Leng	Length of telson from apex to base.	Length
telson Width	Width of telson measured along widest line perpendicular to **telson Leng**.	Length

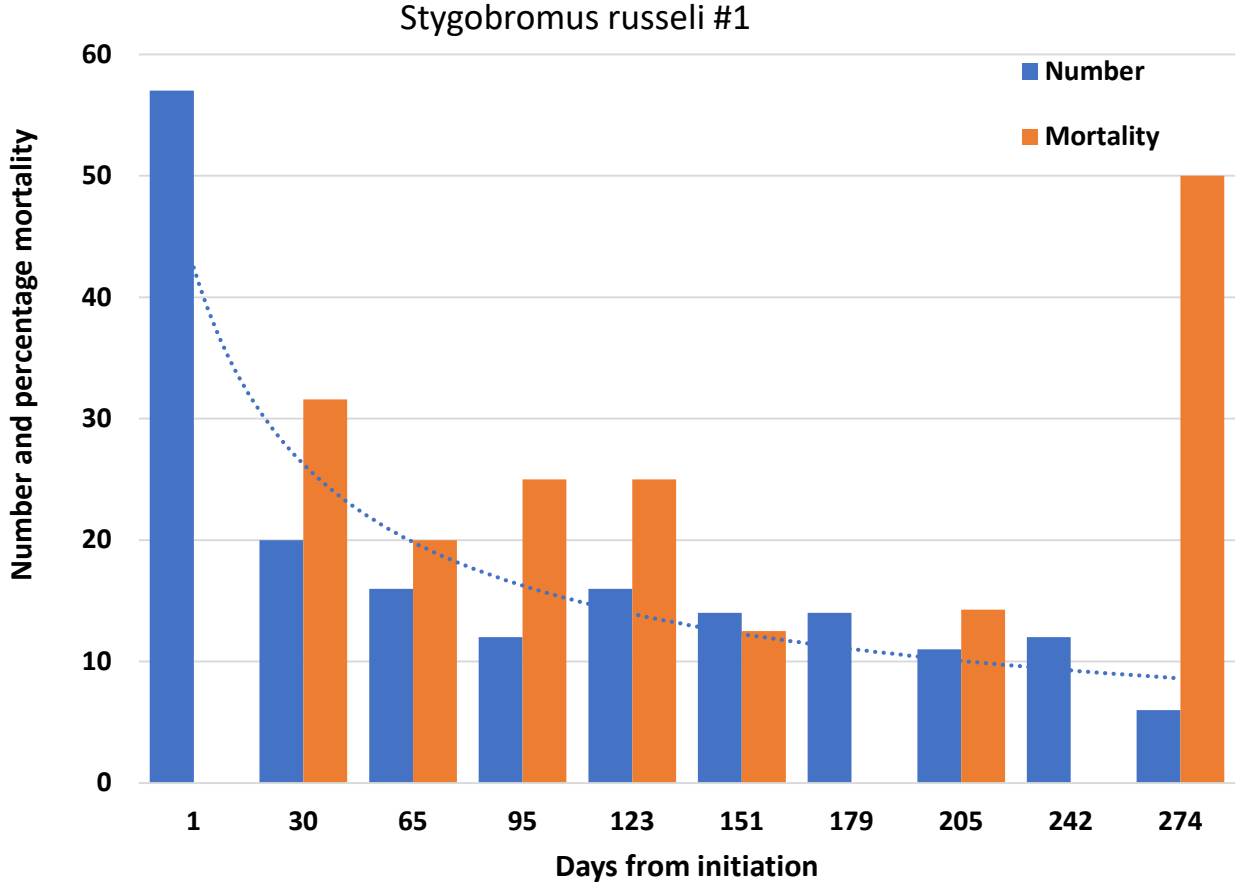
Ind Id	Body length	Size.class	Ant Peduncle 1	Ant Peduncle 2	Gnath 1 CSP	Gnath 1 Prop leng	Gnath 1 Prop wid	Gnath 1 PALM Length	Gnath 1 SC	Gnath 2 CSP	Gnath 2 Prop leng	Gnath 2 Prop wid	Gnath 2 PALM Length	Gnath 2 SC
pecki_15	2.5	2.5	0.61	0.55	2	0.29	0.17	0.15	1	1	0.26	0.16	0.14	1
pecki_17	2.78	3	0.55	0.58	2	0.29	0.2	0.17	1	1	0.27	0.17	0.14	2
pecki_13	2.79	3	0.68	0.64	2	0.33	0.2	0.19	1	1	0.29	0.18	0.15	2
pecki_18	2.87	3	0.63	0.61	2	0.33	0.2	0.18	1	1	0.29	0.18	0.16	2
pecki_16	2.94	3	0.61	0.59	2	0.31	0.19	0.18	1	1	0.29	0.17	0.14	1
pecki_14	2.99	3	0.62	0.61	2	0.32	0.2	0.19	1	1	0.27	0.17	0.14	2
pecki_4	3.34	3.5	0.74	0.77	2	0.41	0.24	0.22	1	2	0.37	0.22	0.19	3
pecki_29	3.47	3.5	0.73	0.71	2	0.39	0.22	0.22	1	1	0.35	0.2	0.18	2
pecki_28	3.58	3.5	0.73	0.72	2	0.41	0.25	0.24	1	2	0.38	0.23	0.21	3
pecki_9	3.69	3.5	0.77	0.79	2	0.4	0.25	0.24	1	2	0.33	0.22	0.2	3
pecki_6	3.7	3.5	0.87	0.84	3	0.46	0.28	0.28	2	2	0.4	0.25	0.21	3
pecki_11	3.84	4	0.82	0.86	3	0.45	0.26	0.27	2	2	0.37	0.23	0.21	3
pecki_10	3.85	4	0.84	0.8	2.5	0.41	0.26	0.26	2	2	0.38	0.22	0.21	3
pecki_27	4.05	4	0.78	0.78	2	0.43	0.26	0.25	1	2	0.37	0.22	0.2	3
pecki_5	4.08	4	0.81	0.89	2	0.44	0.3	0.28	1	2	0.43	0.26	0.24	3
pecki_26	4.17	4	0.87	0.86	3	0.48	0.29	0.29	2	2	0.42	0.26	0.24	3
pecki_23	4.28	4.5	0.9	0.86	3	0.47	0.3	0.27	2	3	0.43	0.26	0.2	3
pecki_12	4.44	4.5	0.97	0.97	3	0.52	0.33	0.29	3	2	0.46	0.28	0.24	3
pecki_19	4.51	4.5	0.88	0.97	3	0.53	0.31	0.34	2	2	0.47	0.27	0.25	4
pecki_22	4.62	4.5	0.84	0.95	3	0.53	0.31	0.32	2	3	0.44	0.28	0.26	4
pecki_24	4.68	4.5	1.01	1.08	3	0.58	0.35	0.34	2	3	0.51	0.3	0.31	3
pecki_20	4.72	4.5	0.98	1.02	3	0.56	0.32	0.33	2	2	0.5	0.31	0.25	3
pecki_41	4.81	5	0.98	0.98	3	0.5	0.32	0.3	2	2	0.45	0.28	0.25	3
pecki_61	4.93	5	1.04	1.12	3	0.6	0.36	0.35	2	3	0.54	0.31	0.31	4
pecki_32	4.95	5	0.9	1.03	3	0.55	0.33	0.31	2	3	0.49	0.29	0.27	4
pecki_21	5.16	5	0.98	0.98	3	0.53	0.34	0.33	2	3	0.5	0.29	0.29	3
pecki_37	5.18	5	1.01	1.07	3	0.55	0.34	0.37	2	2	0.49	0.28	0.29	4
pecki_7	5.2	5	1.02	1.22	3	0.58	0.38	0.35	2	3	0.56	0.34	0.29	4
pecki_31	5.28	5.5	0.98	1.22	4	0.63	0.39	0.4	3	3	0.58	0.34	0.33	3
pecki_38	5.49	5.5	1.17	1.17	3	0.68	0.41	0.42	2	3	0.59	0.35	0.34	3
pecki_2	5.52	5.5	1.24	1.3	3	0.6	0.41	0.41	3	2	0.59	0.37	0.34	4
pecki_39	5.52	5.5	1.26	1.23	4	0.65	0.39	0.42	3	3	0.62	0.36	0.39	4
pecki_8	5.55	5.5	1.19	1.22	4	0.65	0.38	0.39	3	3	0.57	0.33	0.32	4
pecki_3	5.65	5.5	1.16	1.12	4	0.63	0.42	0.42	2	3	0.6	0.34	0.3	3
pecki_46	5.81	6	1.2	1.12	3	0.63	0.37	0.41	2	3	0.57	0.33	0.34	4
pecki_52	5.88	6	1.08	1.2	3	0.62	0.36	0.38	3	3	0.56	0.35	0.33	4
pecki_53	5.95	6	1.32	1.33	4	0.65	0.39	0.41	2	3	0.63	0.36	0.35	4
pecki_25	6.08	6	1.33	1.3	4	0.72	0.42	0.46	3	3	0.66	0.38	0.38	5
pecki_33	6.17	6	1.26	1.43	5	0.78	0.47	0.53	4	4	0.74	0.42	0.46	5
pecki_51	6.18	6	1.15	1.2	3	0.62	0.38	0.36	2	3	0.57	0.33	0.33	4
pecki_48	6.21	6	1.1	1.33	4	0.66	0.39	0.4	3	3	0.61	0.35	0.35	4
pecki_40	6.23	6	1.4	1.41	4	0.69	0.43	0.45	2	3	0.65	0.39	0.37	4
pecki_34	6.29	6.5	1.34	1.46	5	0.73	0.48	0.49	3	4	0.73	0.44	0.46	4
pecki_35	6.31	6.5	1.26	1.15	4	0.69	0.42	0.44	3	3	0.59	0.36	0.36	5
pecki_57	6.39	6.5	1.29	1.45	4	0.72	0.43	0.46	3	4	0.71	0.4	0.42	4
pecki_45	6.45	6.5	1.21	1.35	3	0.64	0.39	0.4	3	3	0.6	0.36	0.37	4
pecki_58	6.69	6.5	1.26	1.51	4	0.81	0.51	0.56	3	4	0.81	0.48	0.52	4
pecki_55	6.82	7	1.48	1.69	4	0.84	0.5	0.54	3	4	0.8	0.46	0.49	5
pecki_44	6.87	7	1.4	1.45	3	0.73	0.43	0.43	3	3	0.68	0.39	0.4	4
pecki_54	7.05	7	1.47	1.63	4	0.85	0.5	0.54	4	4	0.8	0.45	0.49	5
pecki_36	7.14	7	1.48	1.57	4	0.78	0.47	0.48	3	4	0.75	0.42	0.46	5
pecki_60	7.17	7	1.51	1.72	5	0.95	0.59	0.65	4	4.5	0.92	0.56	0.6	5
pecki_30	7.19	7	1.26	1.51	4	0.76	0.46	0.5	3	3	0.69	0.41	0.43	5
pecki_59	7.19	7	1.6	1.66	4	0.8	0.51	0.52	4	4	0.81	0.49	0.48	5
pecki_50	7.78	8	1.6	1.74	4	0.9	0.6	0.58	3	4	0.87	0.53	0.55	4
pecki_43	8.08	8	1.9	1.85	4	0.94	0.58	0.6	3	4	0.89	0.52	0.6	5
pecki_42	8.33	8.5	1.91	1.95	5	0.99	0.62	0.65	3	4	0.99	0.55	0.62	5
pecki_49	8.84	9	1.78	2.02	4	0.93	0.62	0.67	3	4	0.98	0.58	0.59	5

Ind Id	Corp 2 SC	Peripod5 leng	basis5 leng	basis5 wid	Peripod7 leng	basis7 leng	basis7 wid	ur1 peduncle Length	ur1 peduncle spines	ur1 INNER ramus Length	ur1 INNER ramus Spines	ur1 OUTER ramus Length
pecki_15	2	1.06	0.31	0.24	1.59	0.38	0.28	0.31	3	0.25	1	0.21
pecki_17	2	1.12	0.33	0.26	1.52	0.38	0.3	0.3	3	0.26	1	0.22
pecki_13	2	1.13	0.34	0.27	1.66	0.41	0.32	0.35	3	0.26	1	0.23
pecki_18	2	1.13	0.35	0.27	1.7	0.4	0.32	0.35	3	0.27	2	0.23
pecki_16	2	1.13	0.33	0.27	1.56	0.4	0.33	0.32	3	0.25	2	0.22
pecki_14	2	1.1	0.34	0.25	1.63	0.4	0.31	0.31	3	0.25	2	0.21
pecki_4	2	1.48	0.45	0.36	2.16	0.54	0.42	0.49	4	0.31	2	0.24
pecki_29	2	1.38	0.43	0.33	2.04	0.51	0.38	0.41	4	0.34	2	0.29
pecki_28	2	1.56	0.46	0.36	2.14	0.52	0.45	0.45	4	0.34	2	0.31
pecki_9	2	1.47	0.39	0.36	2.21	0.5	0.42	0.38	4	0.31	2	0.27
pecki_6	3	1.75	0.51	0.41	2.29	0.6	0.46	0.49	6	0.35	2	0.28
pecki_11	2	1.63	0.47	0.36	2.05	0.56	0.43	0.42	5	0.32	3	0.31
pecki_10	2	1.53	0.46	0.38	2.14	0.55	0.43	0.43	4	0.34	2	0.27
pecki_27	2	1.46	0.46	0.38	2.21	0.57	0.44	0.43	6	0.35	2	0.3
pecki_5	3	1.7	0.52	0.42	2.61	0.61	0.48	0.39	6	0.3	3	0.22
pecki_26	3	1.71	0.52	0.42	2.55	0.62	0.49	0.48	6	0.37	3	0.31
pecki_23	3	1.85	0.52	0.43	2.32	0.62	0.5	0.48	7	0.38	3	0.34
pecki_12	3	1.85	0.55	0.46	2.65	0.65	0.52	0.54	7	0.4	3	0.32
pecki_19	3	1.93	0.57	0.45	2.77	0.67	0.52	0.51	8	0.4	3	0.35
pecki_22	3	1.88	0.57	0.44	2.72	0.67	0.51	0.52	8	0.38	3	0.33
pecki_24	3	2.05	0.6	0.5	2.94	0.7	0.57	0.58	7	0.44	3	0.38
pecki_20	3	1.98	0.58	0.48	2.86	0.71	0.54	0.55	7	0.39	3	0.35
pecki_41	3	1.8	0.54	0.45	2.61	0.64	0.51	0.53	6	0.39	3	0.34
pecki_61	3	2.2	0.67	0.5	2.83	0.75	0.59	0.63	9	0.43	3	0.38
pecki_32	4	2.09	0.6	0.48	2.78	0.69	0.57	0.56	8	0.41	3	0.34
pecki_21	3	2.04	0.61	0.52	2.92	0.73	0.55	0.55	8	0.43	3	0.29
pecki_37	3	2.03	0.59	0.48	2.85	0.73	0.56	0.58	8.5	0.44	3	0.38
pecki_7	3	2.25	0.66	0.53	3.36	0.81	0.59	0.67	9	0.44	3	0.34
pecki_31	3	2.33	0.71	0.58	3.2	0.83	0.62	0.59	8	0.39	3	0.44
pecki_38	3	1.99	0.66	0.56	3.4	0.85	0.64	0.63	8.5	0.45	3	0.41
pecki_2	3	2.6	0.74	0.56	3.63	0.86	0.59	0.68	9	0.5	4	0.45
pecki_39	4	2.48	0.74	0.57	3.52	0.89	0.66	0.7	10	0.48	3	0.42
pecki_8	3	2.32	0.71	0.56	3.35	0.86	0.62	0.69	9	0.48	4	0.39
pecki_3	4	2.53	0.75	0.59	3.5	0.86	0.65	0.68	8	0.44	4	0.42
pecki_46	3	2.34	0.69	0.54	3.27	0.84	0.62	0.65	9.5	0.47	4	0.41
pecki_52	4	2.3	0.54	0.67	3.12	0.76	0.63	0.63	8.5	0.47	3	0.34
pecki_53	3	2.44	0.73	0.55	3.4	0.83	0.62	0.66	9.5	0.49	4	0.45
pecki_25	3	2.75	0.77	0.6	3.85	0.93	0.69	0.73	11	0.52	5	0.46
pecki_33	4	2.92	0.85	0.65	4.21	0.97	0.7	0.77	11	0.58	4	0.49
pecki_51	3	2.2	0.65	0.53	2.91	0.8	0.58	0.61	8	0.45	3.5	0.41
pecki_48	4	2.46	0.71	0.58	3.56	0.86	0.66	0.71	10.5	0.51	4.5	0.43
pecki_40	3	2.59	0.77	0.58	3.77	0.93	0.65	0.72	10	0.54	5	0.48
pecki_34	4	2.83	0.81	0.65	3.78	0.94	0.67	0.79	11	0.53	4	0.49
pecki_35	4	2.44	0.74	0.61	3.43	0.87	0.65	0.72	9	0.52	4	0.44
pecki_57	4	2.65	0.8	0.62	3.78	0.97	0.67	0.78	9	0.5	5	0.47
pecki_45	3	2.42	0.7	0.56	3.29	0.77	0.66	0.71	8	0.5	4	0.4
pecki_58	4	3.05	0.88	0.66	4.25	1.02	0.71	0.82	11	0.57	5.5	0.51
pecki_55	4	3.28	0.92	0.68	4.33	1.1	0.7	0.88	10	0.6	5	0.51
pecki_44	4	2.31	0.74	0.58	3.42	0.87	0.61	0.71	9.5	0.52	4	0.47
pecki_54	4	3.05	0.95	0.69	4.06	1.08	0.76	0.8	10	0.66	4	0.54
pecki_36	4	3.16	0.85	0.69	4.13	1.02	0.74	0.84	12	0.6	5	0.49
pecki_60	4	3.46	1.03	0.76	4.5	1.21	0.8	0.98	9	0.64	4.5	0.55
pecki_30	4	2.94	0.83	0.72	3.89	1.01	0.79	0.78	12	0.59	5	0.51
pecki_59	4	3.38	0.9	0.65	4.48	1.07	0.75	0.85	10.5	0.61	4	0.52
pecki_50	4	3.34	0.94	0.73	4.71	1.12	0.8	0.88	11	0.67	5.5	0.56
pecki_43	4	3.86	1.08	0.79	4.89	1.26	0.86	1	11.5	0.73	7	0.63
pecki_42	4	3.59	1.1	0.83	5.28	1.22	0.88	0.97	12	0.72	6	0.62
pecki_49	4	4.03	1.15	0.78	5.25	1.32	0.84	1.06	13.5	0.69	6.5	0.59

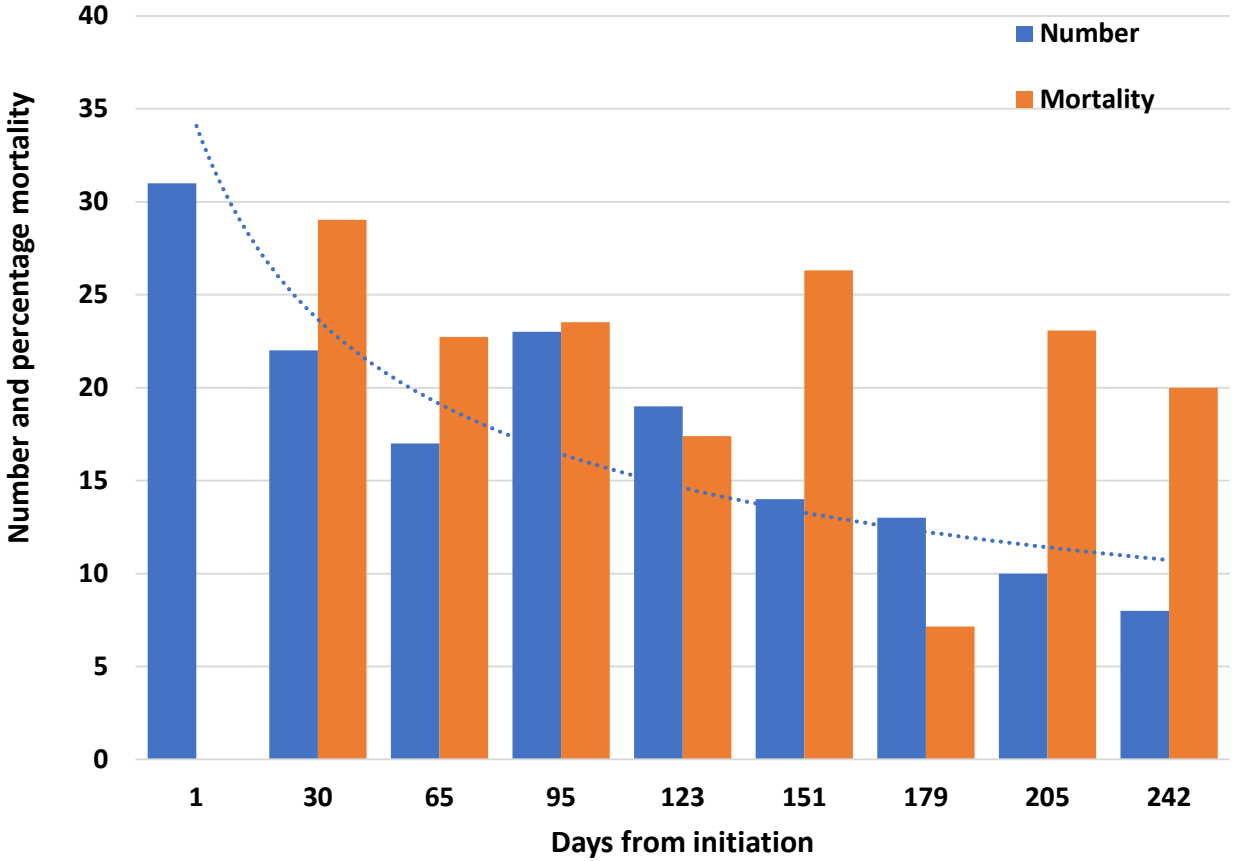
Ind Id	ur1 OUTER ramus Spines	ur2 peduncle Length	ur2 peduncle spines	ur2 INNER ramus Length	ur2 INNER ramus Spines	ur2 OUTER ramus Length	ur2 OUTER ramus Spines	ur3 apical Spines	telson Leng	telson Width
pecki_15	0	0.17	2	0.19	1	0.16	0	2	0.18	0.13
pecki_17	0	0.16	2	0.2	1	0.16	0	2	0.15	0.14
pecki_13	0	0.19	2	0.2	1	0.16	0	2	0.18	0.13
pecki_18	0	0.19	2	0.22	1	0.18	0	2	0.16	0.14
pecki_16	0	0.19	2	0.2	1	0.17	0	2	0.15	0.13
pecki_14	0	0.2	2	0.19	1	0.15	0	2	0.17	0.12
pecki_4	0	0.26	2	0.26	2	0.2	0	2	0.24	0.16
pecki_29	0	0.25	2	0.24	1	0.21	0	2	0.22	0.15
pecki_28	1	0.27	2	0.25	2	0.21	0	2	0.24	0.17
pecki_9	1	0.21	3	0.25	2	0.21	0	2	0.23	0.15
pecki_6	1	0.29	3	0.26	2	0.21	0	2	0.27	0.17
pecki_11	1	0.26	3	0.27	2	0.22	0	2	0.26	0.16
pecki_10	1	0.25	3	0.26	2	0.21	1	2.5	0.22	0.17
pecki_27	1	0.26	3	0.26	2	0.2	0	2	0.26	0.18
pecki_5	1	0.27	3	0.27	2	0.21	1	3	0.25	0.17
pecki_26	2	0.29	3	0.27	2	0.23	1	2.5	0.26	0.18
pecki_23	2	0.3	3	0.28	4	0.24	1	3	0.29	0.19
pecki_12	2	0.31	3	0.31	2	0.24	1	2	0.32	0.21
pecki_19	1	0.35	3	0.35	3	0.28	1	3	0.29	0.21
pecki_22	2	0.31	4	0.31	4	0.24	1	3	0.39	0.22
pecki_24	2	0.34	3	0.33	3	0.27	1	4	0.33	0.2
pecki_20	1	0.33	4	0.3	3	0.26	1	3	0.3	0.2
pecki_41	2	0.32	3	0.31	2	0.24	1	2	0.38	0.2
pecki_61	3	0.36	5	0.35	4	0.29	1	3.5	0.38	0.22
pecki_32	2	0.34	4	0.33	3	0.26	1	3	0.32	0.19
pecki_21	2	0.35	3	0.33	2	0.26	1	3	0.33	0.21
pecki_37	3	0.36	4	0.32	4	0.27	1	3	0.31	0.21
pecki_7	3	0.38	4	0.37	4	0.28	1	4	0.39	0.25
pecki_31	2	0.36	4	0.3	4	0.33	1	3	0.36	0.24
pecki_38	3	0.42	4	0.37	4	0.27	1	4	0.35	0.26
pecki_2	4	0.38	5	0.38	5	0.31	2	4	0.36	0.23
pecki_39	3	0.42	6	0.37	4	0.32	2	4	0.39	0.23
pecki_8	3	0.4	5	0.37	4	0.29	1	3	0.36	0.22
pecki_3	3	0.42	3	0.36	3	0.26	1	4	0.41	0.26
pecki_46	3	0.35	4	0.37	4	0.29	1	3	0.36	0.22
pecki_52	3.5	0.37	4.5	0.39	4	0.3	1	3	0.34	0.23
pecki_53	3.5	0.42	5.5	0.36	4.5	0.32	1.5	4	0.35	0.23
pecki_25	4	0.45	6	0.41	5	0.33	3	4.5	0.43	0.25
pecki_33	4	0.49	6	0.42	5	0.36	3	4	0.42	0.27
pecki_51	2	0.36	3	0.34	4	0.28	1	3	0.32	0.23
pecki_48	4.5	0.41	6.5	0.4	4	0.33	3	4	0.39	0.24
pecki_40	3	0.46	4	0.41	5	0.33	2	4	0.45	0.28
pecki_34	5	0.46	6	0.42	4	0.34	3	4	0.41	0.26
pecki_35	3	0.44	5	0.4	5	0.31	1	4	0.43	0.27
pecki_57	4	0.47	5	0.38	5	0.34	3	4	0.42	0.26
pecki_45	3	0.41	5	0.39	5	0.32	2	3.5	0.37	0.24
pecki_58	5	0.49	7.5	0.38	4	0.36	3	5	0.4	0.26
pecki_55	5	0.47	6.5	0.43	5	0.37	2	4	0.43	0.27
pecki_44	5	0.45	5	0.4	4.5	0.33	2	4	0.45	0.24
pecki_54	3	0.43	4	0.45	6	0.4	3	4	0.49	0.29
pecki_36	5	0.51	5	0.47	5	0.37	3	4	0.44	0.27
pecki_60	5	0.58	5	0.5	4.5	0.4	2	4.5	0.5	0.29
pecki_30	4	0.5	7	0.47	6	0.44	3	5	0.47	0.31
pecki_59	5	0.52	7	0.46	5	0.38	3	5	0.43	0.29
pecki_50	4	0.53	5.5	0.47	6	0.4	3.5	5	0.45	0.29
pecki_43	5	0.65	7.5	0.56	6	0.46	3	4.5	0.55	0.33
pecki_42	6.5	0.62	6.5	0.49	6	0.41	4	5	0.49	0.31
pecki_49	7	0.57	7	0.5	6.5	0.43	4	5	0.56	0.31

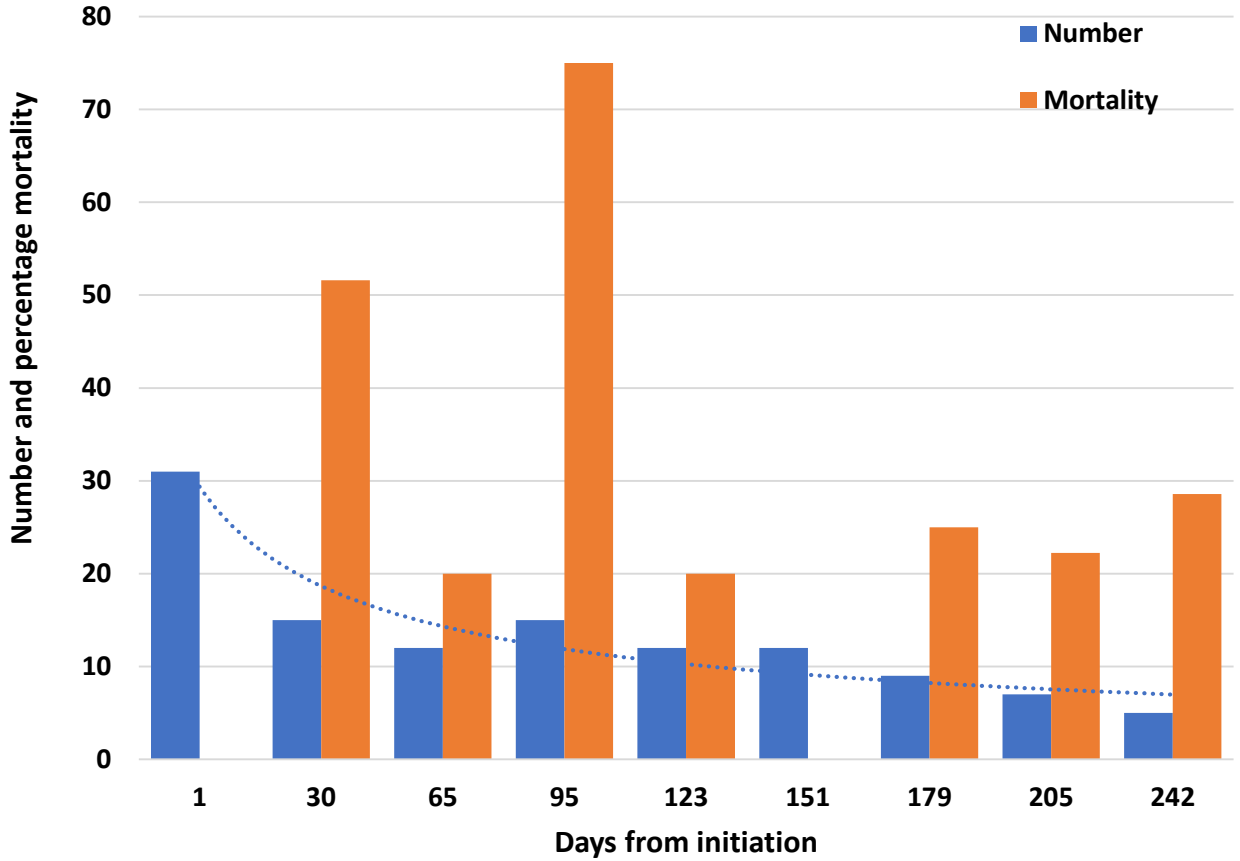
Appendix E





Number of individuals counted during each census, shown as number of days from the initiation of the common garden on 12-Feb-2018. On 25-Ju-2018 Subjects were transferred to a flow-through common garden. The percentage mortality between censuses (accounting for individuals removed or added) is also given. The trendline shows the trajectory of surviving individuals based on a power function.





Number of individuals counted during each census, shown as number of days from the initiation of the common garden on 12-Feb-2018. On 25-Ju-2018 Subjects were transferred to a flow-through common garden. The percentage mortality between censuses (accounting for individuals removed or added) is also given. The trendline shows the trajectory of surviving individuals based on a power function.

Appendix F

specimen	Date instar 1	Size instar 1	Ant1 instar 1	Ant2 instar 1	Days instar1	Date instar 2	Size instar 2	Ant1 instar 2	Ant2 instar 2	Days instar2	Date instar 3	Size instar 3	Ant1 instar 3	Ant2 instar 3	Days instar3	Date instar 4	Size instar 4	Ant1 instar 4	Ant2 instar 4	Days instar4	Date instar 5	Size instar 5	Ant1 instar 5	Ant2 instar 5	Days instar5	Date instar 6	Size instar 6	Ant1 instar 6	Ant2 instar 6
I_unkn_0007	26-Jul	3.09	1.61	0.95	41.00	5-Sep	3.77	1.91	1.12	47.00	22-Oct	4.16	2.18	1.25															
I_unkn_0011	26-Jul	3.10	1.97	1.08	13.00	8-Aug	3.63	2.14	1.20	21.00	29-Aug	4.11	2.33	1.33	54	22-Oct	4.44	2.59	1.42										
I_unkn_0012						26-Jul	3.48	1.84	1.10		10-Sep	3.75	2.11	1.23	78	27-Nov	4.14	2.39	1.38										
I_unkn_0013	26-Jul	2.89	1.62	1.00	12.00	7-Aug	3.44	1.76	1.07	29.00	5-Sep	3.73	2.04	1.23	47	22-Oct	4.19	2.34	1.32										
I_unkn_0015	26-Jul	2.87	1.69	0.97	13.00	8-Aug	3.35	1.81	1.07	47.00	24-Sep	3.83	2.06	1.22	64	27-Nov	4.35	2.37	1.35										
I_unkn_0022	26-Jul	2.61	na	0.88	12.00	7-Aug	3.28	na	1.03	42.00	18-Sep	3.77	na	1.16															
I_unkn_0023	26-Jul	3.01	1.64	0.99	13.00	8-Aug	3.37	1.80	1.09	28.00	5-Sep	4.02	2.09	1.23	47	22-Oct	4.07	2.36	1.37										
I_unkn_0024						26-Jul	3.67	1.95	1.11		22-Aug	4.09	2.25	1.23	33	24-Sep	4.53	2.61	1.38										
I_unkn_0025						26-Jul	3.59	2.11	1.14		7-Aug	4.02	2.24	1.21	65	11-Oct	4.34	2.41	1.36										
I_unkn_0026						31-Jul	3.46	1.82	1.11		24-Sep	3.63	2.01	1.23	64	27-Nov	4.13	2.19	1.32										
I_unkn_0030						26-Jul	3.64	1.98	1.16		10-Sep	3.95	2.03	1.28	52	1-Nov	4.40	2.29	1.38	26	27-Nov	4.67	2.52	1.47					
I_unkn_0031						26-Jul	3.62	2.07	1.12		7-Aug	4.24	2.45	1.32	21	28-Aug	4.56	2.72	1.49										
I_unkn_0033	26-Jul	2.95	1.63	0.92	12.00	7-Aug	3.46	1.73	1.06	28.00	4-Sep	3.83	2.04	1.18	84	27-Nov	4.04	2.34	1.33										
I_unkn_0034																26-Jul	3.96	2.68	1.29	33	28-Aug	4.87	2.94	1.55					
I_unkn_0037	26-Jul	2.86	1.69	0.96	13.00	8-Aug	3.34	1.89	1.10	75.00	22-Oct	3.63	1.94	1.17	36	27-Nov	3.70	2.12	1.28										
I_unkn_0038	27-Jul	3.06	1.67	0.96	12.00	8-Aug	3.33	1.81	1.10	27.00	4-Sep	3.77	2.11	1.24	48	22-Oct	4.46	2.55	1.43										
I_unkn_0039						26-Jul	3.26	1.93	1.03		8-Aug	3.98	2.16	1.24	85	1-Nov	4.50	2.49	1.30										
I_unkn_0040	27-Jul	3.29	1.73	0.99	12.00	8-Aug	3.61	1.85	1.12	64.00	11-Oct	3.88	2.10	1.23															
I_unkn_0042						27-Jul	3.20	1.94	1.02		8-Aug	3.76	2.09	1.18	33	9/10/2018	4.49	2.48	1.38										
I_unkn_0043	27-Jul	3.00	1.69	0.97	12.00	8-Aug	3.50	1.91	1.11	111.00	27-Nov	3.81	2.14	1.27															
I_unkn_0044						27-Jul	3.36	1.80	1.10		22-Oct	3.58	1.94	1.14															
I_unkn_0045	27-Jul	2.91	1.63	0.93	11.00	7-Aug	3.49	1.80	1.09	65.00	11-Oct	3.83	2.13	1.22															
I_unkn_0050						27-Jul	3.21	1.68	1.02		10-Sep	3.66	1.94	1.12															
I_unkn_0052											27-Jul	3.80	2.08	1.18	40	9/5/2018	4.20	2.29	1.31	84	28-Nov	5.00	2.78	1.55					
I_unkn_0060						27-Jul	3.44	1.75	1.06		24-Sep	3.65	2.07	1.12															
I_unkn_0061						27-Jul	3.39	1.90	1.07		8-Aug	3.62	2.05	1.18	29	9/6/2018	3.99	2.21	1.27	82	27-Nov	4.12	2.34	1.25					
I_unkn_0063	27-Jul	2.79	1.57	0.91	12.00	8-Aug	3.18	1.74	1.07	41.00	18-Sep	3.55	2.03	1.19	70	11/27/2018	4.27	2.28	1.33										
I_unkn_0070						27-Jul	3.33	1.78	1.07		5-Sep	3.84	2.07	1.25	47	10/22/2018	4.72	2.45	1.46										
I_unkn_0071											23-Aug	3.81	2.14	1.20	13	9/5/2018	4.02	2.33	1.32	47	22-Oct	4.56	2.46	1.42		27-Nov	5.17	2.90	1.61
Stygsp_47						16-Oct	3.37	1.71	1.08		19-Dec	4.10	2.02	1.22	55	2/12/2018	4.36	2.25	1.31	42	26-Mar	5.30	2.61	1.46					
		Size instar 1	Ant1 instar 1	Ant2 instar 1	Days instar1	Date instar 2	Size instar 2	Ant1 instar 2	Ant2 instar 2	Days instar2	Date instar 3	Size instar 3	Ant1 instar 3	Ant2 instar 3	Days instar3	Date instar 4	Size instar 4	Ant1 instar 4	Ant2 instar 4	Days instar4	Date instar 5	Size instar 5	Ant1 instar 5	Ant2 instar 5					
	mean	2.96	1.68	0.96	14.46		3.44	1.86	1.09	48.08		3.84	2.10	1.22	50.71		4.27	2.40	1.35	52.33		4.75	2.61	1.45					
	range	2.61-3.29	1.57-1.97	0.88-1.08	11-41		3.18-3.77	1.68-2.14	1.02-1.2	21-111		3.55-4.24	1.94-2.45	1.12-1.33	13-85		3.7-4.72	2.12-2.72	1.27-1.49	26-84		4.12-5.3	2.34-2.94	1.25-1.55					
	std	0.16	0.10	0.05	7.68		0.15	0.12	0.04	24.28		0.18	0.11	0.05	19.24		0.24	0.16	0.06	22.68		0.37	0.20	0.10					
	n	13	12	13	13		27	26	27	13		29	28	29	21		22	22	22	6		6	6	6					

Appendix G

Loadings for principal components 1 and 2 for character states of *Stygobromus pecki* immature stages of growth. Principal component 1 was used to find instar estimates based inflection points of a smooth spline of the ranked component.

Character	PC1	PC2
ur1.peduncle.spines	0.576	0.423
ur1.OUTER.ramus.Spines	0.343	-0.372
ur2.peduncle.spines	0.311	-0.565
ur2.INNER.ramus.Spines	0.304	0.433
ur1.INNER.ramus.Spines	0.228	-0.014
ur2.OUTER.ramus.Spines	0.210	-0.243
Gnath.2.SC	0.203	0.261
ur3.apical.Spines	0.194	-0.097
Gnath.2.CSP	0.188	0.047
Peripod7.leng	0.179	-0.032
Gnath.1.SC	0.173	-0.146
Gnath.1.CSP	0.169	-0.093
Corp.2.SC	0.148	-0.061
Peripod5.leng	0.136	-0.027
Ant.Peduncle.2	0.068	-0.016
Ant.Peduncle.1	0.057	0.002
basis7.leng	0.045	0.005
basis5.leng	0.038	0.001
ur1.peduncle.Length	0.036	-0.008
Gnath.2.Prop.leng	0.035	-0.014
Gnath.1.Prop.leng	0.034	0.001
basis7.wid	0.029	0.014
basis5.wid	0.029	0.008
Gnath.1.PALM.Length	0.024	-0.006
Gnath.2.PALM.Length	0.023	-0.017
ur1.INNER.ramus.Length	0.023	0.004
ur2.peduncle.Length	0.022	-0.001
Gnath.1.Prop.wid	0.021	-0.004
ur1.OUTER.ramus.Length	0.021	0.001
Gnath.2.Prop.wid	0.020	-0.011
telson.Leng	0.019	0.015
ur2.INNER.ramus.Length	0.017	0.008
ur2.OUTER.ramus.Length	0.015	0.001
telson.Width	0.010	0.008

January, 2019

Life-history aspects of the Comal Springs dryopid beetle (*Stygoparnus comalensis*) and notes on life-history aspects of the Comal Springs riffle beetle (*Heterelmis comalensis*)

Prepared by:
Ely Kosnicki, PhD – Senior Invertebrate Ecologist
Eric Julius – Aquatic Ecologist
BIO-WEST, Inc.
1405 United Drive, Suite 111
San Marcos, TX 78666

Prepared for:
The Edwards Aquifer Authority
900 E. Quincy Street
San Antonio, TX 78215

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Purpose statement

The objectives of this project were to provide a better understanding of the life histories of the Comal Springs dryopid beetle (*Stygoparnus comalensis*) and to contribute information towards the improvement of captive propagation of this species that is in-line with the goals and objective of the Edwards Aquifer Habitat Conservation Plan. More specifically, the goals of this project were to better understand habitat requirements, sexual dimorphism, mating and oviposition requirements, egg incubation rates, larval habitat requirements, larval growth, and adult response to flow.

Studies conducted and main findings

- Identification of natural habitats and subject acquisition
    - Root mats (particularly sycamore) associated with spring upwellings were found to be reliable habitats to retrieve subjects through use of wood lures.
- Identification of sexually dimorphic characters (including the Comal Springs riffle beetle)
    - In living adults, the visible structure of the 8th abdominal sternite consistently distinguish male *S. comalensis* while the fused gonocoxites of the ovipositor consistently distinguish female *S. comalensis*.
    - Morphological investigations of *Heterelmis comalensis* revealed that the visible structure of the 8th abdominal sternite in living adults consistently distinguish female and male subjects.
- Mating and oviposition
    - Mating chambers were constructed to accommodate mating pairs with cotton cloth used to catch eggs. Although eggs were observed to be oviposited underwater, it is still unknown if females actively seek an emergent environment to oviposit.
- Egg production and incubation
    - At the time this report was prepared 173 eggs were produced from 15 females, with eggs 22 hatching; however, a number of eggs were still developing.
    - Incubations times were 82 ± 15 days.
- Larval habitat and growth
    - Larvae exhibited a burrowing behavior in conditioned poplar wood dowels.
    - Larvae were not fully developed at the time this report was prepared, but it was estimated that larvae reached the 4th instar; it was estimated that larvae undergo 6 instars before pupation.
- Adult response to flow (including the Comal Springs riffle beetle)
    - Adult beetles tend to remain within a food resource when they are initially placed in one, regardless of flow. *Stygoparnus comalensis* tends to move towards flow under slower-flow conditions, while *H. comalensis* tends to move with flow under similar conditions.
- Many questions remain, especially since most of the study was discontinued before completion. In particular more information is needed in regard to: fecundity, optimal conditions for egg incubation, complete larval development, pupation, and adult longevity.

Executive summary

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. Some of the species of concern still pose several substantial questions concerning refuge cultivation; particularly the invertebrate species. The Comal Spring dryopid beetle (*Stygoparnus comalensis*) is a federally endangered species that is adapted to subterranean habitats associated with Edwards Aquifer springs. *Stygoparnus comalensis* is the only species described for the genus and, therefore, little is known about the life history and the environmental requirements of this species.

During the process of obtaining specimens, specific locations at Comal Springs were identified as habitats where *S. comalensis* could be sampled, consistently. The use of poly-cotton lures was not an effective means of sampling adults; adults were obtained on coarse woody debris that were directly placed on or in spring upwellings that appeared to be associated with the roots of sycamore trees that grew adjacent to the springs.

Male and female adults could be told apart by observing the 8th and 9th abdominal sternites beneath the 5th ventrite, for males and females, respectively. These morphological features were readily viewable in living subjects with the use of proper lighting techniques. Measures showed that females and males did have significantly different body measures; however, there was too much overlap to utilize measurements in a reliable way for discerning sex. The use of the 8th abdominal sternites were also found to be reliable characters for separating female and male Comal Springs riffle beetles (*Heterelmis comalensis*). Review of the literature showed that these characters have been used to separate sexes of other species of riffle beetles.

An oblique plane apparatus was implemented to determine if females oviposited above or below the surface of the water. Attempts to use this device turned out to be difficult to utilize as eggs were never recovered. Smaller mating chambers were constructed to mimic the upwelling of the spring systems with a food resource that was partially emergent. The food resource consisted of a conditioned poplar dowel loosely wrapped in poly-cotton lure. Although, the oviposition behavior of the female was never observed, eggs were regularly collected from a number of mating pairs and groups. Eggs were not glued or attached to any substrate and appeared to be produced one at a time. Eggs sank and were usually found below the water line entangled in cotton fibers or discovered loose.

We collected a total of 173 eggs from 15 females. However, not all females produced eggs and several appeared to be prolific; one female produced 47 eggs in six months and was still alive at the time this report was written. Because of the limited time to study this organism, we can only make crude estimations with regard to the fecundity of this species.

Eggs were transferred to a terrestrial humid habitat, consisting of a bed of aquarium rocks raised above a water level with a conditioned leaf-base layer, a conditioned poplar dowel, and other conditioned leaves forming a tent over the eggs. Eggs hatched over a period of 82 ± 15 days and larvae were found feeding on leaves and dowels.

About four instars were delineated from measurements of the larvae over time. Larvae were estimated to undergo six instars within a period of 134 days by extrapolating to the maximum lengths reported in the literature. It is still a question as to how many instars larvae go through and how long they spend in each instar. There is no information regarding the final instar from this study as it was concluded before larvae were given the chance to fully develop.

After several checks on larval growth it became apparent that larvae were burrowing into the poplar dowel, creating galleries. It is surmised that this behavior is reminiscent to their natural habitat conditions and that woody material, possibly fibrous roots such as those of sycamore trees at Comal Springs. It is suggested that the burrowing and gallery formation within woody material serves as a means for larvae to survive in a submerged habitat and is likely that they utilize the galleries as chambers for pupation.

A variable-flow artesian-spring emulator (VFASE) was constructed to simulate a spring system with varying levels of flow. The VFASE was used to test the response of *S. comalensis* and *H. comalensis*, separately, under varying flow regimes with differing locations for food resources. In general, *Stygoparnus* moved against the flow towards a food resource while *Heterelmis* moved in the direction of flow to a food resource. Both species tended to stay in a food resource if placed in one at the beginning of the trial.

Although many new insights regarding the life history of *S. comalensis* have been revealed during the course of this study, many questions remain unanswered. In general, more time is needed to study the life cycle of this animal. Information regarding the optimum larval habitat, number of instars, length of time to pupation, pupation requirements, adult longevity, and fecundity still remain in question.

Introduction

Stygoparnus comalensis life histories

The Comal Springs dryopid beetle (*Stygoparnus comalensis*) is a federally endangered species (USFWS 1997) adapted to subterranean habitats associated with Edwards Aquifer (EA) spring systems. *Stygoparnus comalensis* have been recovered from a limited number of perennial Edwards Aquifer springs (Comal, Fern Bank, and Sessom Springs in Hays and Comal counties, Texas). At the initiation of this study, it has rarely been encountered, with less than 80 adults collected or observed since the species was described 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 the only species of the genus *Stygoparnus* (Barr and Spangler 1992), therefore, conservation of this species should be considered particularly important. However, studies on *S. comalensis* are difficult due to its rarity and the lack of suitable surrogate species.

Like other dryopid beetles, adult *S. comalensis* are aquatic and similar to adult elmid 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 hydrophobic 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 (Brown 1987, Ulrich 1986). It was proposed by Barr and Spangler (1992) that larvae lived in air pockets at the ceilings of subterranean spaces, noting that they have functional spiracles. Larvae collected in drift float and appear caught on the water-surface tension, suggesting that they may reside in terrestrial or semiaquatic habitats.

Identifying adult female habits and behavior related to oviposition is of great importance towards understanding the life-histories of this species. In general, little is known about the life history and

development of *S. comalensis*. Information on oviposition, clutch size, incubation times and egg size are unknown. Additionally, there is insufficient information on larval development, though it is hypothesized that it requires up to 2 – 5 years before pupation, similar to other dryopid species (Ulrich 1986). However, a single *S. comalensis* larva produced at the San Marcos Aquatic Resources Center (SMARC) grew from 2 to 10 mm in 9 months, suggesting larval development may require only one year. Thus far, captive-produced and wild-caught larvae have failed to pupate in captivity; it is uncertain if pupation takes place above the water line like other dryopids or in a submerged habitat.

Stygoparnus comalensis and Heterelmis comalensis response to flow

Extensive field collections by Bowles et al. (2003) showed that pupae of *Heterelmis comalensis* were rarely sampled but were found in January, April, July, and October, indicating that they are non-seasonal as is similar for emergence patterns of other elmid species (Shepard 2002). Other field studies have shown that *H. comalensis* is restricted to active springs (Cooke et al. 2015) and laboratory studies have shown that adult beetles tend to move in the direction of flow (Cooke et al. 2015), but may move towards flow (BIO-WEST 2002), presumably, when conditions approach stagnation (see Cooke et al. 2015). To our knowledge, no attempts have been made to study *S. comalensis*' response to flow. Considering the important role hydrology plays as part of the natural habitat of both species, new information concerning flow-conditions should be of value.

Study objectives

- Develop a better understanding of the natural habitat of *Stygoparnus comalensis* at Comal Springs.
- Identify a discernable way to distinguish between sexes of both *Stygoparnus comalensis* and *Heterelmis comalensis*.
- Develop a better understanding of mating and oviposition characteristics.
- Estimate fecundity.
- Determine egg incubation times and conditions needed to hatch eggs.
- Identify conducive conditions for rearing larvae.
- Track larval growth, estimate number of instars, and determine the length of time larval development precedes pupation.
- Investigate behavioral responses of *Stygoparnus comalensis* and *Heterelmis comalensis* to varying flow conditions.

Methods

Identification of natural habitats and subject acquisition

Poly-cotton lures (PCLs) following Gibson et al., (2008) were first employed to capture adult *S. comalensis.* However, it was quite evident that hand picking from logs that were placed over specific spring upwellings quickly became a more reliable method for obtaining subjects. Additional scouting was performed by snorkeling localities for active springs that had not been traditionally sampled or were otherwise unknown, as an attempt to find alternative sample habitats. Fifty percent of collected adults were separated and transported back to the SMARC where they were maintained within custom aquaria and fed detrital material.

Identification of sexually dimorphic characters (including the Heterelmis comalensis)

To determine if sexual dimorphic characters could be used to differentiate sexes, nine female and six male preserved *S. comalensis* adult specimens were examined and measured for various body parts

similar to the measurements used to distinguish sexes in *Heterelmis comalensis* (BIO-WEST 2017). After specimens were photographed and measured, their genitalia were dissected to determine their sex. During this process and through interactions with living subjects, other features were inspected to see if there were any repeatably recognizable features that could be used to quickly identify the sex of a subject without having to restrain it, take a photograph, then measure.

ANOVA was used to test for differences among males and female measured body parts. A Bonferroni correction was applied to *p*-values to control for type-I error and AIC was used to determine which of the characters would make the best candidates for delineating sexes. Percentiles were used to find size ranges that may be useful for separating males and females. Validation was performed by applying these ranges to new living subjects.

Mating and oviposition

There has been question regarding the habitat of larvae and of female oviposition behavior. The morphology of larvae suggests that they require a direct connection to an air source due to the fact that they have open spiracles that do not appear to be specifically modified for underwater respiration (Bar and Spangler 1992; E. Kosnicki personal observation). To test if the female left the water to oviposit above the water surface as other dryopid beetles, we tested an oblique plane apparatus (OPA) (**Fig. 1**). The OPA was filled with biogenic material consisting of conditioned leaves, PCL, and poplar dowels, packed within a matrix of rocks. The OPA was placed within a flow-though tank at an angle so that half of the apparatus was above the water line while the other half was submerged. Flow-through water was provided at the bottom of the apparatus with Edwards Aquifer well water.

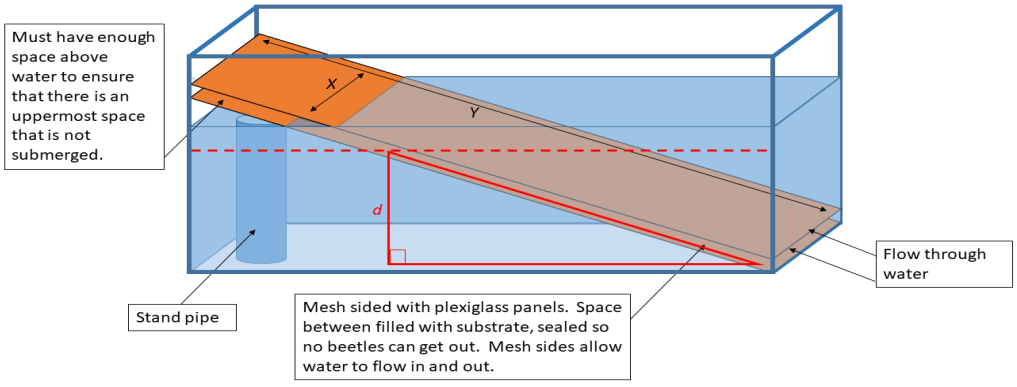


Figure 1. Schematic of tank system holding the OPA. The two primary dimensions have been assigned to either the X or Y axes. The Y axis will be utilized for Pythagorean calculation of depth (*d*); an example of which is depicted by the red right triangle and red dashed line. Design by M. Worsham.

Subjects for the OPA were chosen by placing six adults in a Petri dish. Individuals were allowed to interact until copulatory behavior was observed. After a pair was in amplexus, they were placed into the middle of the OPA at the water line. The OPA was inspected ca. every two weeks for eggs and the location of the adults.

After implementation of the OPA experiment we were able to reliably sex adult *S. comalensis* and were able to pair them for additional mating experiments. Mating chambers were constructed for the

purpose of housing a mating pair and were designed to simulate upwellings with substrate and debris that were both submerged and emergent so that females could oviposit above water if they chose. Each chamber was constructed by dry-fitting two 1-inch PVC tees together with a 100-micron mesh screen placed across the perpendicular opening of each tee. Thus, a screen was fitted on the bottom and across the side-opening outflow, creating a narrow vertical chamber when placed upright. A cap was dry-fit to the top of the chamber. Chambers were connected side to side at the base with a closed connection at one end. A water line was attached to the other side and water was effectively carried vertically through each chamber, flowing out of the side-opening into the sump.

Chambers were packed with a combination of PCL, conditioned dowel, and gravel. The bottom of the chamber was lined with a PCL so that other materials would fit inside of it. A layer of gravel was then placed inside the PCL as a base layer. The conditioned dowel was loosely wrapped inside a second PCL. The dowel-PCL wrap was placed "standing up" vertically within the chamber so that the top would be emergent of the water line. Additional gravel was added to pack the remaining empty space around the dowel. A mating pair or small group (≤ five individuals) was then added within a space between the dowel and its PCL wrapper with a pipette. Once all chambers were packed, the flow line was attached and set to gently flow through each one.

Mating chambers were inspected ca. once a month for eggs and the condition of the adults. Chambers were disconnected from the water source and each other, then placed into a bucket so that the waterline would still be at the same level as it was before disconnecting. In the laboratory, the wrapped dowel was first extracted and placed into a sorting tray. The remaining contents of the chamber were gently poured into a sorting tray and both screens of the chamber's interior were inspected for adult beetles. Water was flushed through both screens (outside-in) in order to recover any eggs that were not caught in the PCL lining layer. The condition of the adults was first recoded, then they were placed into a watch glass with a piece of gravel and water and set aside. The contents of the mating chamber were then inspected under ca. 7.5X magnification for eggs. With this method it was not possible to identify if eggs were oviposited above or below the water. When eggs were found within fibers of the PCL, that section of PCL was cut out using a small pair of dissecting scissors. When eggs were found outside of the PCL, they were pipetted and transferred to a small section of cut PCL. Eggs were then transferred to rearing chambers by grabbing an end of the cut PCL section containing the eggs and placing it flat on top of leaf material prepared in the rearing chamber (see description below). After the contents were thoroughly examined, the container was cleaned, replenished, and repacked with habitat and adults.

Egg production and incubation

To estimate the number of eggs produced per female, 10 females used for the sexual morphometrics part of this study were dissected and their eggs were counted. Number of eggs found in utero were compared to various body measurements to see if there was a relation between female size and number of eggs produced.

In addition, we counted total number of eggs produced from each female in our mating chambers. Many of the chambers contained only one female so we were able to associate number of eggs with a single subject over a period of time. Inspections of mating chambers were recorded on datasheets and records were entered into a MS Access database (2016) so that the length of time between inspections could be queried (**Appendix A**).

Rearing chambers were constructed from plastic sandwich boxes modified with a screen on the bottom of one side. A water line was pushed through a hole in the lid on the opposite side of the screen,

allowing water along the bottom of the container. A ca. two cm layer of aquarium gravel was used to cover the bottom but was graded into a slope so that there was a lower layer on the screen side. A conditioned wooden dowel was then placed on the gravel slope so that the lower end of the dowel would come into direct contact with water and act as a wick to supply moisture to other substrates that were exposed to it. Conditioned sycamore leaves were then placed as a base layer adjacent to the dowel onto which the cut PCL sections with eggs were placed (**Fig. 2A**). After placement of eggs, conditioned walnut, pecan, and sycamore leaves were placed over the dowel and base-sycamore leaf layer, acting as a tent (**Fig. 2B**). This configuration provided eggs and hatching larvae with a humid terrestrial environment. Information related to the origin of the eggs was recorded on datasheets and entered into the database so that we could keep track of egg development and associate the eggs with the parents (**Appendix B**).

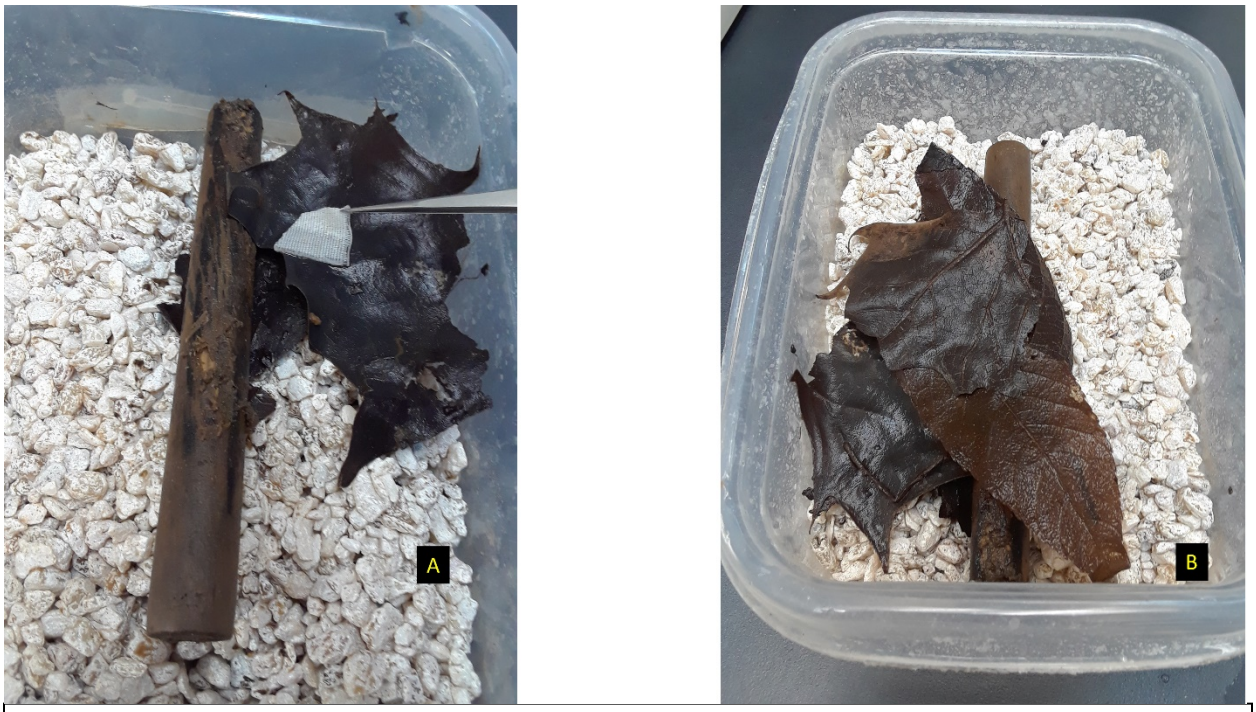


Figure 2. Humid terrestrial habitat used for hatching *Stygoparnus comalensis* eggs and rearing larvae. (A) fresh eggs on a piece of a cut-out poly-cotton lure, being placed on a conditioned sycamore leaf next to a conditioned poplar dowel; (B) mix of conditioned leaves placed over the dowel, forming a tent over the eggs.

Larval habitat and growth

After hatching, larvae were left in the same rearing chamber and monitored ca. every month to observe their general condition and to be photographed for measurements. Searching for larvae was performed by carefully removing each leaf layer and inspecting both sides for larvae. The dowel was also inspected for larvae, but mostly on the outside. Towards the end of this study, it became apparent that the larvae were burrowing into the dowel, but a protocol for searching for burrowing subjects was not developed at the time this report was created (see results below). General observations on behavior were also made in an effort to better understand the habits of this life stage.

Because larvae were rare to work with and delicate, effort was made to disturb them as little as possible; many early measures were not taken by restraining subjects in a standardized fashion. Therefore, some photographs of subjects captured an incomplete view, or were taken at angles difficult to take accurate measures. Our goal was to provide larvae with opportunities to grow rather than take precise measures. However, when possible, 12 characters were measured on each larva (**Appendix C**).

All 12 measures were separately ranked among all subjects, including repeated measures of the same individuals over time. A second derivative of a smooth spline was used to find inflection points, representing the rate of change of a rate of change that was equal to zero. Instar breaks where subjectively selected, using the curve of the second derivative descended from positive to zero as guidance for potential separations between instars. Groups of individuals between inflection points were considered to belong to the same instar. This process was performed for each of the 12 measured characters. The spline and derivatives were calculated using the *features* package for R statistical software version 3.4.1 (R Development Core Team 2017).

Adult response to flow (including the Heterelmis comalensis)
To conduct a deeper investigation into the response of *H. comalensis* to flow and to conduct the first test of flow response of *S. comalensis*, we constructed a Variable Flow Artesian Spring Emulator (VFASE) that was designed to mimic flow conditions of upwellings at Comal Springs.

The VFASE consisted of a series of four chambers fitted together with a 300 – 500 gph submersible water pump. The water pump was fitted with a 1" ball valve to control flow. The first three chambers (A, B, and C) were 7" in length, each, consisting of 1" PVC that was divided into a top and bottom section, dived by ¼" mesh inside of a slip coupling with a union fitting on the other end. The top chamber (D) was 2" in length and not divided into sections. The chambers were fit together to form a continuous pipe. In this way, the VFASE could be disassembled so that sections of each chamber could be independently inspected for subjects exposed to different flow conditions (**Appendix D**). A plastic container ("terrarium") was attached to the top of chamber D, using a 1" PVC bulkhead fitting at one end and a standpipe was fitted with a bulkhead at the other end (**Appendix D**). The water pump base was placed into a ca. 70 L flow-through container that was ca. 40 L full. A PVC stand was constructed to secure the VFASE while it was in operation.

Sterilized gravel was packed into sections of opposing chambers before connecting together. Food resources consisted of a poplar dowel and leaves that were wrapped with a PCL; food components were conditioned and were similar to the description of the food source used for the mating chambers. Three treatments were implemented to test beetle behavior related to flow-regimes and with the location of food resources, for each species separately. Treatment 1 consisted of the food resource placed in the middle (top section of chamber B) of the VFASE with a slow-flow regime. Treatment 2 exhibited a slow-flow regime with food resources placed at the top and bottom, chamber D and top section of chamber A, respectively. Treatment 3 exhibited a medium-flow regime with food resources placed at the top and bottom (same locations as treatment 2). In each treatment, test groups of 6 adult beetles were placed within the middle of the VFASE (top section of chamber B). Three groups of *S. comalensis* and four groups of *H. comalensis* were run as separate trials so that there three trials of each treatment-group combination of *S. comalensis* and four trials of each *H. comalensis* (**Table 1**). After the

Table 1. Number of subjects of dryopid and riffle beetle adults used for VFASE experimentation exposed to low and medium flow regimes. All subjects were placed in the middle of the VFASE in test groups of individuals. Three groups of *S. comalensis* and four groups of *H. comalensis* were run.

		Number of individuals	
Flow regime	Food	*Stygoparnus*	*Heterelmis*
Low	Middle	16	24
	Top and bottom	18	23
Medium	Top and bottom	16	23

beetles and food resources were put into place and chambers were assembled, the VFASE was placed into the flow-through container and the pump was turned on with the valve slightly open to allow water to slowly fill all the chambers. After the cambers were full, the valve was closed and the pump was shut off to allow test subjects to acclimate for > 10 mins. After acclimation, the pump was turned on and the valve open slightly. A flow meter was placed at the opening to the terrarium to help gauge the appropriate amount of discharge. Discharge was then measured by using a stop watch to time how long it took to fill a beaker to 500 mL. Five discharge measures were taken and averaged for each trial. Medium-flow regime trials were acclimated in stages, allowing the beetles to acclimate to the low-flow regime for > 10 before increasing flow to the medium level.

Based on the day of the week, trials were allowed to run for 3 – 4 days (because we did not have access to the facilities on weekends). Test groups alternated the starting treatment in order to account for blocking effects (e.g. group two started with treatment 2 and ended with treatment 1). At the end of a trial, a final averaged-discharge measure was taken. The valve was then closed and the VFASE was disassembled from the top to the bottom and the location of adults were recorded as individual responses. The middle of each chamber-section from which a subject was retrieved was considered the distance traveled from the starting position. Subjects recovered closer to the flow source were considered to have moved in a negative direction while subjects recovered further from the flow source were considered to move in a positive direction. Individuals not recovered were considered missing and excluded from the analysis. An unbalanced 1-way ANOVA was used to test for differences between all six species-treatment combinations. A Tukey test was used to compare means among species-treatment pairings.

Results and discussion

Identification of natural habitats and subject acquisition

PCL's were not an effective means of attracting adult *S. comalensis*. During the process of snorkeling to find new habitat, productive springs that had not been traditionally used to capture dryopids were discovered by inspecting pieces of coarse woody debris on upwellings. These spring upwellings were evidentially associated with the fibrous root systems of sycamore trees and likely contained air pockets as evident by the bubble streams associated with the springs (LBG-Guyton Associates 2004). Barr and Spangler (1992) originally hypothesized that the larvae resided in these types of air pockets. Although we have not caught any larvae by this method, we can say that adults are clearly associated with these habitat types and provides evidence to support the original hypothesis. (See further discussion about habitat requirements in conclusions below).

Identification of sexually dimorphic characters (including Heterelmis comalensis)

Morphology

After close inspection of preserved specimens and living subjects that we knew were female or male, based on copulatory behavior, and the viewing of photos taken previously to 2018, distinguishable

features beneath ventrite 5 became apparent. Males were observed to have a darkened Y-like structure. This feature was not apparent or lightly represented in females. However, females had a visible lance-like structure beneath ventrite 5 that was usually off of center. Dissections and slide mounts of these features were made and photographs were sent to a number of beetle morphology specialists. Additional literature review and conversations with specialists revealed that these structures represented the 8th abdominal sternite in the male and the 9th abdominal sternite in the female, also known as the gonocoxite (Lawrence et al. 2010). See illustrated details (**Appendix E**).

After developing a handle of these sexually dimorphic features in *S. comalensis* a similar inspection of *H. comalensis* was conducted. Male *H. comalensis* displayed the same feature of the 8th abdominal sternite as seen in *S. comalensis*. The females of *H. comalensis*, did not have viewable gonocoxites as in the dryopid, but did have a well developed 8th abdominal sternite with a long anterior strut that extended to ventrite 2. The anterior strut of abdominal sternite 8 in elmids and dryopids is sexually dimorphic with the female strut usually much longer than the male's (Čiampor 2001; Kodada et al. 2009; Kodada et al. 2013; Yoshitomi and Jeng 2013) and has been used in other elmid species to delineate sex without dissection (Fernandes et al. 2010). See illustrated details (**Appendix E**).

Measurements

After several measurements of individual *S. comalensis*, it was determined that the width of ventrite 1 was difficult to measure due to the placement of the legs and the convex nature of the animal's venter. The pronotum width was compromised in several specimens, due to damage. Also, the total length, including the head, was not a precise measurement since individuals have the tendency to retract their heads. Therefore, these measures were not considered for further analysis.

The elytron length was the only measure to show promise for distinguishing between sexes (**Table 2**; **Fig. 3**). Using the 10th and 90th percentiles for females and males, respectively, we can expect females to have an elytron length > 2.06 mm and males < 2.05 mm. From these ranges, our living female subjects would have been misidentified six out of 11 times (55%) and our males three out of nine times (33%). Including all 35 specimens and subjects with measured elytra (20 females; 15 males), the percentiles shift to the 30th and 70th, representing females > 2.00 mm and males < 2.00 mm, respectively. However, this gives a misidentification for ca. 30% of encountered individuals, which is not an acceptable error rate. These ranges are not supported by the original description; Barr and Spangler (1992) found males to be slightly, though not significantly, larger than females.

The only other measure to show relatively good distinction between females and males was the length of ventrite 4 (**Table 2**). This variable was not found to be significant after a Bonferroni correction was applied, but probably would if more specimens were added to the analysis. Combining these two measures for sex determination could be useful if morphologically distinguishable characters are not available. However, from our experience it is rare to encounter an adult *S. comalensis* (or *H. comalensis*) that is not readily identifiable as a female or male using the morphologies described above. In general, the width measurements were poor for distinguishing between the sexes, although the width of ventrite 5, length of ventrite 1, length of ventrite 4 may prove to be significant with additional specimens (**Appendix F**).

For both species, and probably many lighter colored species within Elmidae and Dryopidae, having the correct lighting to illuminate the eighth abdominal sternite or gonocoxites of female dryopids, is the surest way to identify the sex of these individuals. Measurements should be considered as a secondary method, as is the case in taxonomy.

Table 2. ANOVA and AIC results performed for 11 measurements to test for differences between nine females and six males.

	Female	Male	F-value	p-value	AIC
Length ventrite 1	0.42 ± 0.03	0.39 ± 0.02	3.801	0.073	23.314
Length ventrite 2	0.31 ± 0.02	0.28 ± 0.03	6.818	0.022	20.837
Length ventrite 3	0.24 ± 0.03	0.21 ± 0.03	3.747	0.075	23.362
Length ventrite 4	0.22 ± 0.03	0.17 ± 0.03	8.117	0.014	19.884
Length ventrite 5	0.43 ± 0.04	0.43 ± 0.02	0.122	0.733	27.022
Width ventrite 2	1.02 ± 0.07	0.99 ± 0.05	0.915	0.356	26.141
Width ventrite 3	0.97 ± 0.07	0.96 ± 0.04	0.084	0.777	27.065
Width ventrite 4	0.84 ± 0.07	0.83 ± 0.04	0.005	0.944	27.156
Width ventrite 5	0.67 ± 0.04	0.62 ± 0.03	7.584	0.016	20.268
Length elyton	2.15 ± 0.10	1.98 ± 0.08	11.788	0.004*	17.480
Length pronotum	0.88 ± 0.05	0.83 ± 0.03	4.556	0.052	22.654
* - Significant after Bonferroni correction.					

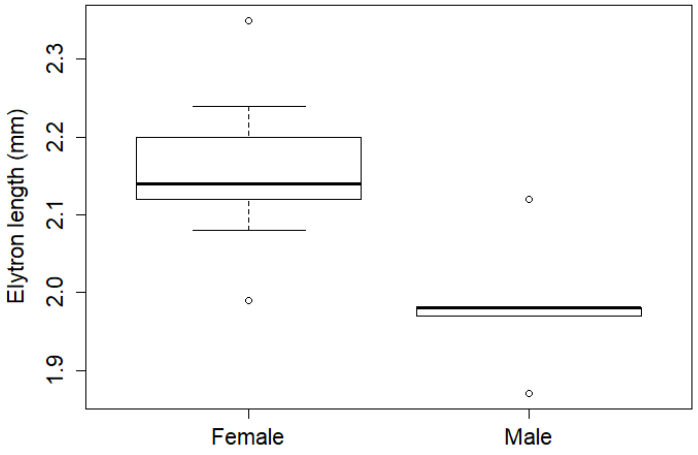


Figure 3. Box plot of elytron lengths of females and males.

Mating and oviposition

The OPA experiment ran for two months from December 4, 2017 through February 7, 2018, after which time the experiment was discontinued due to the mortality of the female. The female was dissected and three eggs were found within her body cavity. During inspections, both adults were always found near the bottom of the apparatus, evidently seeking out flowing water. Eggs were never found during the run of this experiment and so it is unknown if the female oviposited or if we could not find eggs due to the design; OPA was primarily too big for efficient searching for eggs. There were edges and crevasses that may have trapped eggs as water flowed out of the OPA when it was removed from the holding tank; eggs trapped in these locations would have been overlooked because eggs cannot be seen easily without the assistance of magnification.

The use of the mating chambers designed and built soon after the decommission of OPA proved to be excellent mesocosms for collecting eggs and studying egg production. Because we had so few individuals and because males and females were reliably identifiable, we named all of our subjects (**Fig. 4**). Several

packing strategies were implemented; however, the one described in the methods was determined by practice to be the most effective for recovering eggs. Eggs were frequently attached to the fiber of the PCL, but did not appear to be glued or affixed, other than being caught in the fiber due to entanglement. Other eggs were found loose at the bottom of the sorting tray. Additionally, we observed the oviposition of 4 eggs within a flow-through tube that adults were placed within. This suggests that females will oviposited while submerged. Considering the location that we found more of the eggs in the mating chambers, we cannot conclude that females leave the water to oviposit; however, more work should be conducted to better understand oviposition behavior.

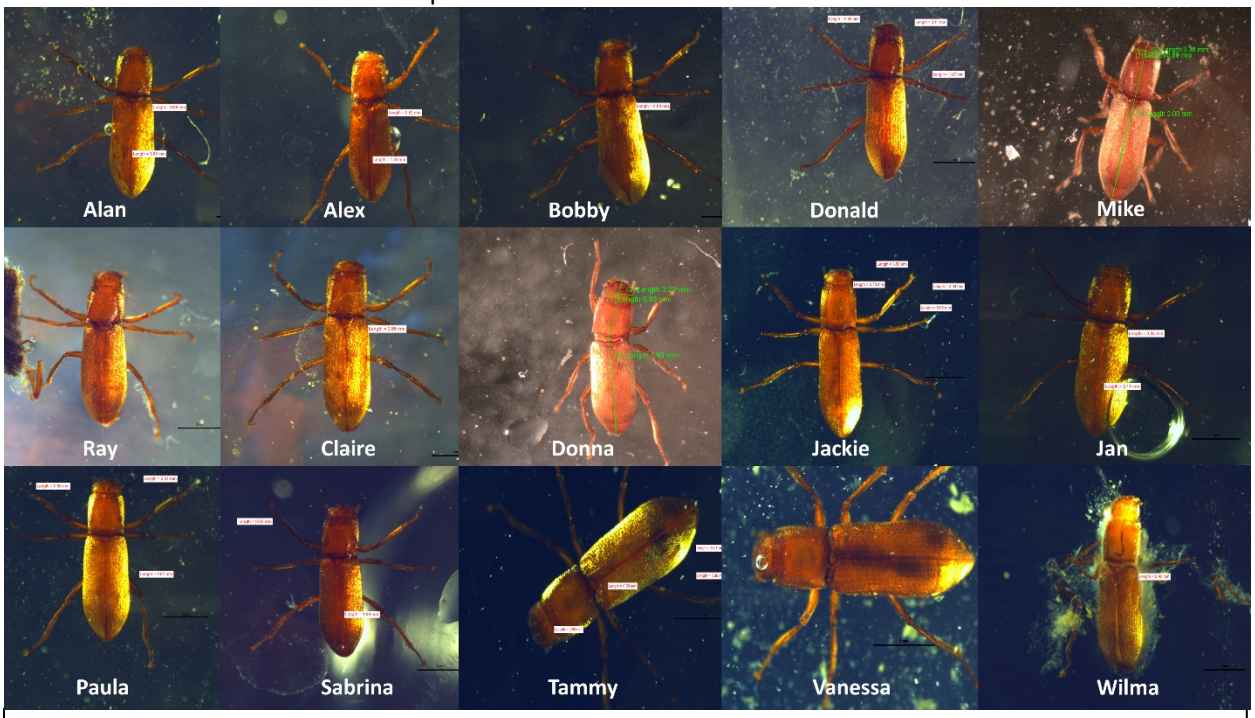


Figure 4. Meet the beetles! Individuals were named as they were paired and placed in mating chambers. Inevitably, naming the beetles helped us better observed individual performance. Unfortunately, not all of them survived.

Egg production and incubation

Eggs counted from preserved females were not found to be related to female size (**Fig. 5**). After discovering that females continue to produce eggs over many months, this was not a surprise and implies that females must develop eggs at some rate to replenish those that have been fertilized and oviposited. An antidotal note, females that had 10 and 14 eggs within their body cavity appeared to be holding the maximum capacity of developed eggs their bodies could support; both of these females were recovered dead from the refuge. It is possible that they had not successfully mated and therefore did not oviposit; however, more work must be done to have a better understanding of mating and oviposition behavior in addition to captive longevity in order to better understand captive propagation.

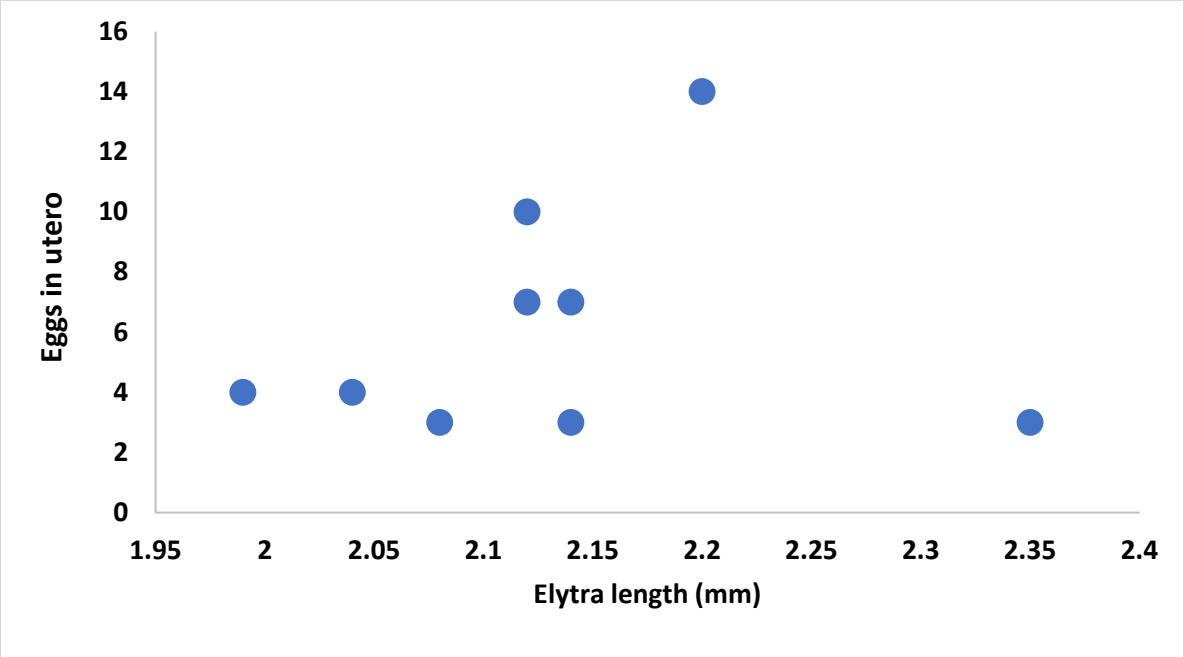


Figure 5. Number of eggs per size of female based on elytron length.

Throughout a six-month period of time we tracked 15 females within the mating chambers. At present we cannot make any creditable estimates on fecundity because several adult females have continued to reproduce through-out this study and we do not know how long they may live in captivity, let alone undisturbed in their natural habitat. At best, we can use the egg production of Nancy as case study of what we have seen as the most productive female. Nancy produced 47 eggs within 228 days (and still alive at the time this report was produced). If we extrapolate these numbers to a hypothesized 21 months as has been the maximum time an adult has been observed to live in captivity (Barr and Spangler 1992), we can estimate a total of 130 eggs as the potential number of eggs produced per female. This is barely an educated guess because rates of egg production may reach optimums and minimums at various stages of an adult female's life cycle. For instance, we may have observed Nancy during an optimum period of egg production. On the other hand, Clair was observed with a partner for 82 days then placed into a chamber with 2 males and another female for an additional 52 days, but never produced eggs and is still alive. It is possible in her case that she ran out of eggs before we utilized her for this experiment or perhaps, she was undergoing an egg regeneration period and will start to produce eggs later. We can only speculate about fecundity at this stage because we simply have not had the time to properly study this aspect of the animal's life history.

Of the 100 eggs we observed all the way to hatching or death, 22% hatched (22 larvae were produced) over a period of 82 ± 15 days (**Fig. 6**). This may be somewhat of an overestimate due to the fact that when we first started to observe hatching, it was at a rate of > 60 days, so we adjusted the schedule to check eggs after 70 days of incubation; however, even after extending the time between checks, some eggs required a longer period to hatch after the 70-day check. From our observations we can say eggs require between 2 – 3 months of incubation time before hatching under the conditions we reared them. It is also possible that some eggs may go through a period of aestivation or delayed development and therefore may take longer to hatch. We have an additional 73 eggs in rearing chambers that may still hatch, but not before this report is due, bringing the total number of eggs produced during this study to

173. It can also be said that there is no certainty that we recovered all eggs that were oviposited during our investigation, so numbers are likely higher than what we observed.

Causes of egg mortality are unknown, however, we have seen indications of contamination, presumably by fungus (?) shown by the appearance of a dark spots (**Fig. 7**). Eggs found with this coloration have never been observed to hatch or develop any further, although they may persist in this state for months without additional degradation.

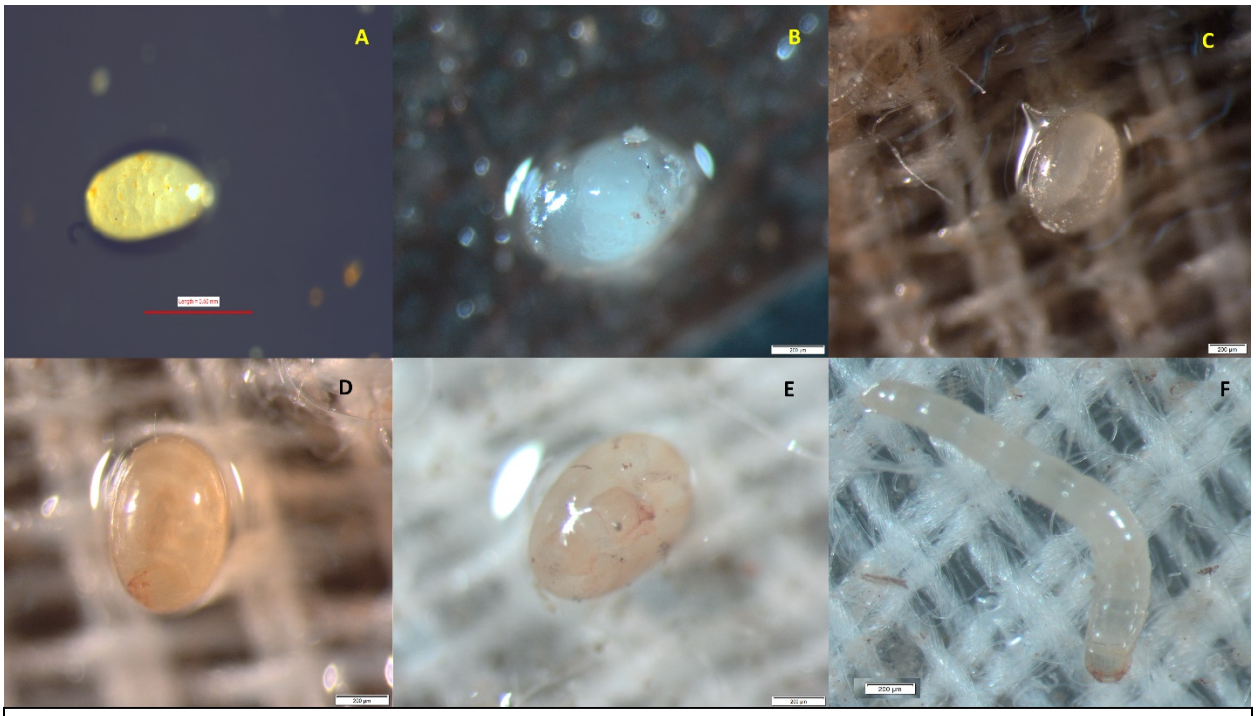


Figure 6. Progression of *Stygoparnus comalensis* egg development. (A) Early stage of cellular division after 30 days; (B) small embryo; (C) medium embryo; (D-E) well developed embryos at 84 days of observation; a developed head capsule, legs, and body segments are visable; (F) first instar larvae.

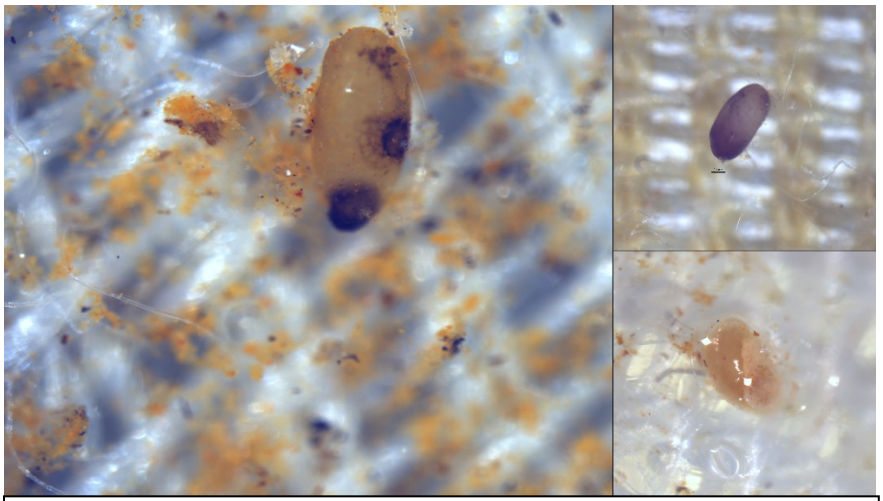


Figure 7. A few examples of contaminated eggs.

It is of interest to better understand if the humid terrestrial conditions are necessary for eggs to hatch or if they can hatch submerged. We originally constructed a rearing chamber to answer this question, but the design turned out to be difficult to work with (submerged eggs roll around and are hard to find mixed within a matrix of detritus), and it was surmised that if larvae did hatch, they may have drowned. A second experiment to test submerged egg hatching success was implemented by using a mating chamber. Instead of packing the chamber with bio-media as with the adults, the chamber was left empty, except for a few conditioned leaves that were lined along the inside of the pipe at the water interface. The idea is that if eggs hatch and larvae float, the larvae will be able to grab onto the leaf material, similar to how we imagine this might take place in Comal Springs. This experiment is still running so we will not know the results before this report is finished.

Larval habitat and growth

On our last complete check of eggs and larvae on December 3, 2018, we observed 11 larvae, including 2 new hatchlings. We were unable to account for 9 individuals, suggesting about 50% mortality. However, it is surmised that at least a few individuals were not observed because they had burrowed into the wood dowel and could not be seen. During the process of searching for larvae, burrowing behavior was noted from several early-instar subjects that were partially or completely embedded within the spongy wood of the dowel (**Fig. 8**). Later instar larvae were also found to excavate a galley that extended the length of the dowel (ca. 8 cm) (**Fig. 9**). When individuals were found within the wood, they were measured, but the entire extent of the galleries were not excavated because we did not want to excessively damage their habitat. We suggest that multiple larvae can occupy these galleries, concurrently, and that the suggested mortality we observed was not as high because of the "hide-and-seek" nature of rearing this animal.

Figure 8. A first instar larvae exhibiting burrowing behavior.

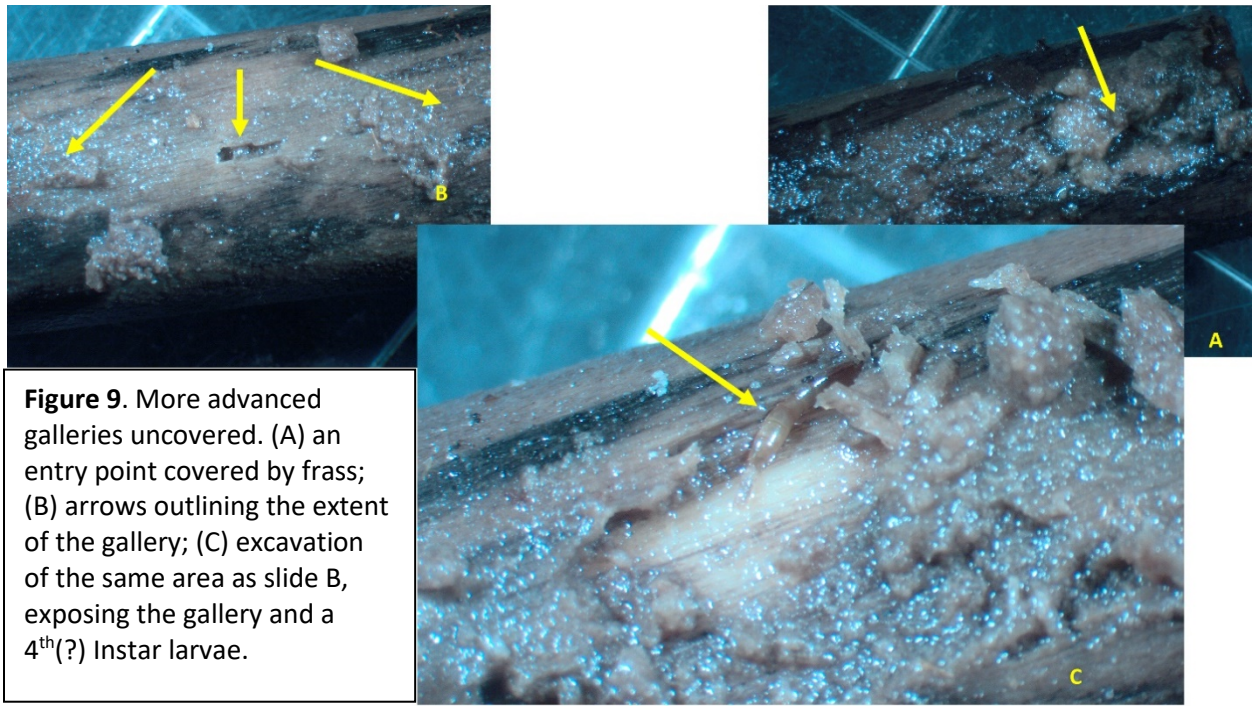


Figure 9. More advanced galleries uncovered. (A) an entry point covered by frass; (B) arrows outlining the extent of the gallery; (C) excavation of the same area as slide B, exposing the gallery and a 4th(?) Instar larvae.

In addition to being a food source, it is likely that the galleries formed within the wooden dowel may serve several additional purposes. Galleries formed within submerged coarse-woody material may create air pockets that will allow larvae to respire, which could explain why larvae do not show any morphological adaptations to submerged respiration. Furthermore, this behavior strongly implies that larvae will pupate within the galleries created by their feeding habits. Future work should try to gain a better understanding of the types of the woody material utilized by the larvae and how conditioning phases relate to larval life-history aspects.

Examination of the inflection points used to help delineate instar, indicated that total body length gave the best representation of instar delineation (**Appendix G**). Four instars were estimated where the second derivative descending from > 0.3 was equal to zero (**Fig. 10A**). Fitting an exponential function to the body sizes delineated for these 4 instars and using the body length range 8 – 10 mm as the last instar size (Barr and Spangler 1992), we solve for number of instars as 6 (5.7 – 6.3) (**Fig. 10B**). Using the time of our observations to estimate number of days per instar, we have the average of 22.4 days between instars. Adding these lengths of time to instar estimate we would expect larvae to reach the final instar within 134 days after hatching. It is expected that individuals will remain in the final instar for a longer period of time, assimilating energy towards reproduction and adult tissue maturation before pupating and it is unwise to make predictions on how long they will remain in that stage with the current information. Although these are very tentative estimates (very tentative), it does compare similarly to the estimates of the *H. comalensis*; it was estimated to reach its 7th and final larval instar in ca. 120 days (BIO-WEST 2017).

More data should be gathered in order to make better estimates. Our graphs also imply that we may have missed an instar between our delineated 3rd and 4th instars; one subject placed in instar 3 may be the true instar 4 and our delineated 4th instar may actually be instar 5. There is also a question to the legitimacy of the two individuals that were delineated as the first instar; it is possible that the photos for these were taken with an incorrect setting and the instar we delineated as the 2nd is really the 1st.

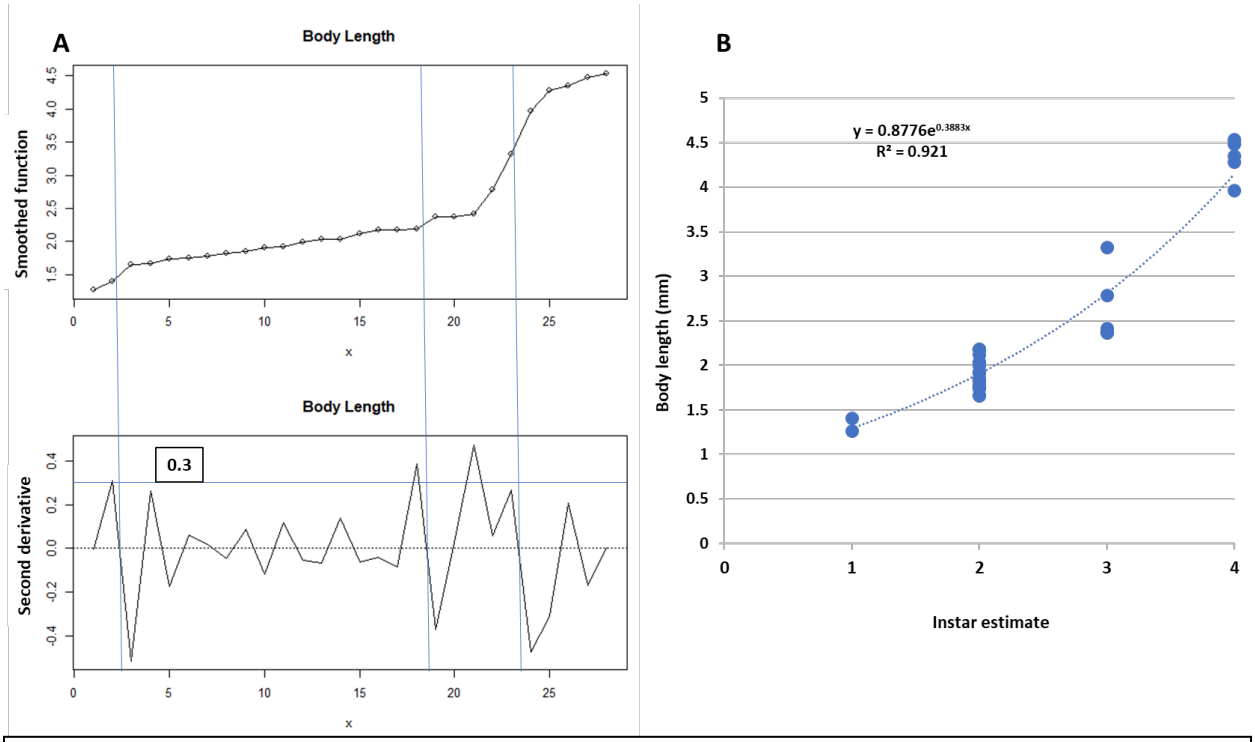


Figure 10. Body lengths used for estimated instars I – IV. (**A**) The second derivative where descending from > 0.3 to equal to zero, grades individuals into an instar. (**B**) Exponential function used to extrapolate to the total number of instars based on maximum body lengths of 8 and 10 cm.

Note on conditioned leaves and poplar dowels

Conditioned leaves and poplar dowels at present are the primary food sources for *Stygoparnus* reared in captivity. Sycamore leaves tend to degrade at a slower rate and maintain their structure better than pecan or walnut; however, larvae were found feeding on the biofilm of all leaf types and it was not noticed if one leaf type was favored. Because of the slower degradation rate of sycamore leaves, it is recommended that these leaf types are used for structural purposes (as a base layer for rearing chambers, for instance). More work is clearly needed to ascertain whether or not one food source is preferred over another or if one is a more effective resource for growth and overall beetle health.

Monitoring of the poplar dowels indicates that they go through several phases during conditioning. After about one month of soaking in a flow-through system of Edwards Aquifer well water, the dowels develop a gooey film ("snotty stage") that does not appear to be healthy for adult beetles. As a consequence, we have not tested the efficacy of this stage of conditioning with larvae, presuming that it would not be a safe resource. After the snotty stage, dowels appear to be in a favorable state as a food source by adults and larvae. Additionally, we think that further conditioning of the dowel, leading to a soft-spongy pulp, may be important to promote larval burrowing. We do not fully understand the importance of wood burrowing but it does appear to be a characteristic behavior of *S. comalensis* larvae; even the leaves show signs of gallery formation (**Fig. 11**) and we believe that a soft spongy-wood resource that may serve more than one purpose for the developing larvae (see comments above).

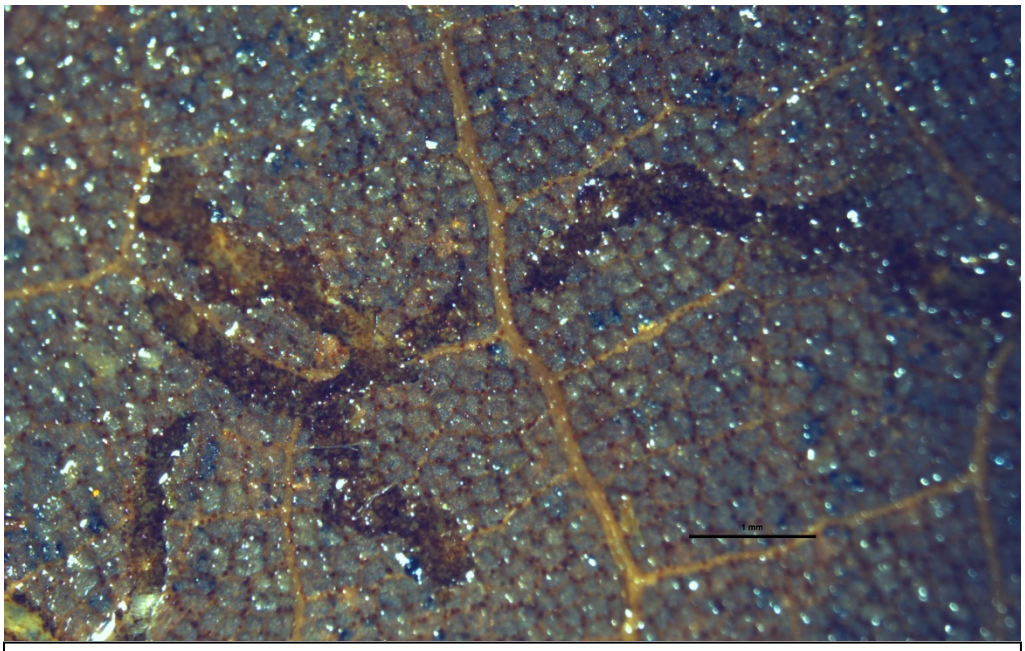


Figure 11. Feeding trails from *Stygoparnus comalensis* larvae, giving a surficial visualization of gallery formation.

Adult response to flow (including Heterelmis comalensis)
Discharge across all low-flow trials was 7.90 ± 1.76 mL/s (n=14) and the medium-flow discharge was 45.04 ± 9.89 mL/s (n=7). Preliminary analyses did not show differences among species and flow-regime; flow-regimes for each treatment compared similarly among trials for each species.

Results from the VFASE experiments indicated that beetles had differing responses among the treatment regimes (*F*-value = 20.47; *df effect* = 5; *df error* = 114; *p*-value < 0.001). Mean separation showed that the *S. comalensis* medium-flow treatment was not different from any of the *H. comalensis* treatments. Low-flow regimes with beetles starting in the food resource showed that both species tended to stay within the resource. There was no difference between the two *H. comalensis* treatments with food at the bottom and top; adults tended to move with flow. The *S. comalensis* low-flow treatments with beetles in food and food at the bottom were not found to be different. All other treatment combinations were found to be different from each other (**Fig. 12**).

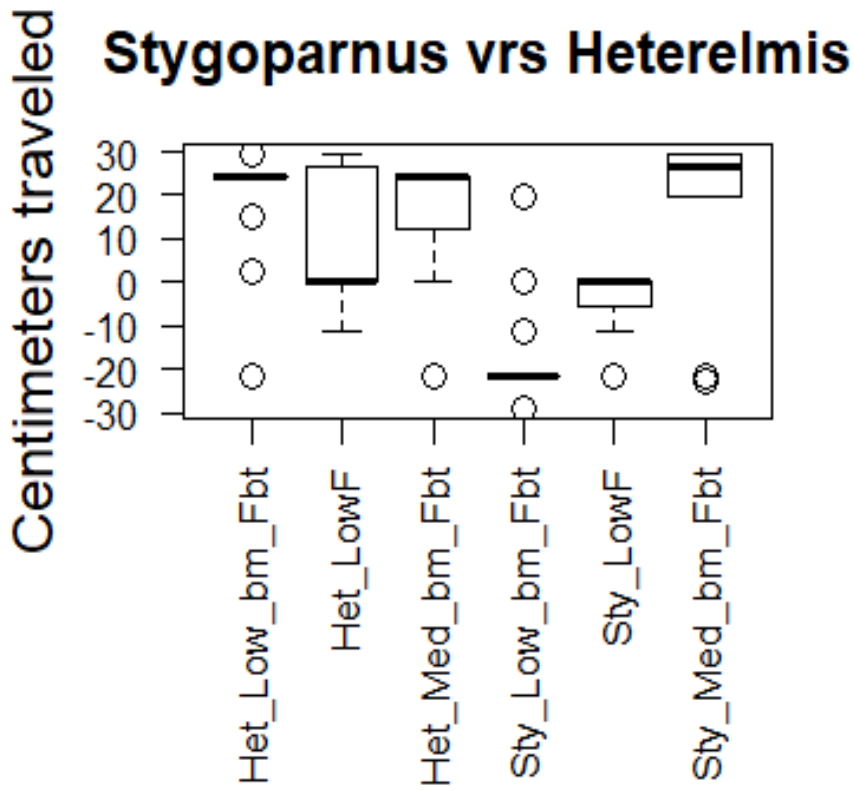


Figure 12. Comparison of movements of adult *Stygoparnus comalensis* and *Heterelmis comalensis* response to flow-regimes with food resources at the middle or top and bottom of the VFASE. All adults were placed in the middle of the VFASE and negative distances traveled indicate movement against flow while positive movements indicate movement with flow. Het = *Heterelmis comalensis*; Sty = *Stygoparnus comalensis*; Low = low flow; LowF = low flow with food in middle; bm_Fbt = beetles middle food top and bottom.

Most adult beetles remained within the food resources when they were placed in it to start the experiment, though *H. comalensis* had a slight tendency to move with the flow, while *S. comalensis* had a slight tendency to move against it. When beetles were exposed to a medium-flow regime and food resources placed at the top and bottom of the VFASE, both species were pushed towards the top and were usually found in the food resource or the terrarium, with a few exceptions. For low-flow treatments with food resources at the top and bottom, *H. comalensis* tended to move with the flow to the top food resource while *S. comalensis* tended to move against the flow to the bottom food resource. It is possible that the flow regime consistent with our low-flow treatment was overpowering for riffle beetles, but not dryopids. Or it may show a difference in behavior related to flow, where *S. comalensis* instinctively moves towards the flow while *H. comalensis* moves with the flow. Future work should include flow-regimes less than our low-flow range of 7.90 ± 1.76 mL/s. The implication from the result of this study and from other general observations of adult behavior is that flowing systems are important to *S. comalensis* and that they will seek out flowing water; therefore, rearing containers should have some source of flow so that adults feel at home once they finish pupating.

Conclusions

Some of the important findings as a result of our investigations include the knowledge of specific habitats where *S. comalensis* can be found. It is surmised that other locations with similar and differing conditions may exist, since the distribution of this species appears to be more extensive than *H. comalensis*. A better understanding of habitat conditions will surely lead to the identification of new localities. Determining the sex of individuals is quite easy given an understanding of the morphologies and utilizing a correct lighting system. Production of eggs is easily generated as adults need no encouragement to mate. At this time eggs should be harvested and transferred to a terrestrial habitat as described here except that we would emphasize an increased use of conditioned poplar dowels as this food source evidentially serves as housing. Eggs hatched within such an environment probably do not need further attention; however, we do not know the habitat requirements needed for pupation. At this time, it is not recommended to inventory larvae until a time for pupation and adult development is estimated. Habitat quality should be monitored to ensure a healthy environment for maturing beetles. Replenishing conditioned sycamore leaves covering the dowels appears to prevent overgrowth of disruptive fungi and replenishing the conditioned dowels is also recommended.

Many questions still remain and will be important with regard to developing a self-propagating refuge. A better estimate of the number of larval instars before pupation and information on the last larval instar is needed. At present there is no information on pupation and habitat conditions for this life stage are only speculative. Furthermore, we need a better understanding of female reproductive potential; how many eggs can a female produce and over what period of time?

The most immediate question regards the best habitat conditions for hatching eggs and developing larvae. At present, eggs are hatched in a humid terrestrial environment, but it is expected that submerged eggs hatch and possibly at a more successful rate. A better understanding of the woody habitat-food resource for larvae is needed. It has been surmised that plant roots, particularly sycamore, are the primary food source for the larvae in the wild (Gibson, personal communication) which is congruent with our observations in the field. However, if this is an important food-habitat resource do they utilize living as well as dead roots? Can they form galleries within submerged root or wood systems or must they reside in a more terrestrial habitat above the surface of the water? If eggs sink and females do not appear to migrate to the surface to oviposit, how do larvae make it to their food-habitat resource?

A potential explanation to these last questions is that adults prefer to reside within woody debris or root systems. We have not witnessed gallery formation or burrowing behavior from adults, but it is possible that they prefer to crawl between the bark and vascular cambium or other woody crevasses where eggs could be dropped. In the field, after removing a piece of wood from a spring, more adults would "appear" the longer the wood was inspected out of water; presumably, adults came out of crevasses in response to becoming emergent. From our findings, these spaces would be ideal habitat for hatching eggs. Dense sycamore root mats could provide a similar habitat and it is probable that these types of root systems associated with spring activity are the primary habitat of *S. comalensis*. It is strongly recommended that efforts should be made to preserve and protect these habitats.

Acknowledgements

Thanks goes to the USFWS San Marcos Aquatic Resources Center for help and support for this study. Thanks goes to M. Worsham for providing the construction of the OPA and contributing to the design of

the OPA experiment and literature review. Extra special thanks go to J. R. Gibson whose insights were truly inspirational for several of the methods developed during this project. This project was funded under USFWS cooperative agreements F17AC00030 and F18AC00065 in support of compliance with the Edwards Aquifer Habitat Conservation Plan.

References

Barr, C. B., and P. J. Spangler. 1992. A new genus and species of Stygobiontic dryopid beetle, Stygoparnus comalensis (Coleoptera: Dryopidae), from Comal Springs, Texas. Proceedings of the Biological Society of Washington 105:40-54.

BIO-WEST. 2002. Comal Springs riffle beetle laboratory study: evaluation under variable flow conditions. Final Report. Prepared for the Edwards Aquifer Authority. 30 pp.

BIO-WEST. 2017. Comal Springs riffle beetle (*Heterelmis comalensis*): life history and captive propagation techniques. Final Report. Prepared for the Edwards Aquifer Authority. 36 pp.

Bowles, E. B., C. B. Barr, and R. Stanford. 2003. Habitat and phenology of the endangered riffle beetle *Heterelmis comalensis* and a coexisting species, *Microcylloepus pusillus*, (Coleoptera: Elmidae) at Comal Springs, Texas, USA. Archiv für Hydrobiologie 156:361-384.

Brown, H. P. 1987. Biology of riffle beetles. Annual Review of Entomology 32:253-73.

Cooke, M., G. Longley, and R. Gibson. 2015. Spring association and mircohabitat preferences of the Comal Springs riffle beetle (*Heterelmis comalensis*). The Southwestern Natrualist 60:110-121.

Čiampor Jr., F. 2001. Systematic revision of the genus *Graphelmis* (Coleoptera: Elmidae) I. Redescription of the genus and description of four new species. Entomological Problems 32:17-32.

Fernandes, A. S., M. I. Passos, and N. Hamada. 2010. A new species of *Hintonelmis* Spangler (Coleoptera: Elmidae: Elminae) from Central Amazonia, Brazil. Zootaxa 2353:43-48.

Fries, J. N., J. R. Gibson, and T. L. Arsuffi. 2004. Edwards Aquifer spring invertebrate survey and captive maintenance of two species. Report for U. S. Fish and Wildlife Service. Austin Ecological Services Field Office, Austin, Texas

Gibson, J. R., S. J. Harden, and J. N. Fries. 2008. Survey and distribution of invertebrates from selected Edwards Aquifer springs of Comal and Hays counties, Texas. Southwestern Naturalist 53:74–84.

Kodada, J., M. A. Jäch, and F. Čiampor Jr. 2009. Review of the genus *Drylichus* Heller (Insecta: Coleoptera: Dryopidae). Zootaxa 2057:43-58.

Kodada, J, M. Kadubec, and F. Čiampor Jr. 2013. *Geoparnus loebli*, a new species of terrestrial dryopid from Peninsular Malaysia (Coleoptera: Dryopidae). Zootaxa 3646:68-74.

Lawrence, J. F., R. G. Beutel, R. A .B. Leschen, and A. Ślipiński. 2010. 2. Glossary of Morphological Terms. *Coleoptera, Beetles, Volume 2, Morphology and Systematics (Elateroidea, Bostrichiformia, Cucujiformia partim)*.

LBG-Guyton Associates. 2004. Evaluation of augmentation methodologies in support of in-situ refugia at Comal and San Marcos springs, Texas. Final Report prepared for the Edwards Aquifer Authority. 192 pp.

Resh, V. H., D. B. Buchwalter, G. A. Lamberti, and C. H. Eriksen. 2008. Aquatic insect respiration. Pages 39–53in An introduction to the aquatic insects of North America. 4th ed (R. W. Merrit, K. W. Cummins, and M. B. Berg, editors). Kendall Hunt Publishing, Dubuque, Iowa.

Shepard, W.D. 2002. Elmidae. *In*: R. H. Arnett, M. C. Thomas, P. E. Skelley, and J. H. Frank (eds.), *American Beetles Vol 2*. (pp. 117-126). Boca Raton, FL: CRC Press LLC.

Gary W. Ulrich. "The Larvae and Pupae of Helichus Suturalis Leconte and Helichus Productus Leconte (Coleoptera: Dryopidae)." *The Coleopterists Bulletin*, vol. 40, no. 4, 1986, pp. 325–334.

Yoshitomi, H. and M.J. Jeng. 2013. A new species of the genus *Dryopomorphus* Hinton (Coleoptera, Elmidae, Larainae) from Laos. Elytra, Tokyo 3:45-51.

Appendix A

Mating Chamber 9					
Date	**Fathers**	**Mothers**	**# Eggs**	**EggBundle Code**	**Notes**

Appendix B

Rearing Chamber 1

Date	EggBundle Code	# Eggs	Condition	Notes

Rearing Chamber 2

Date	EggBundle Code	# Eggs	Condition	Notes

Appendix C

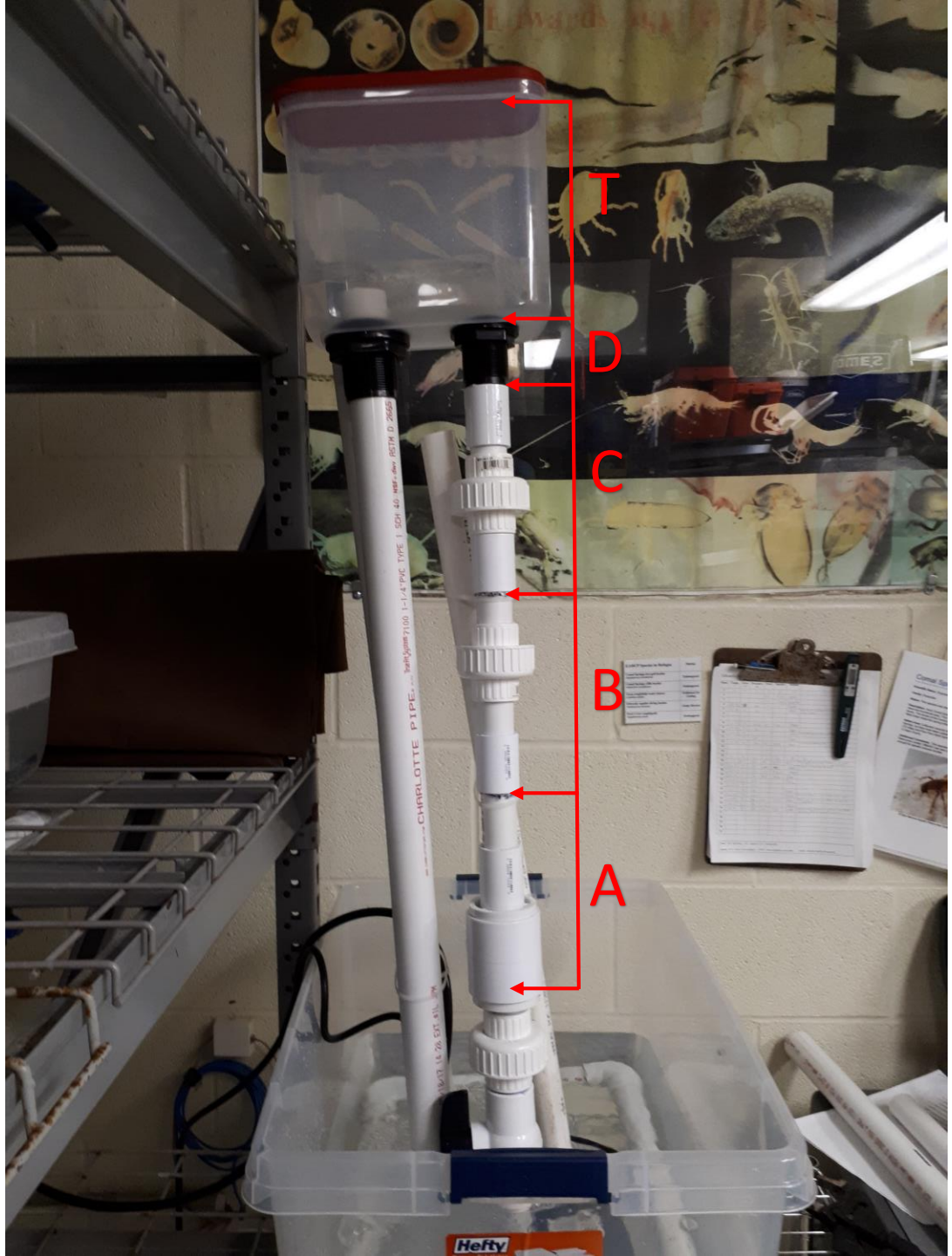
Larval *Stygoparnus* Growth and Accession Datasheet					Date:____________________	
Larvae ID	**X** If First Measure	**X** if Dead	PhotoCode(s)	Rearing chamber	Egg bundle code (If first measure)	Date entered into db
BodyLength	HCW	PrNW	PrNL	MsNW	MsNL	MtNW
MtNL	Ab1W	Ab1L	Ab9W	Ab9L	Notes	
Larvae ID	**X** If First Measure	**X** if Dead	PhotoCode(s)	Rearing chamber	Egg bundle code (If first measure)	Date entered into db
BodyLength	HCW	PrNW	PrNL	MsNW	MsNL	MtNW
MtNL	Ab1W	Ab1L	Ab9W	Ab9L	Notes	
Larvae ID	**X** If First Measure	**X** if Dead	PhotoCode(s)	Rearing chamber	Egg bundle code (If first measure)	Date entered into db
BodyLength	HCW	PrNW	PrNL	MsNW	MsNL	MtNW
MtNL	Ab1W	Ab1L	Ab9W	Ab9L	Notes	

Acronyms for body measures that were taken from photos of developing larvae.

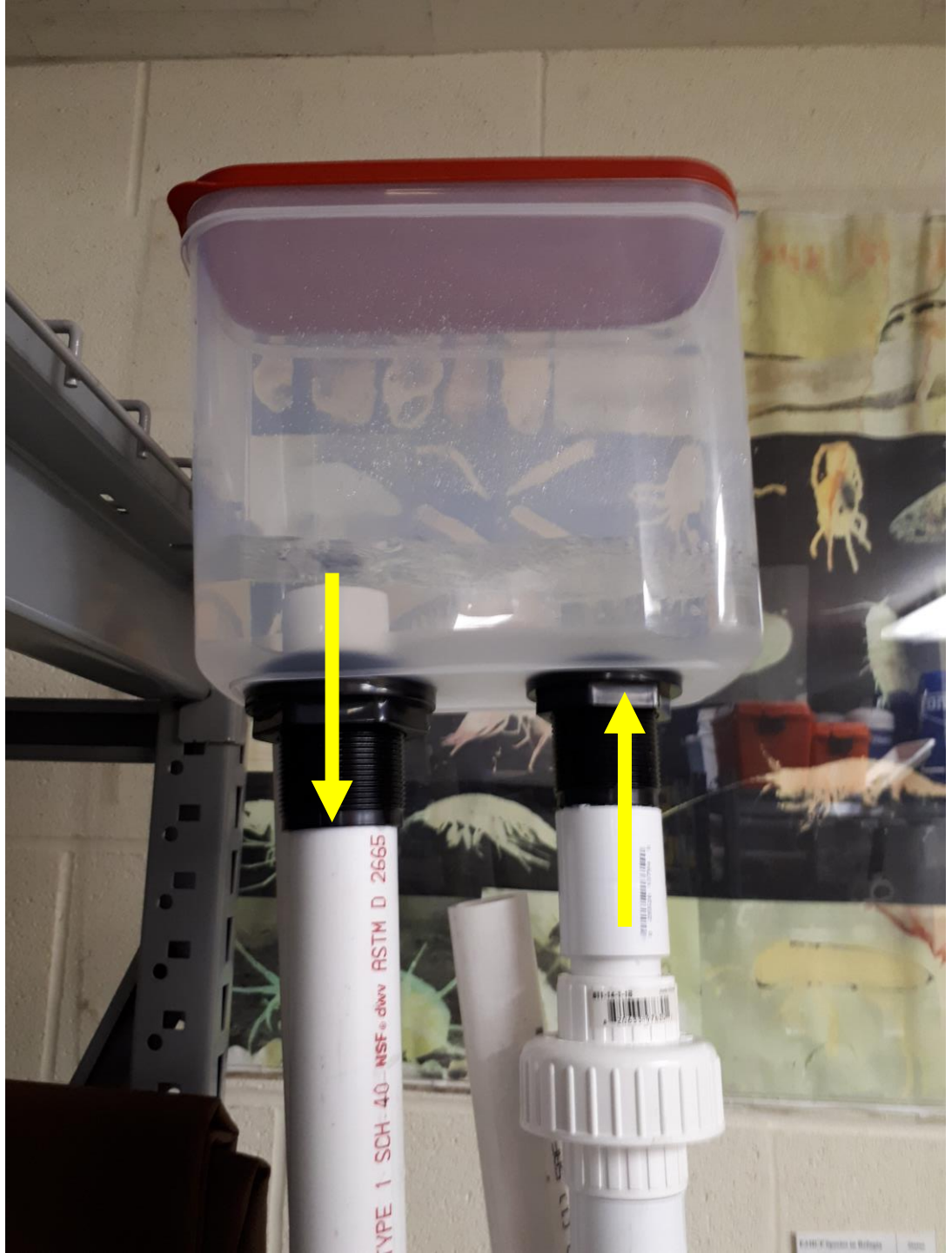
BodyLength	Length of body (mm)
HCW	Head capsule width (mm)
PrNW	Pronotum width (mm)
PrNL	Pronotum length (mm)
MsNW	Mesonotum width (mm)
MsNL	Mesonotum length (mm)
MtNW	Metanotum width (mm)
MtNL	Metanotum length (mm)
Ab1W	Width of abdominal segment 1 (mm)
Ab1L	Length of abdominal segment 1 (mm)
Ab9W	Width of abdominal segment 9 (mm)
Ab9L	Length of abdominal segment 9(mm)

Appendix D

The Variable Flow Artesian Spring Apparatus (VFASE), showing the configuration of the chambers in relation to each other and the terrarium at the top.



The VFASE terrarium section showing the direction of flow.

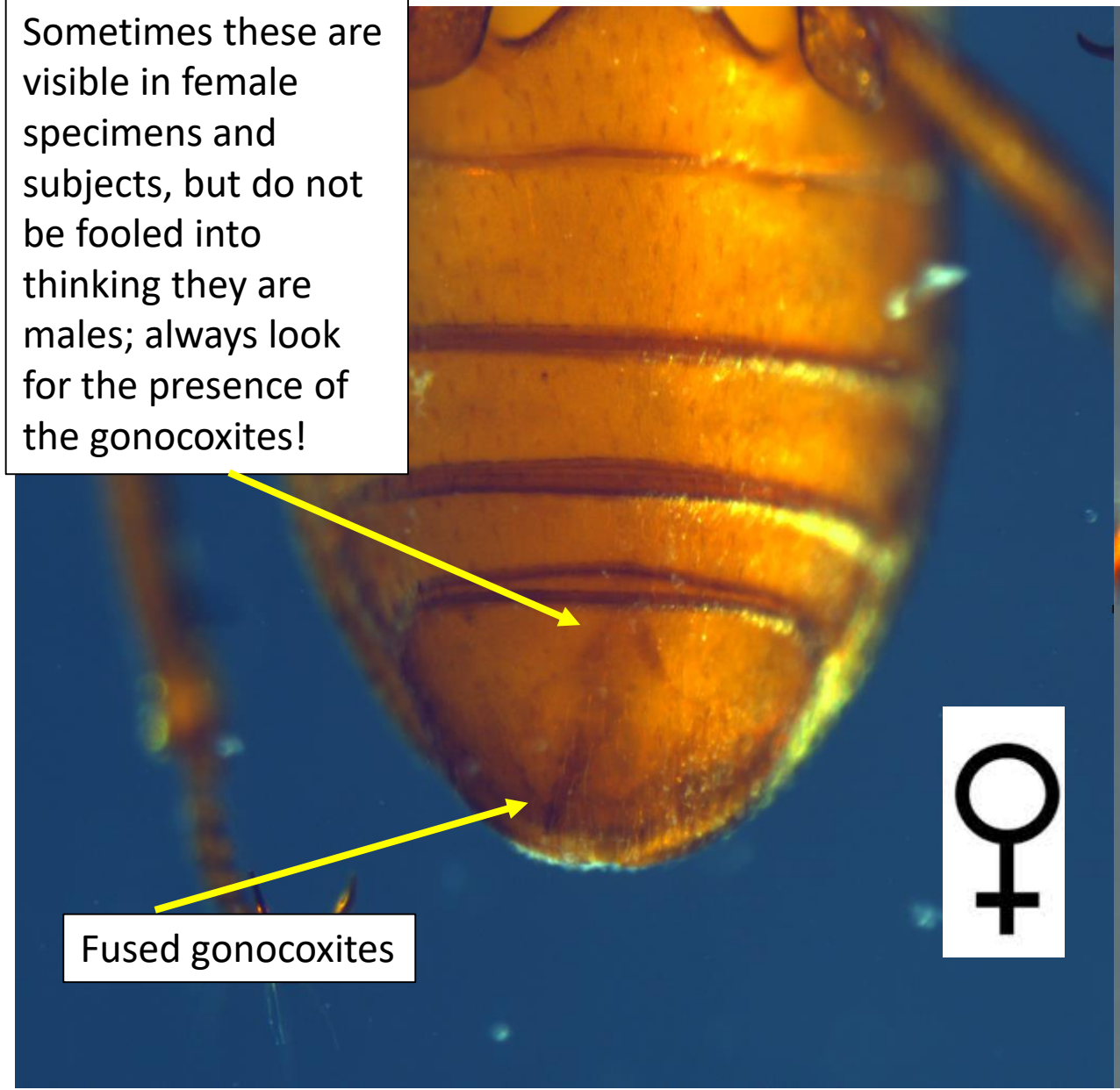


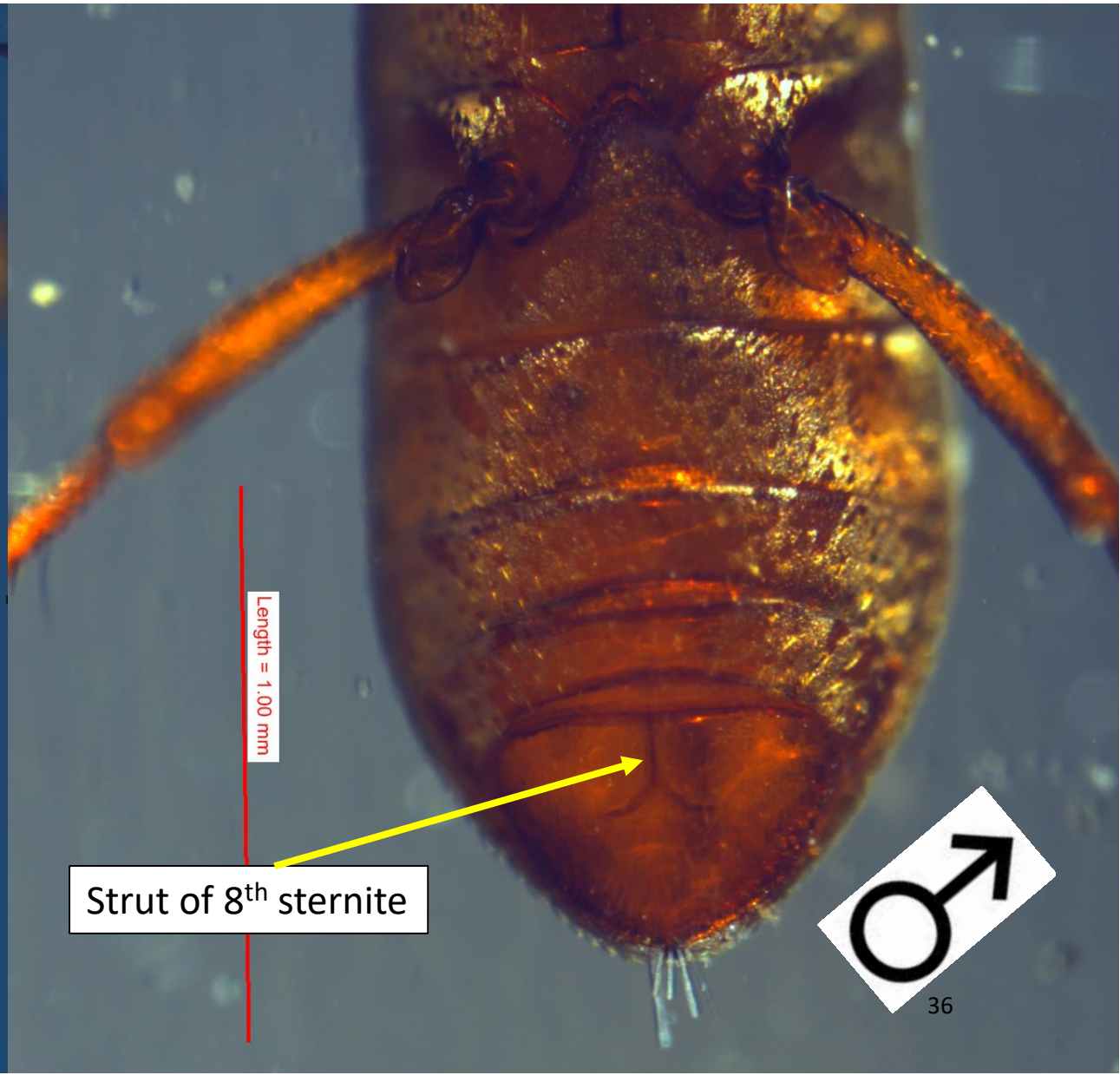
The VFASE water pump in operation.

Appendix E

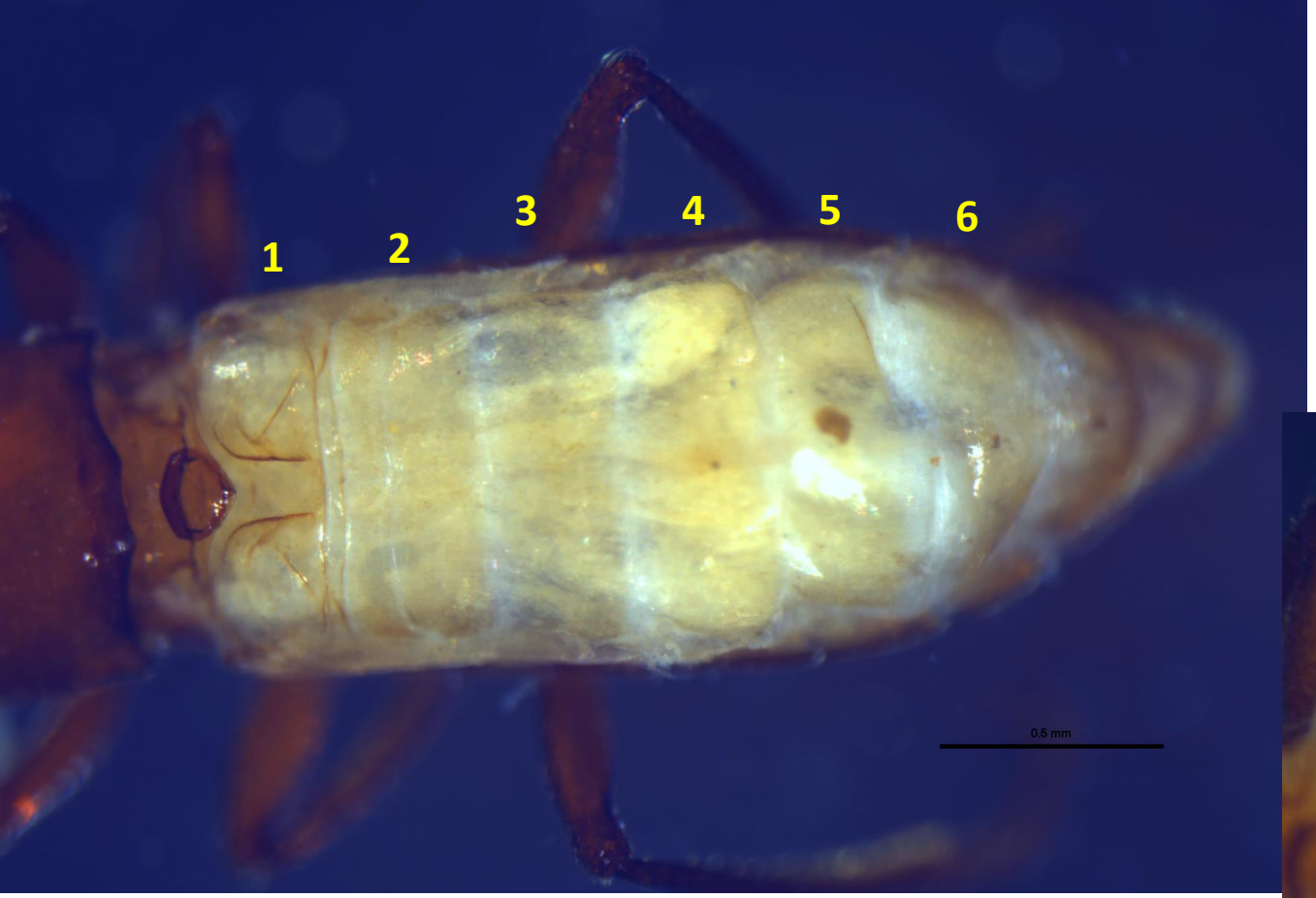
Stygoparnus comalensis

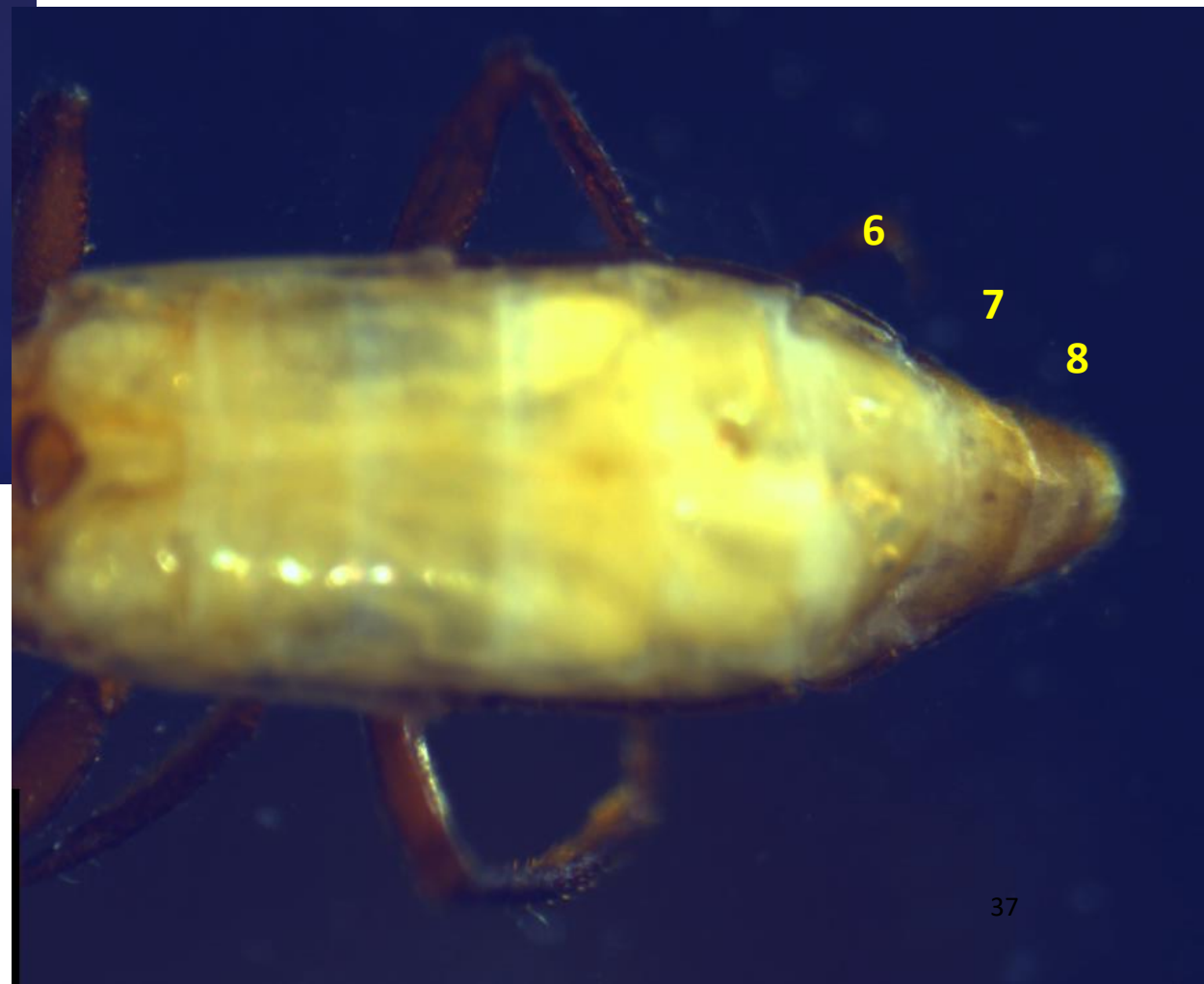
Remnants of 8th sternite.
Sometimes these are visible in female specimens and subjects, but do not be fooled into thinking they are males; always look for the presence of the gonocoxites!

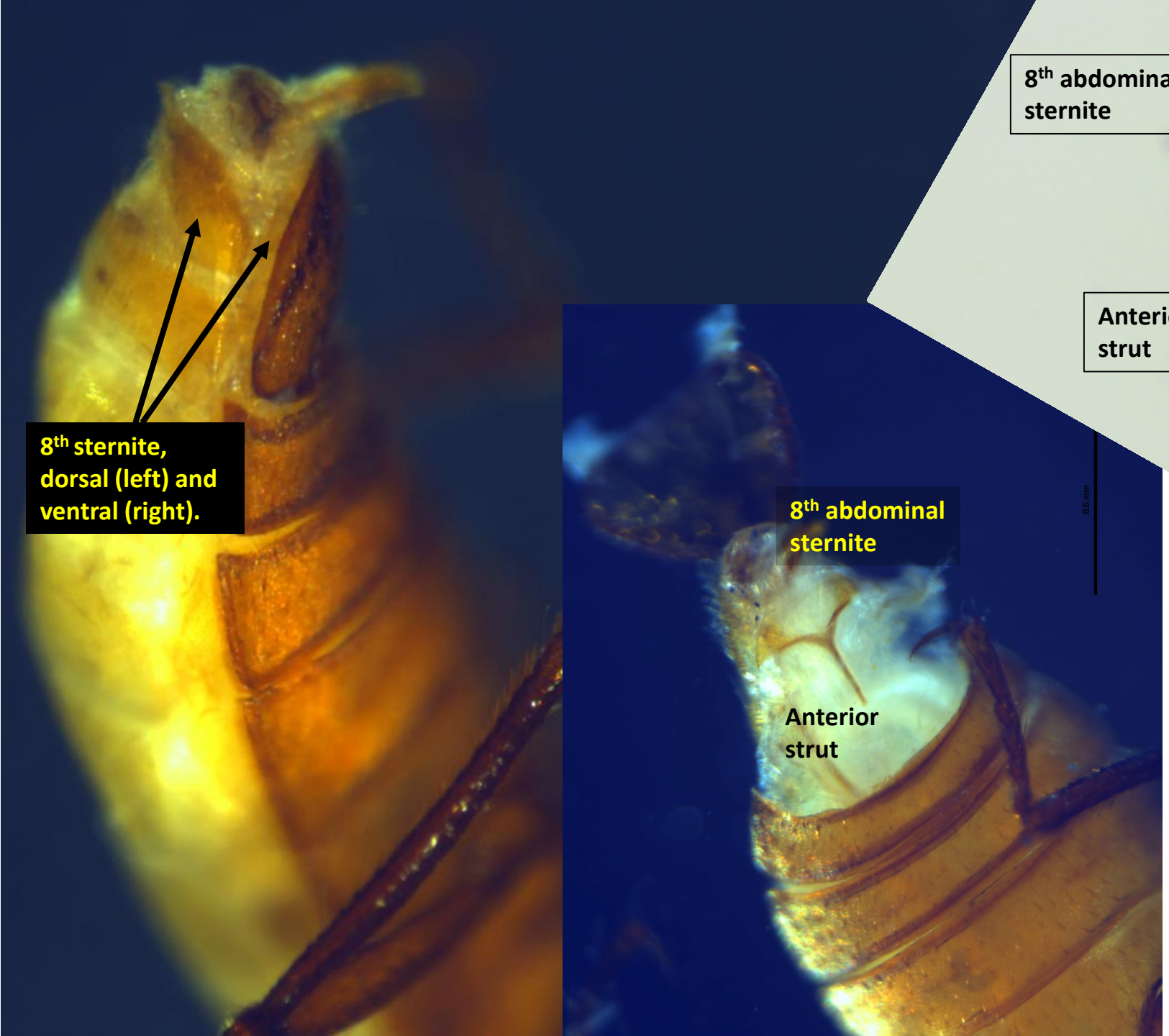


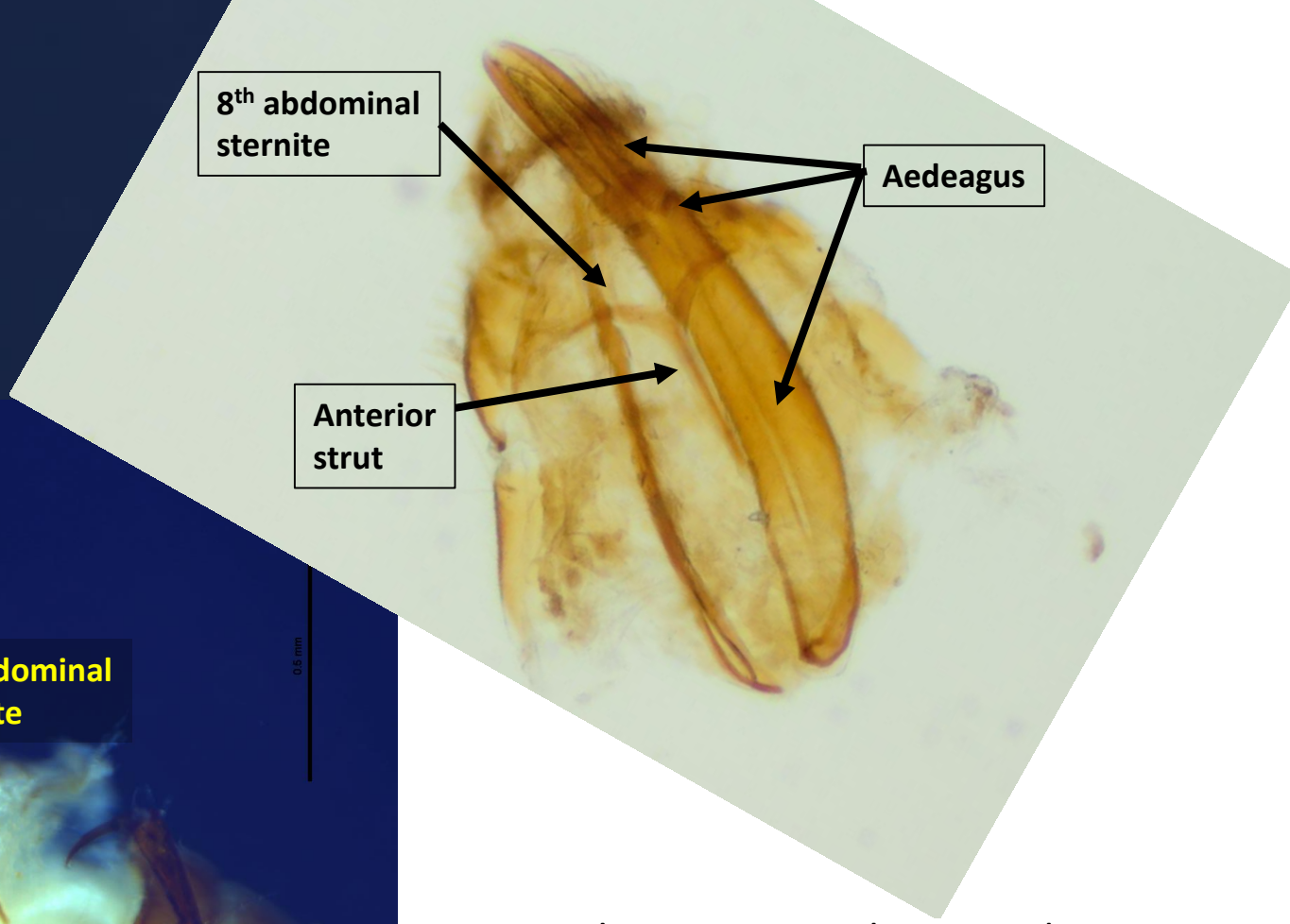


Using the dorsum of the abdomen we can count the abdominal segments, showing 8.



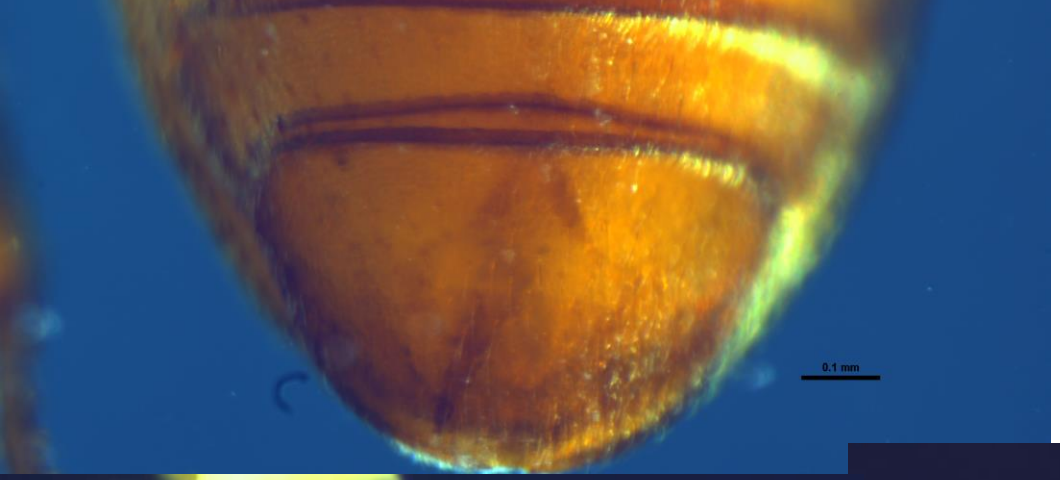


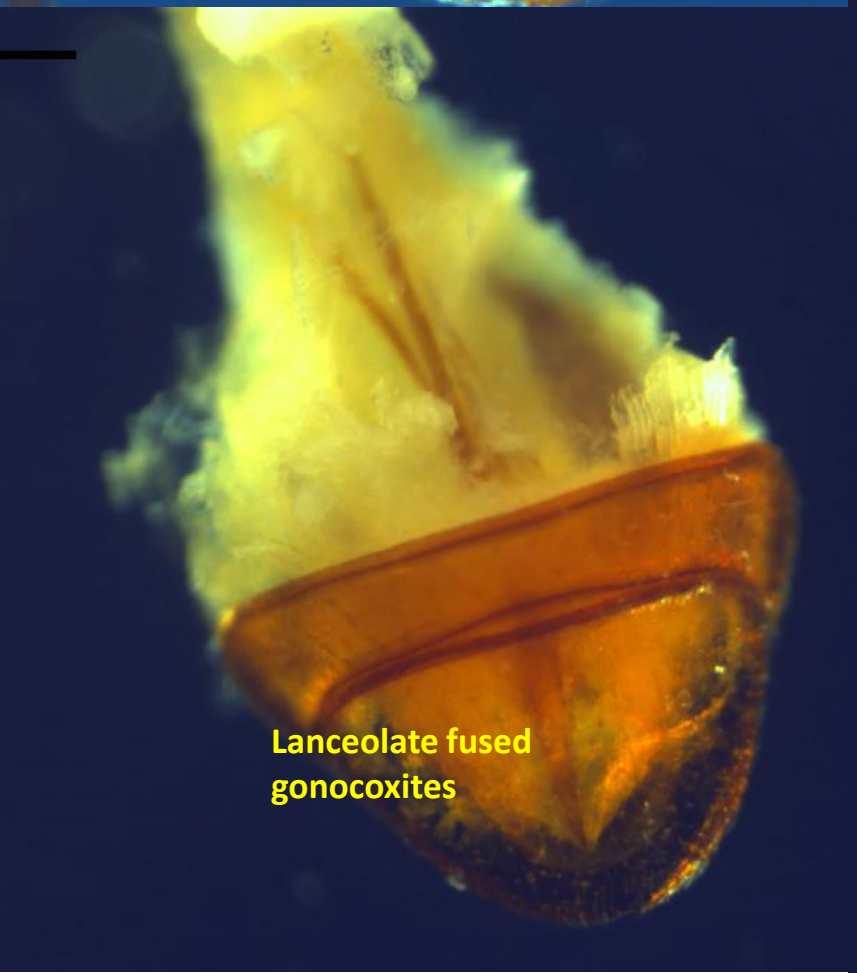


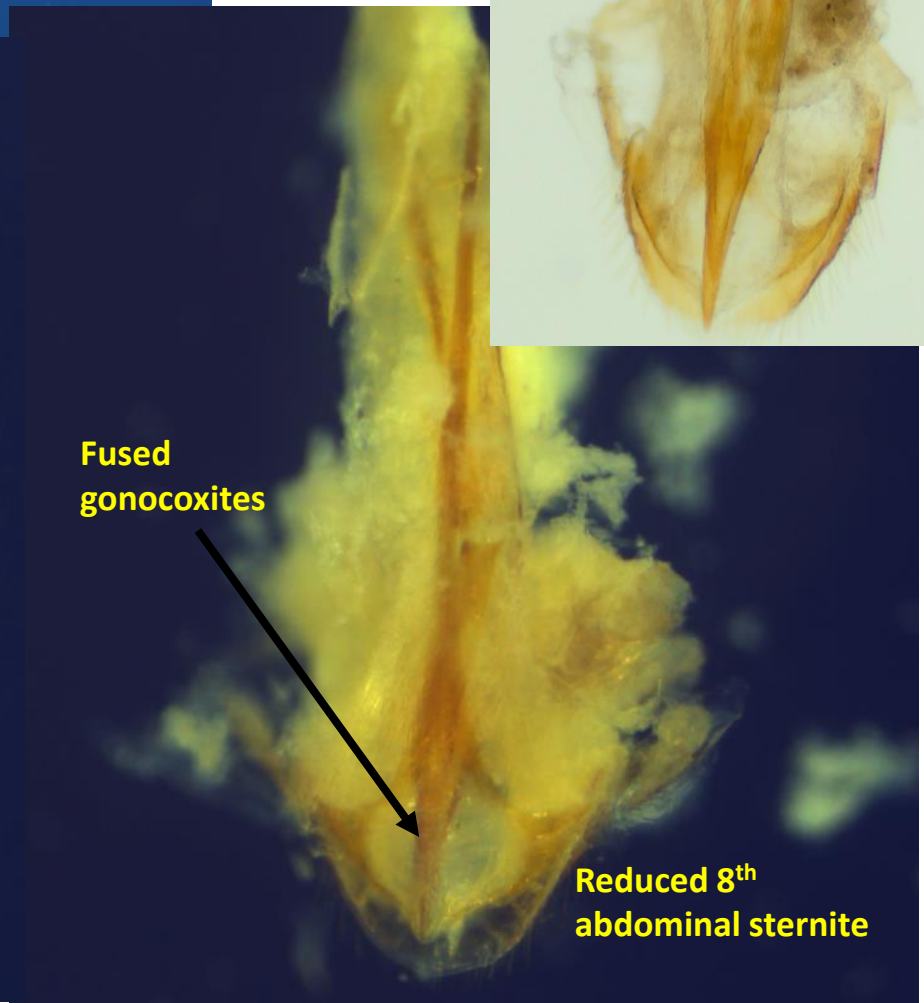


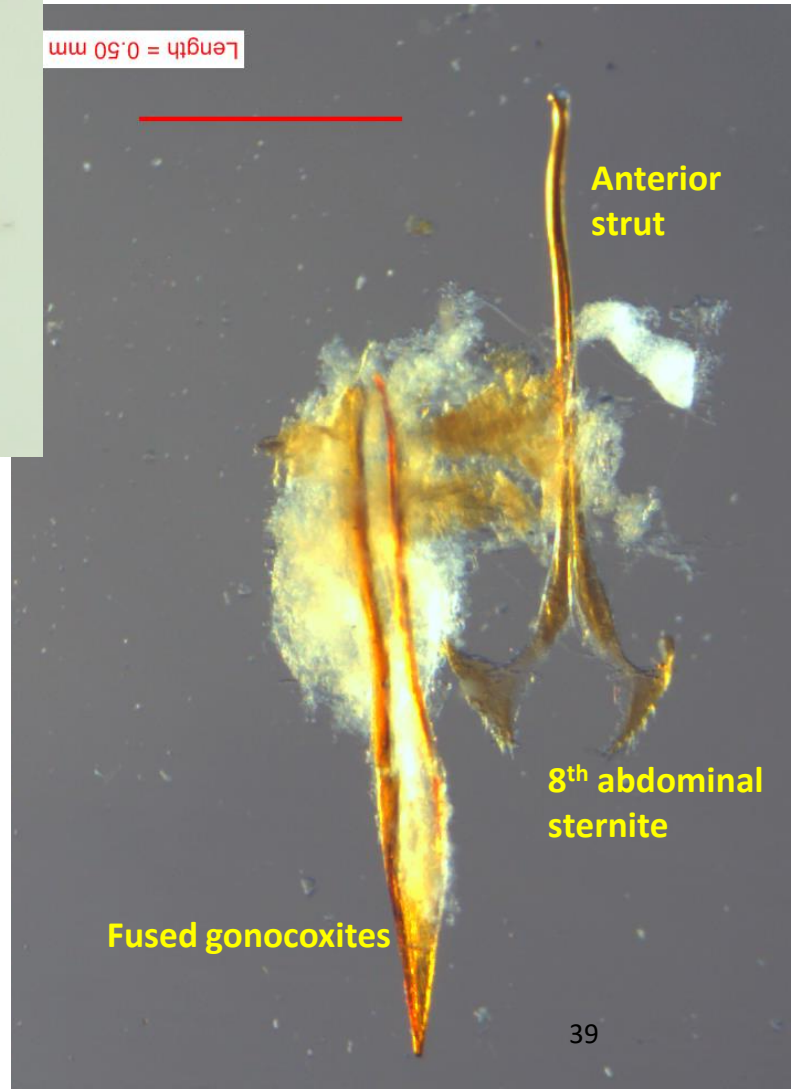
Rotate sideways to see the ventral counterpart of the 8th segment. Expose the ventral region to have a better view of the 8th sternite and anterior strut. Third image is the cleared last segments of the abdomen, showing the male genitalia in relation to the 8th sternite.

Dissection of the female showing key features from the ventral perspective. Note the remnants of the 8th abdominal sternite lay ventral to the fused gonocoxites, which are part of the ovipositor. The cleared slide mount shows how they fit together, internally.



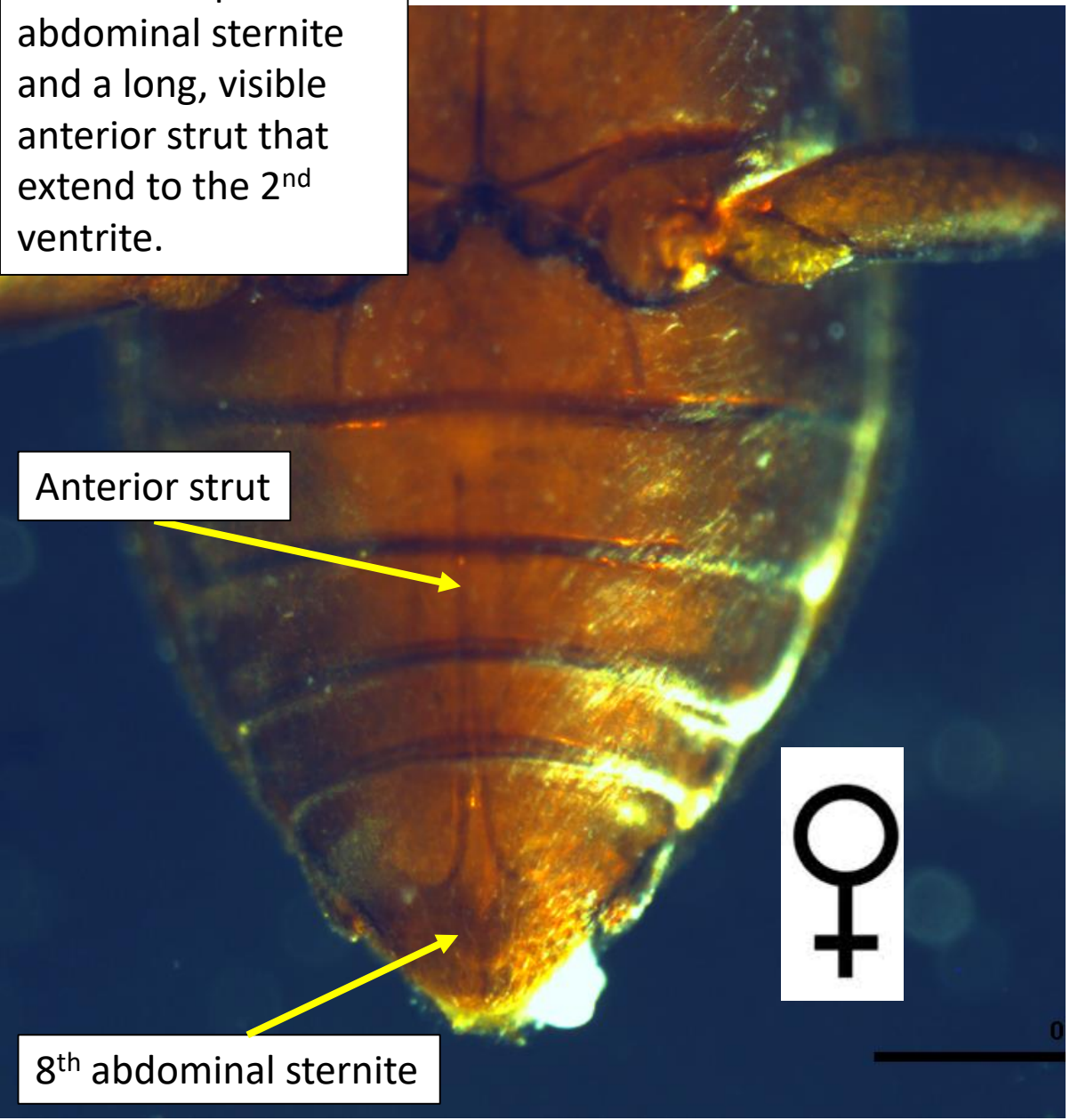


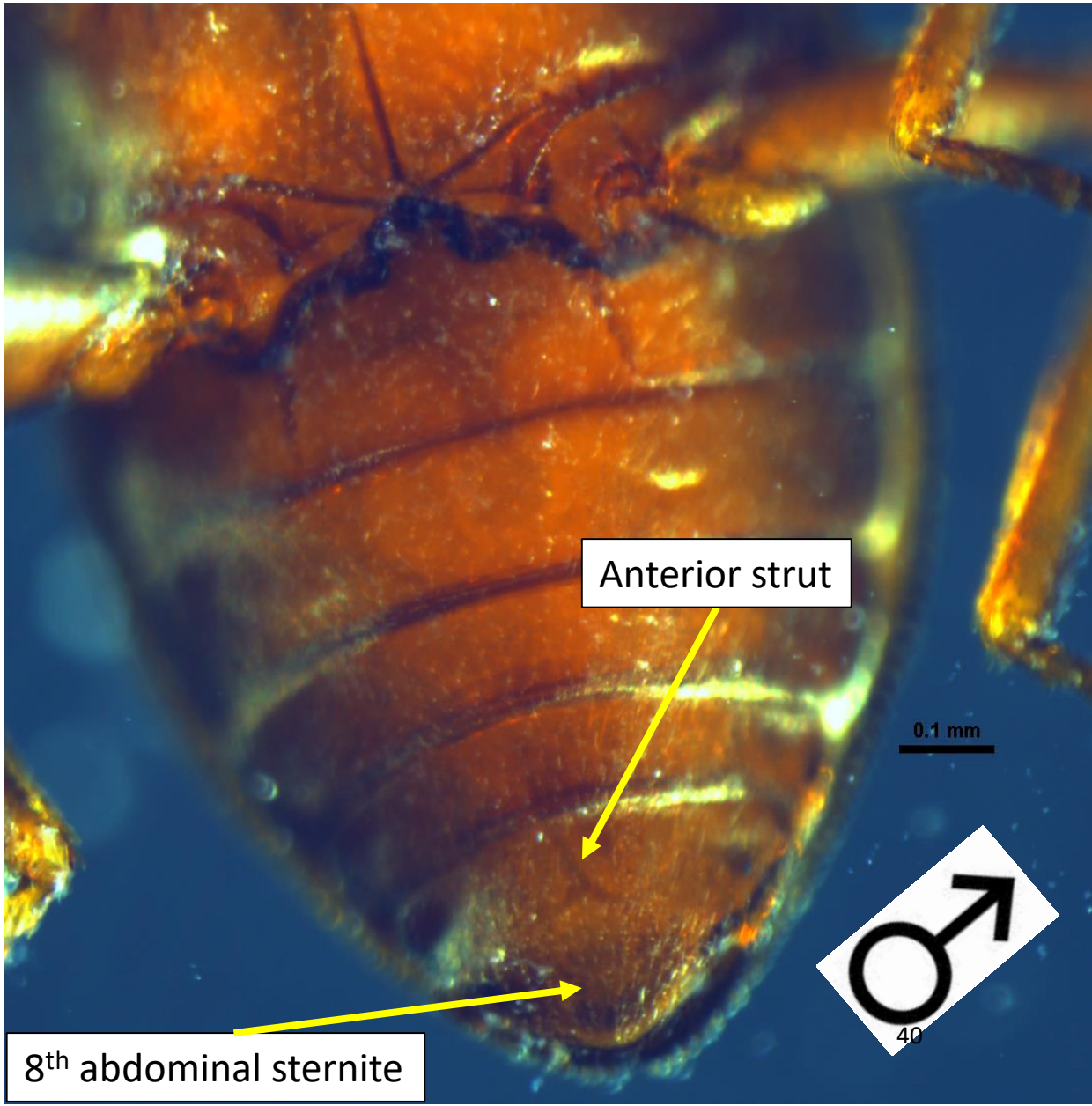




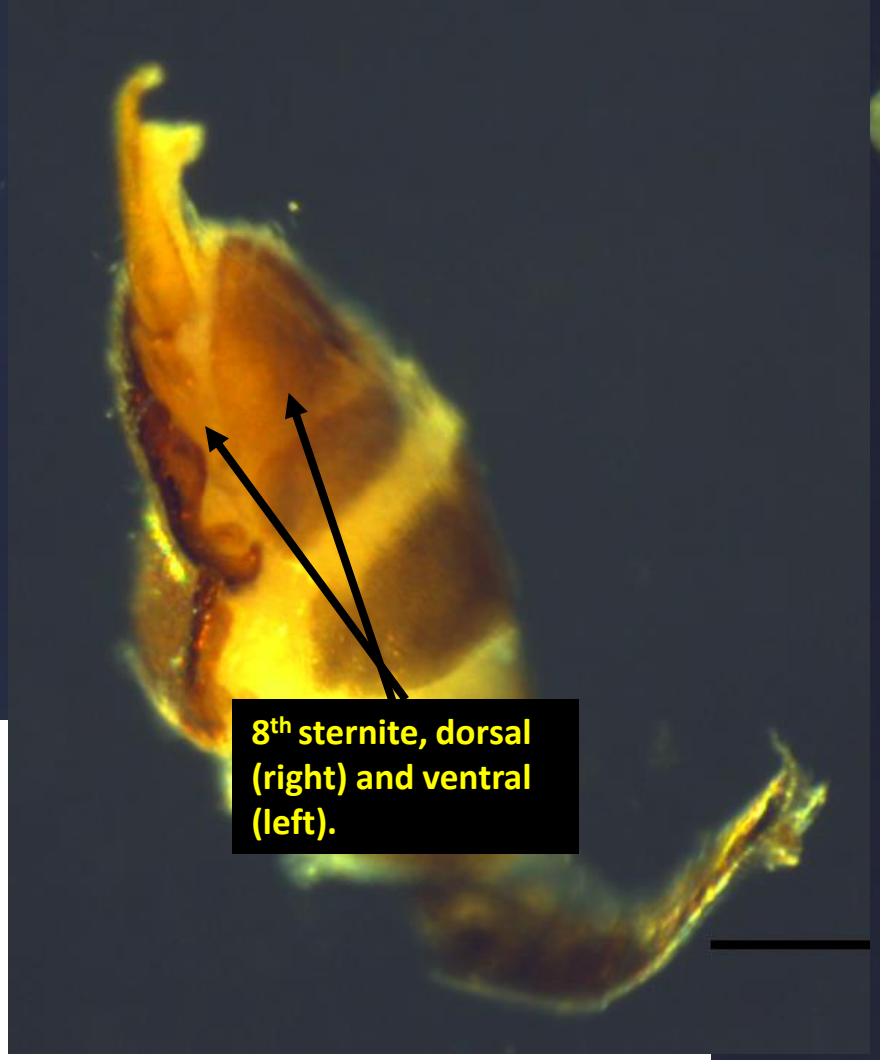
Heterelmis comalensis

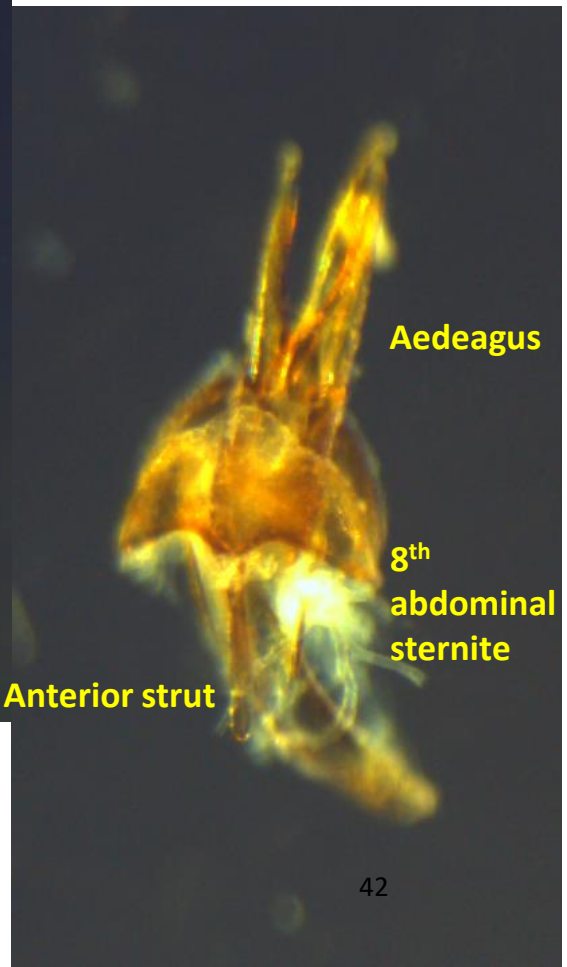
In the Comal Springs riffle beetle we see a well developed 8th abdominal sternite and a long, visible anterior strut that extend to the 2nd ventrite.



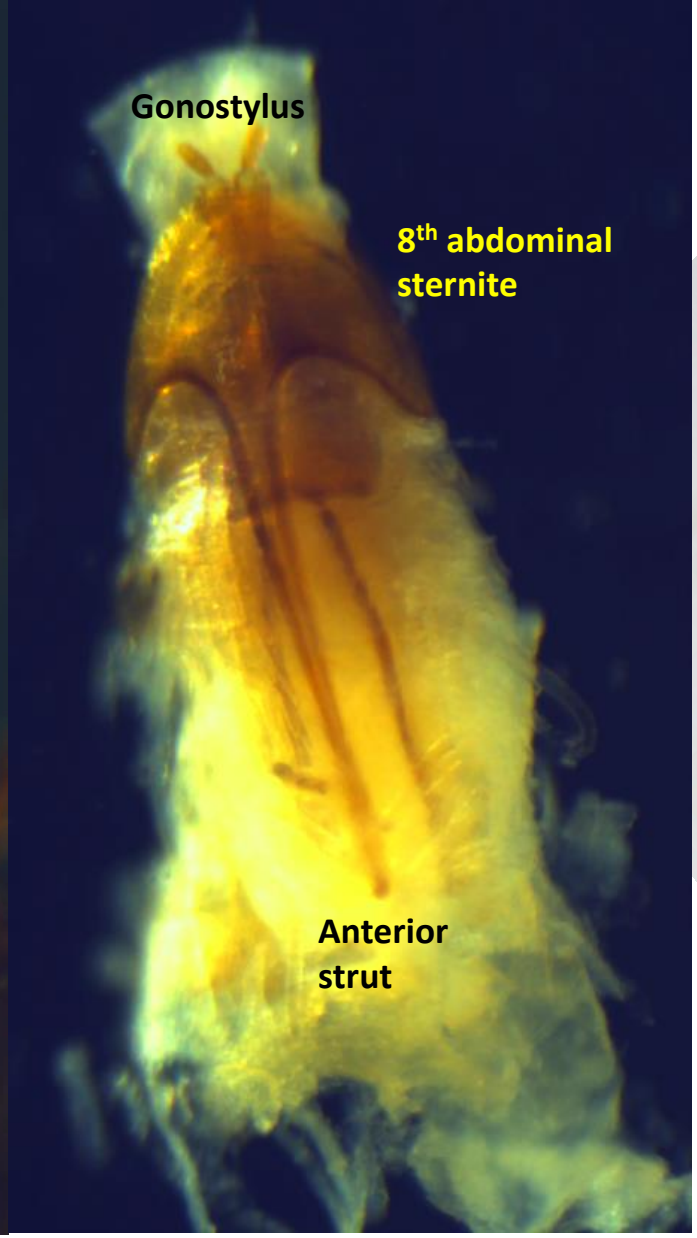


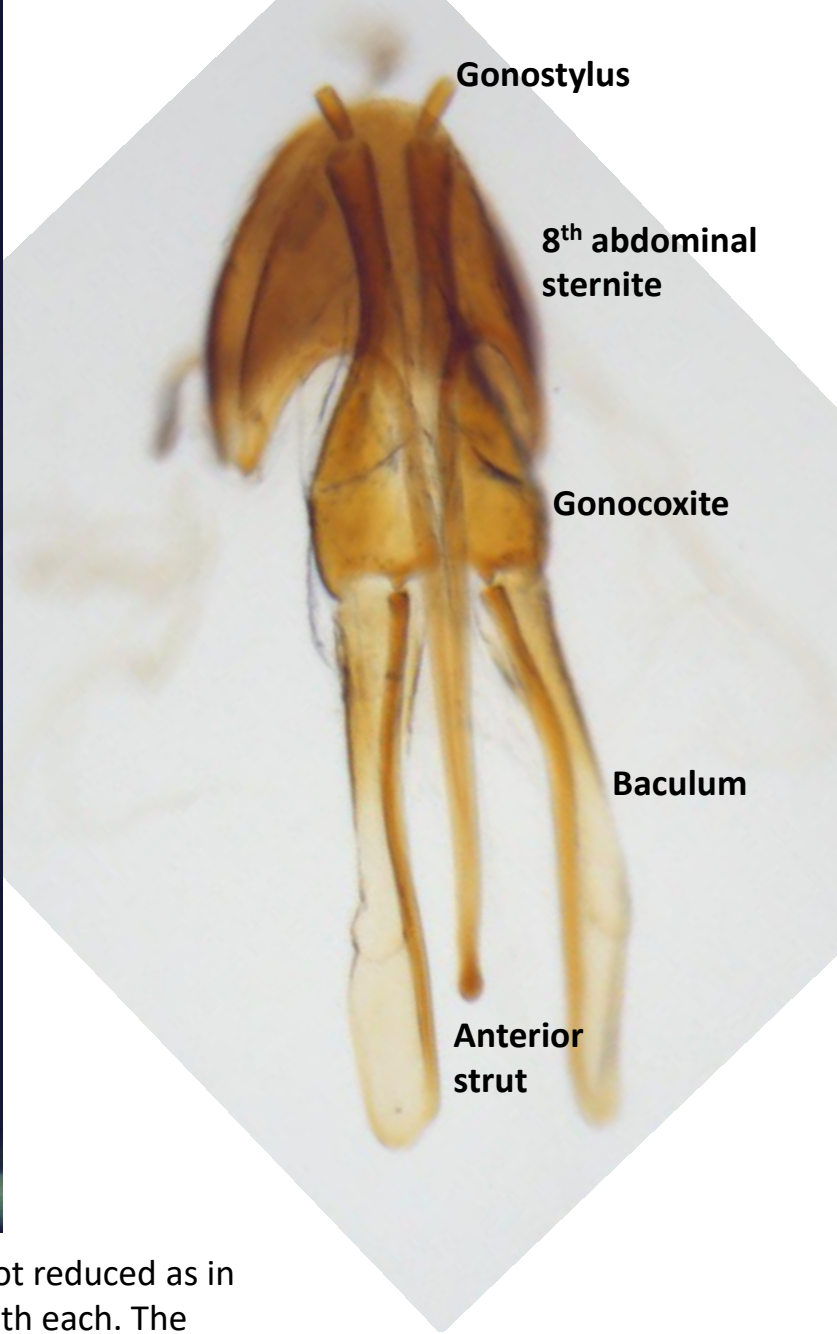
As with the dryopid, use the dorsum of the abdomen to count the abdominal segments, showing 8.





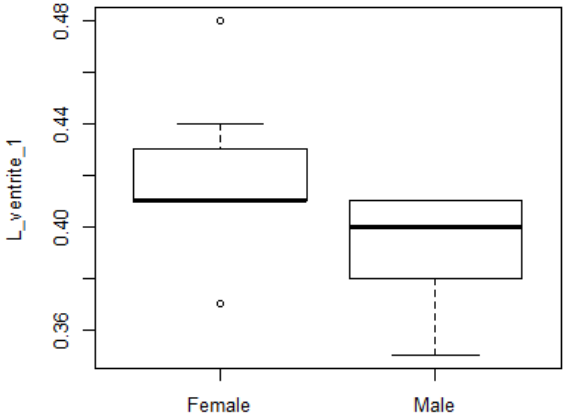
Rotate sideways to see the ventral counterpart of the 8th segment. Expose the ventral region to have a better view of the 8th sternite and anterior strut. Fourth image is the cleared last segments of the abdomen, showing the male genitalia in relation to the 8th sternite.

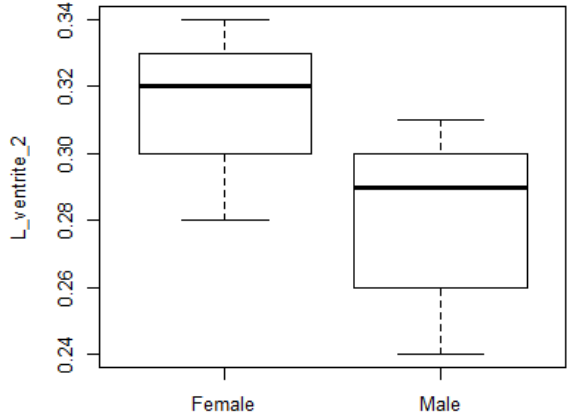




Dissection of the female showing key features from the ventral perspective. Note the 8th abdominal sternite is not reduced as in Comal Springs dryopid beetle. Also, the gonocoxites are not fused and have a gonostylus at the tip associated with each. The gonostyli and gonocoxites along with baculum are parts of the ovipositor. The anterior strut of the 8th abdominal sternite, as with the dryopid, extends through most of the abdomen.

Appendix F


L_ventrite_1
0.48
0.44
0.40
0.36
Female
Male


L_ventrite_2
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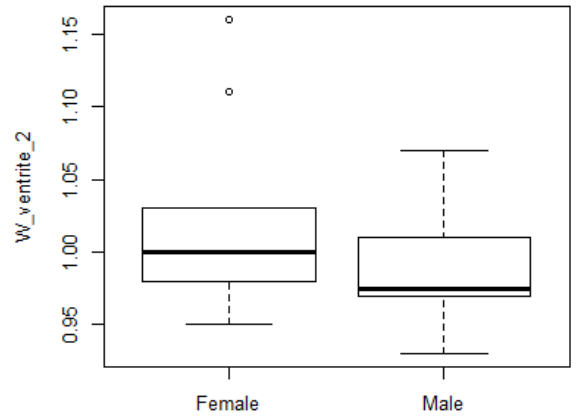
0.32
0.30
0.28
0.26
0.24
Female
Male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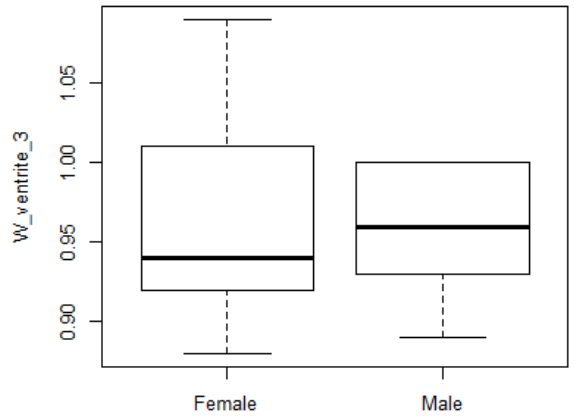
1.10
1.05
1.00
0.95
Female
Male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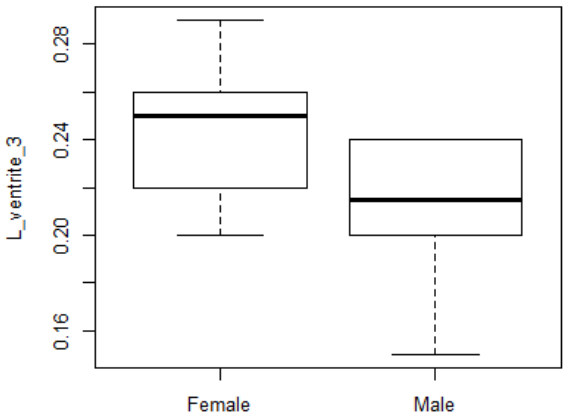
1.00
0.95
0.90
Female
Male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0.24
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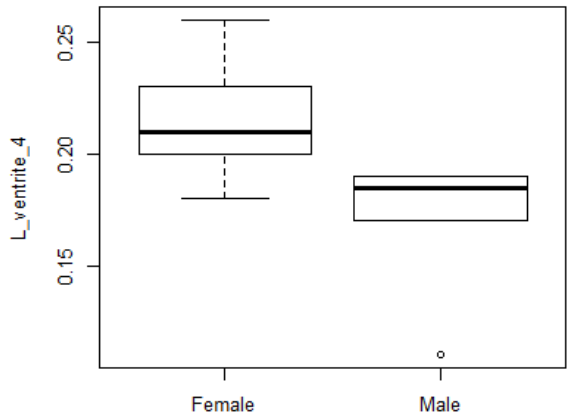
0.16
Female
Male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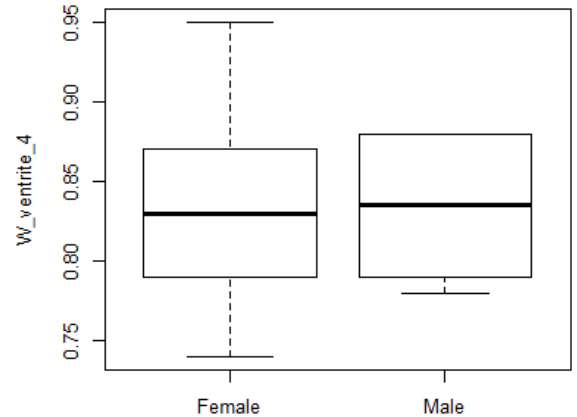
0.20
0.15
Female
Male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0.85
0.80
0.75
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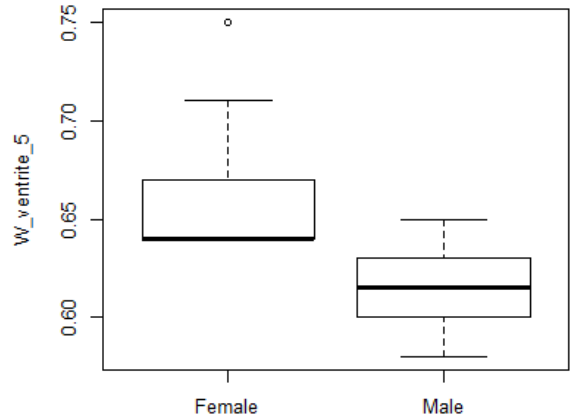
Male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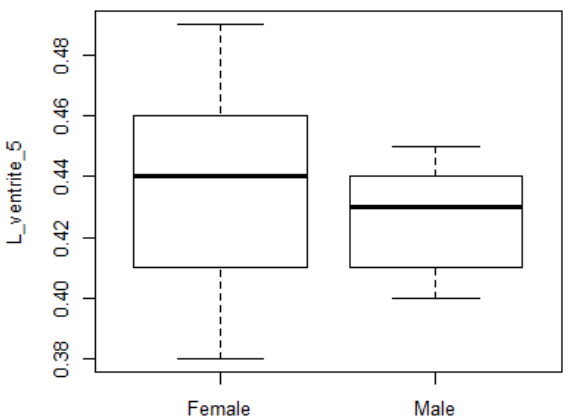
Female
Male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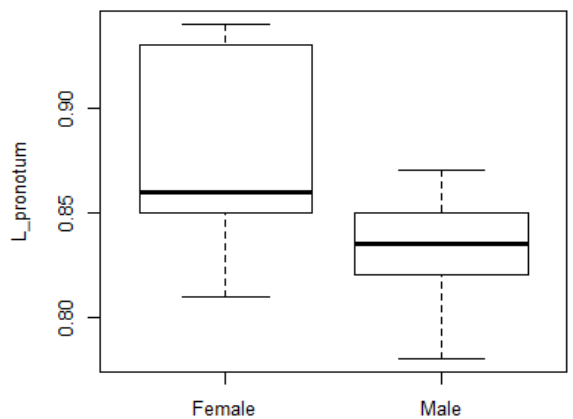
Female
Male


L_pronotum
0.90
0.85
0.80
Female
Male

Appendix G

```
[1] -0.0008676147 0.3085324703 -0.5132622666 0.2625165961 -0.1708041178 [6] 0.0606998753 0.0180046167 -0.0427183421 0.0868687518 -
0.1187566651 [11] 0.1181579087 -0.0538749697 -0.0646580300 0.1385070896 -0.0633703286 [16] -0.0410257754 -0.0845265699 0.3851320548
-0.3700016495 0.0268745433 [21] 0.4725034765 0.0571115509 0.2650503201 -0.4693128314 -0.3077989946 [26] 0.2065088097 -0.1702362444
0.000436167
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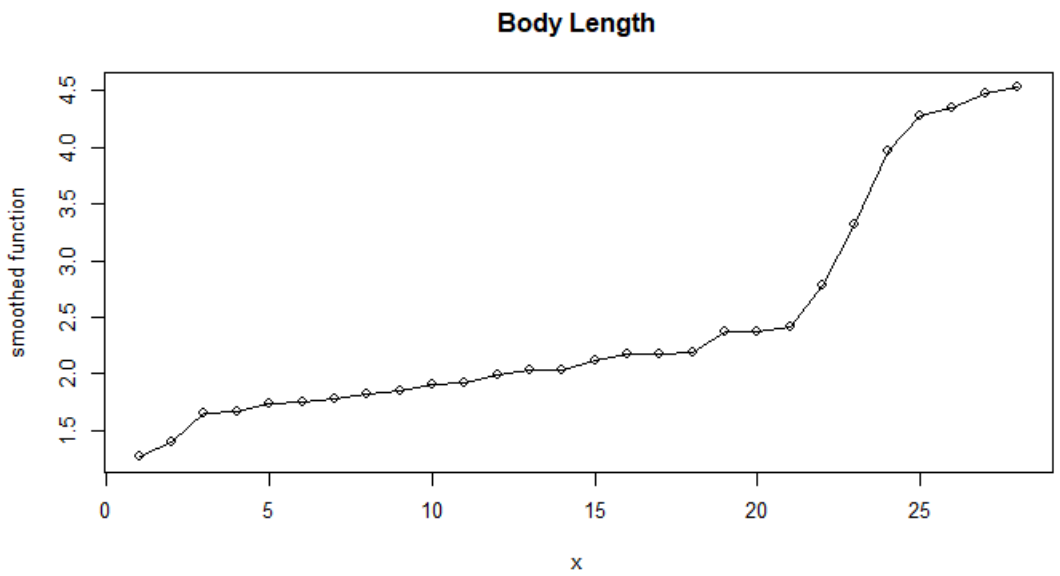

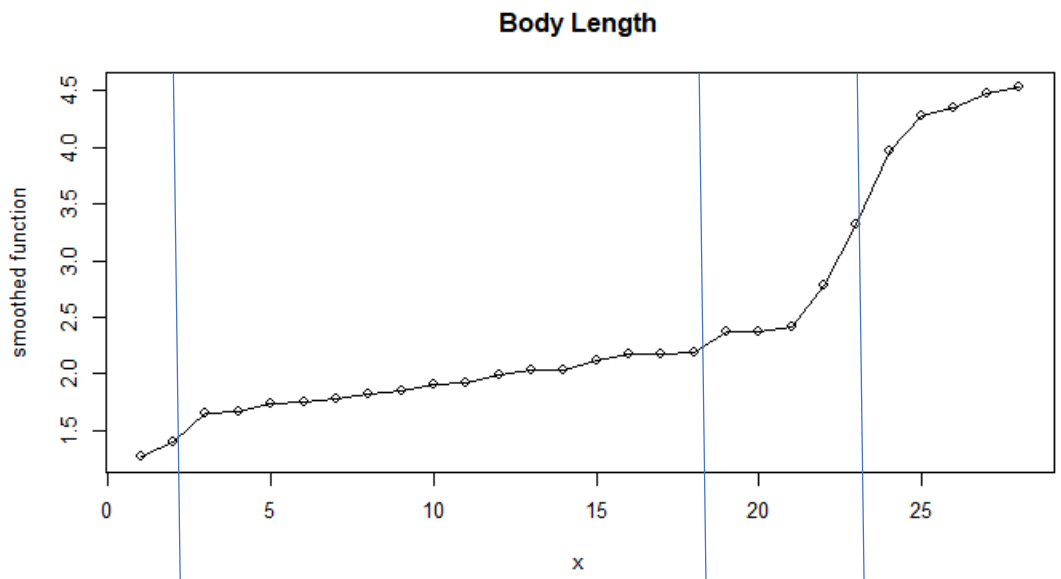

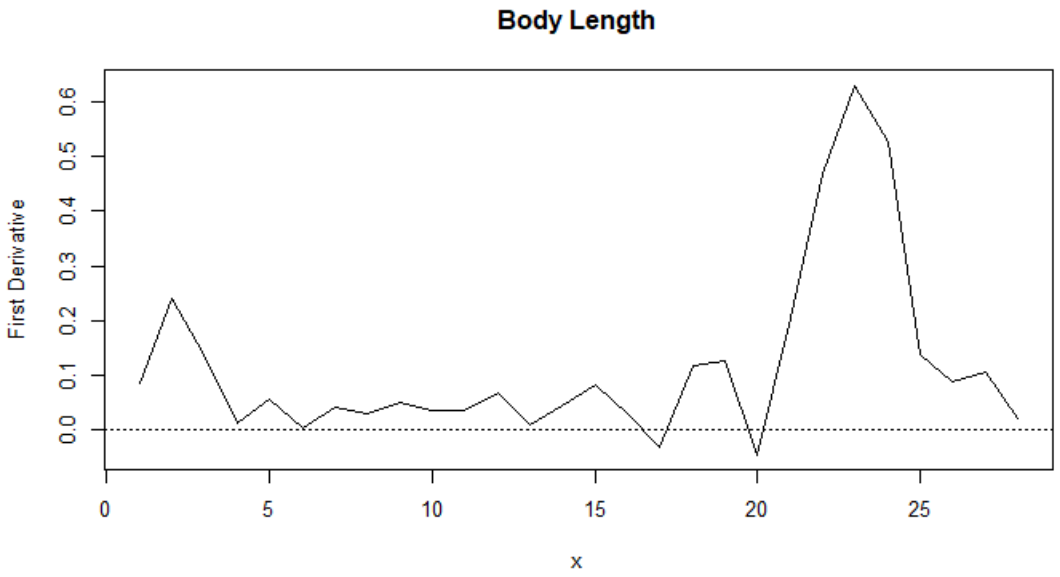

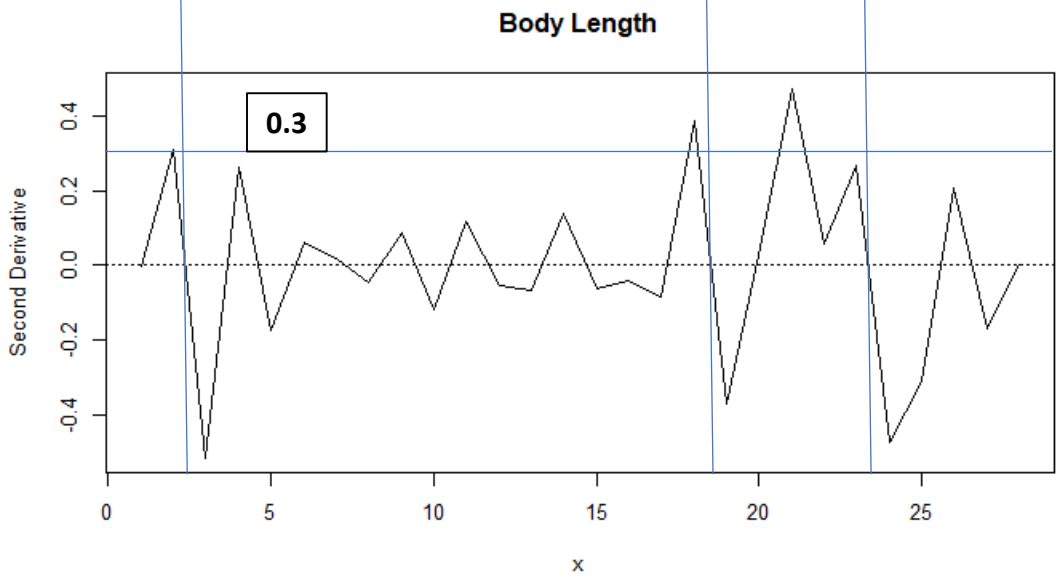

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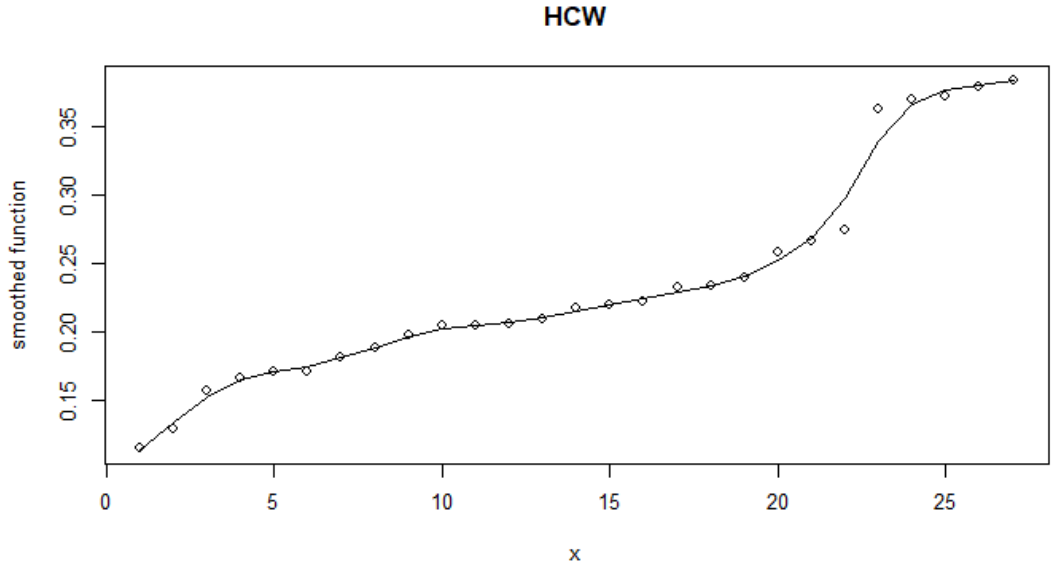

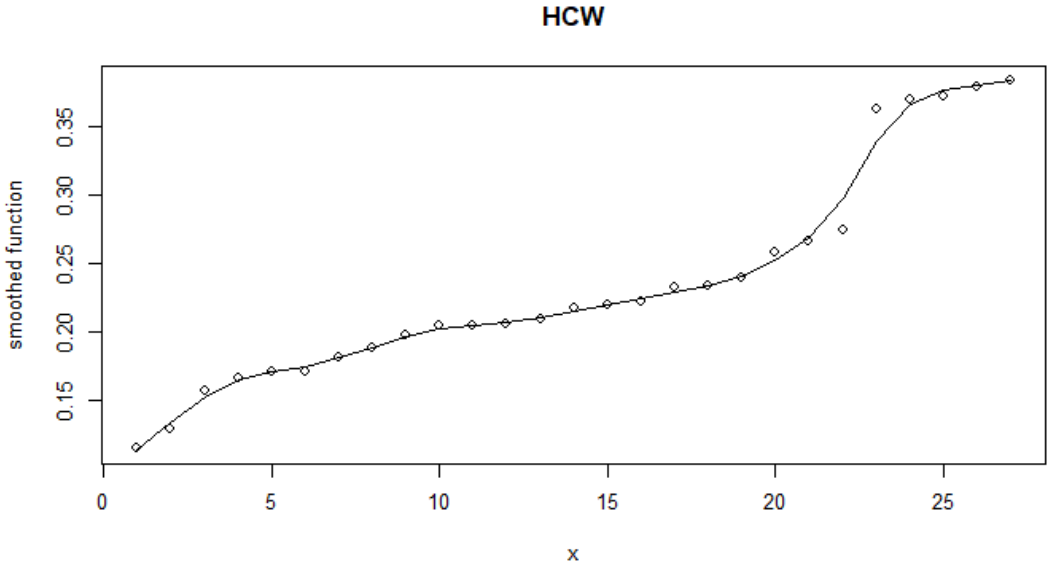

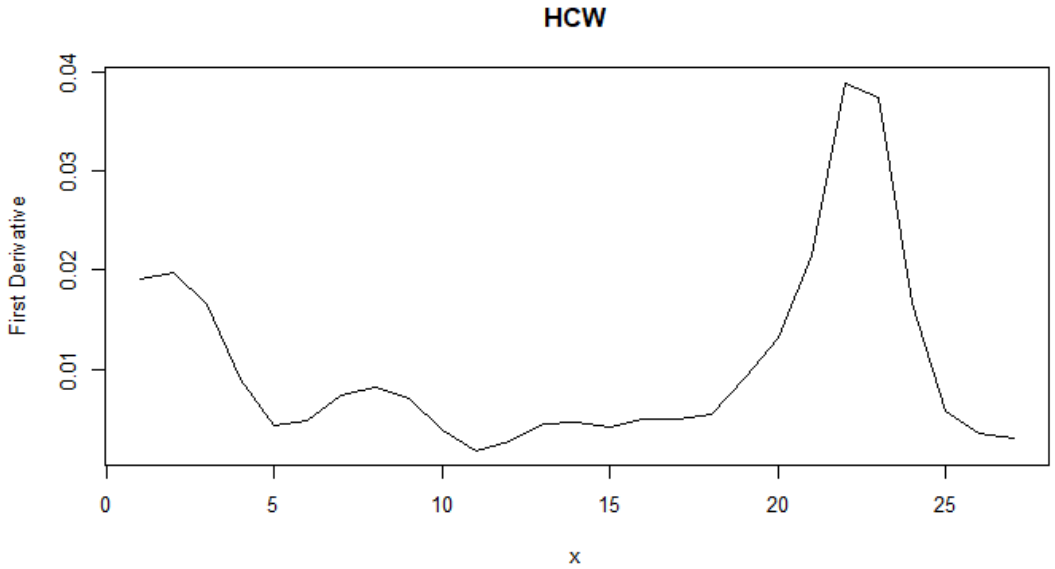

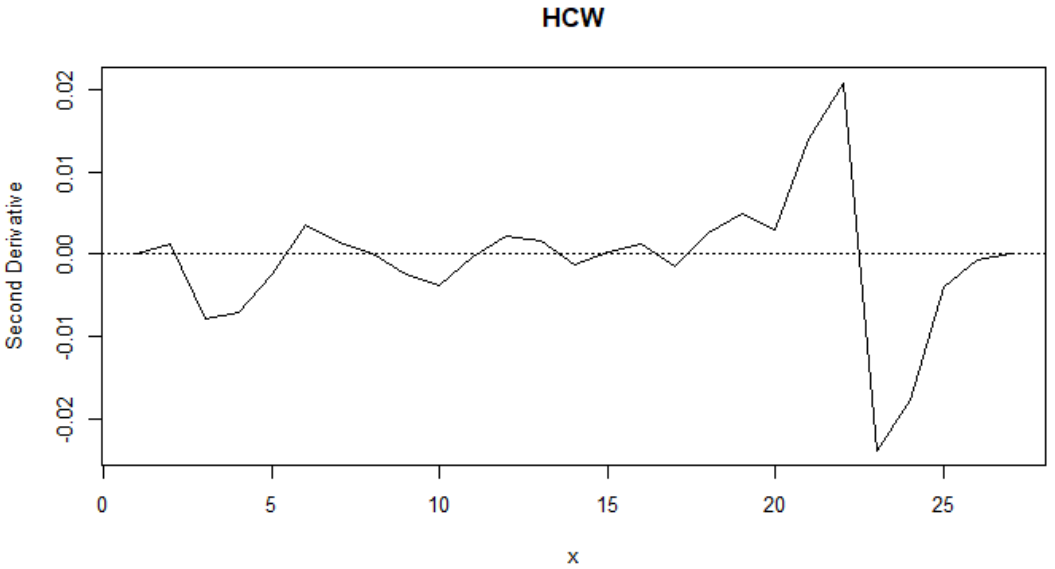

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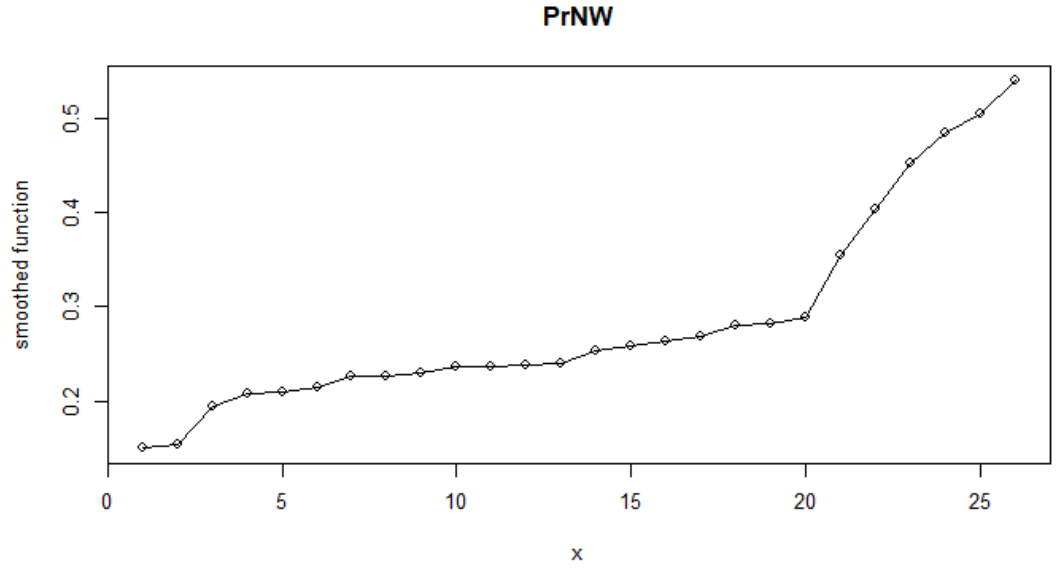

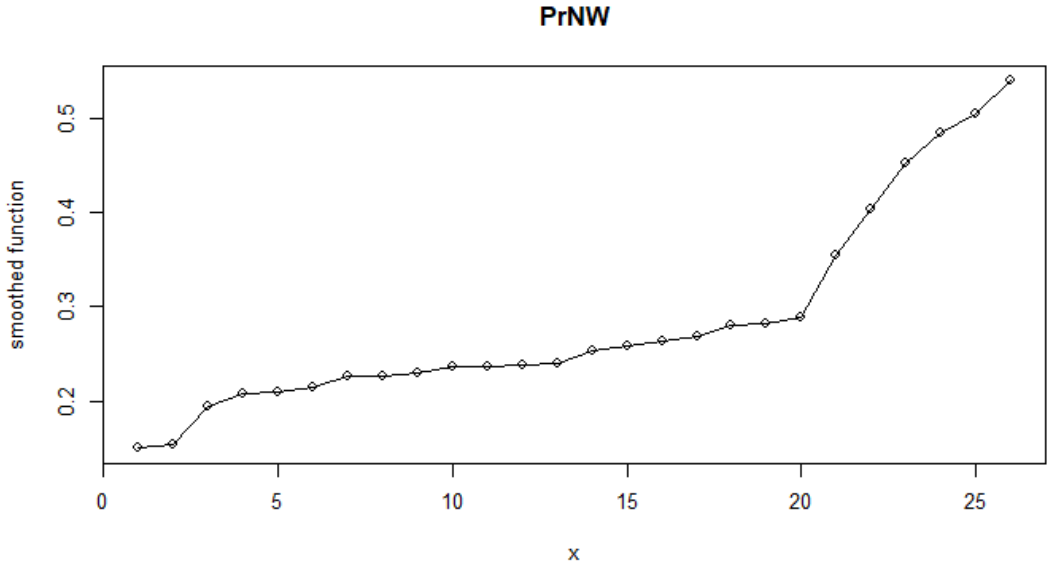

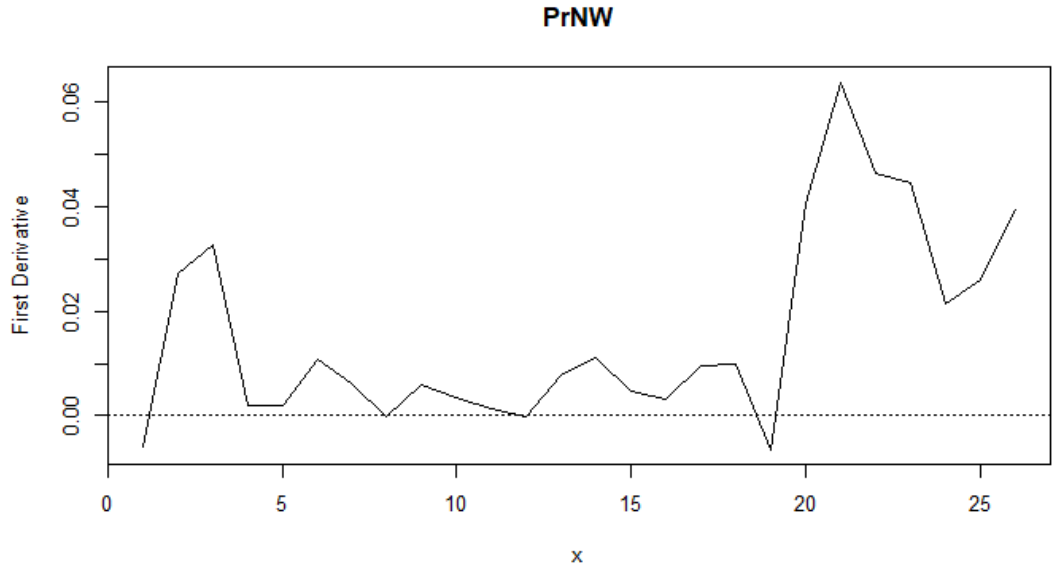

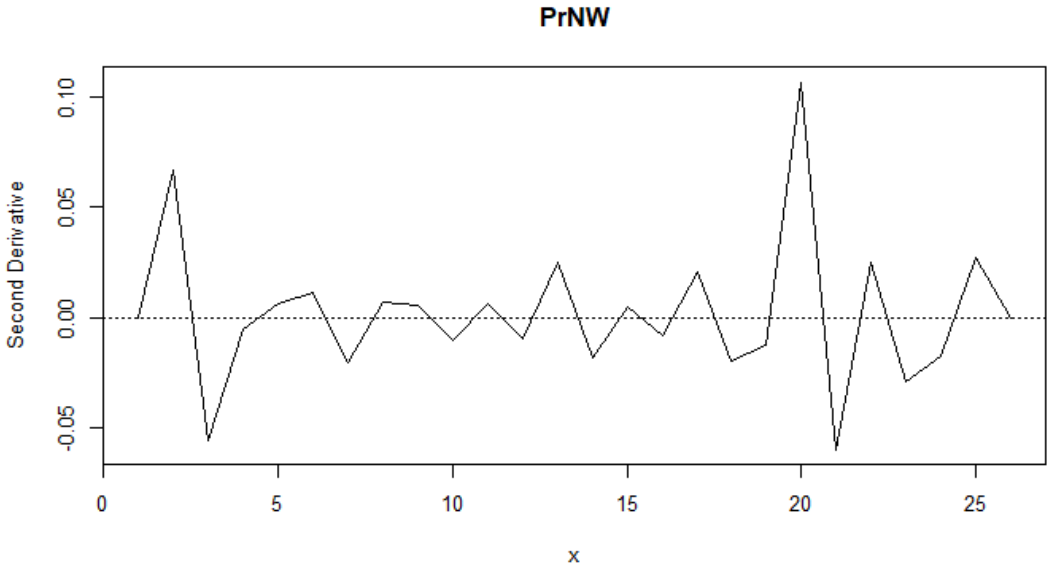

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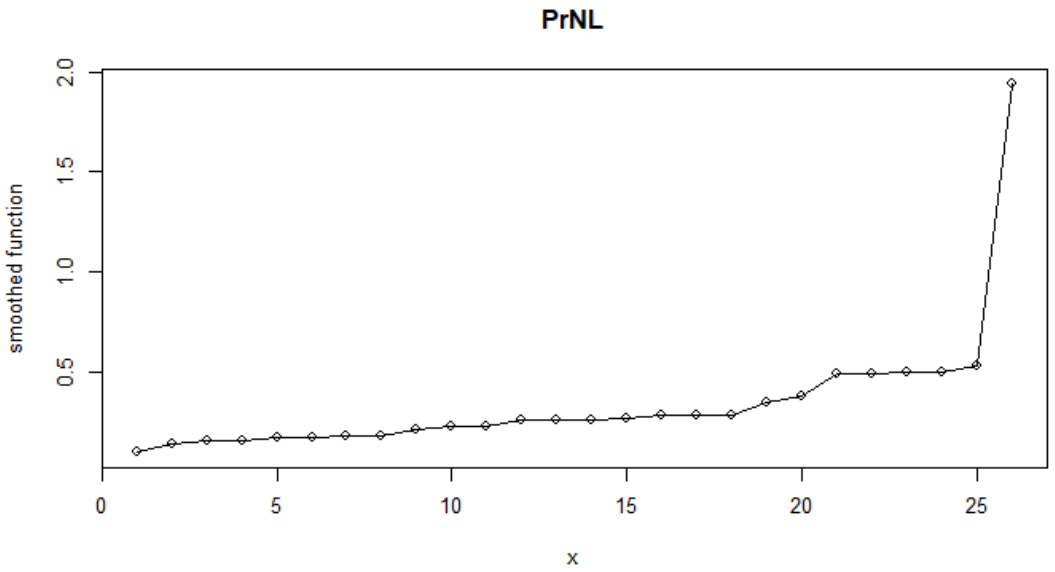

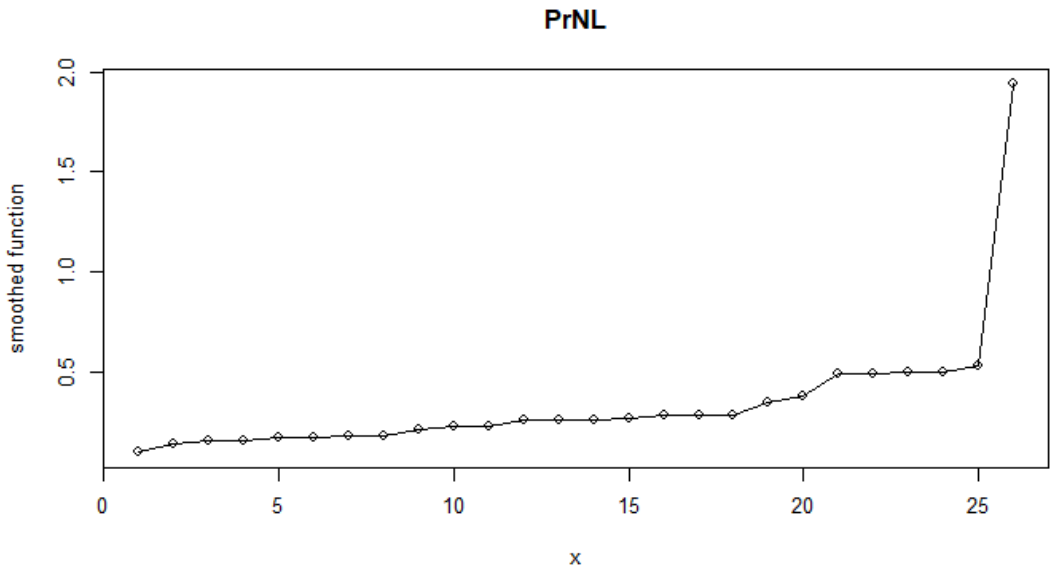

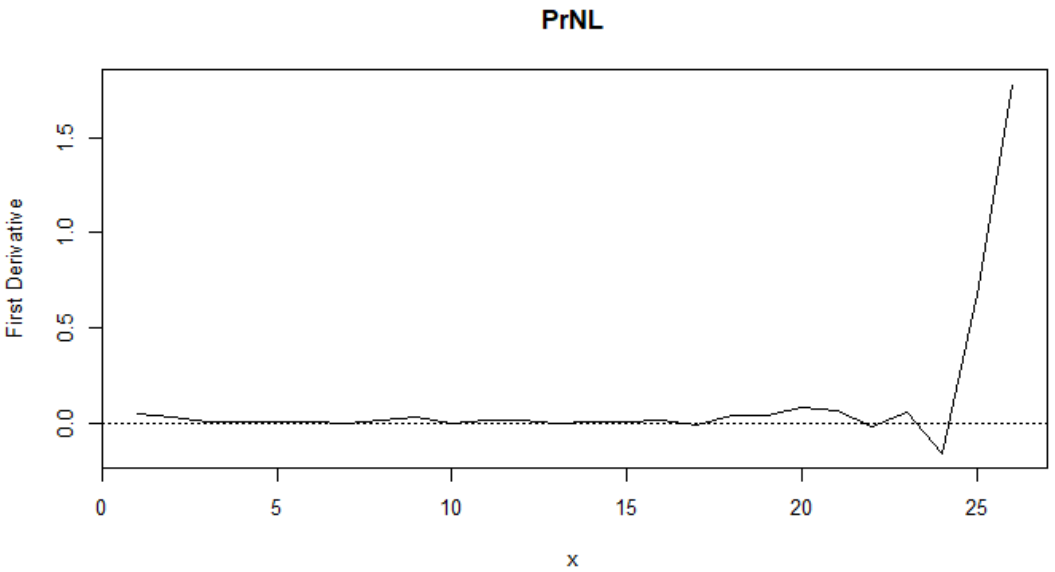

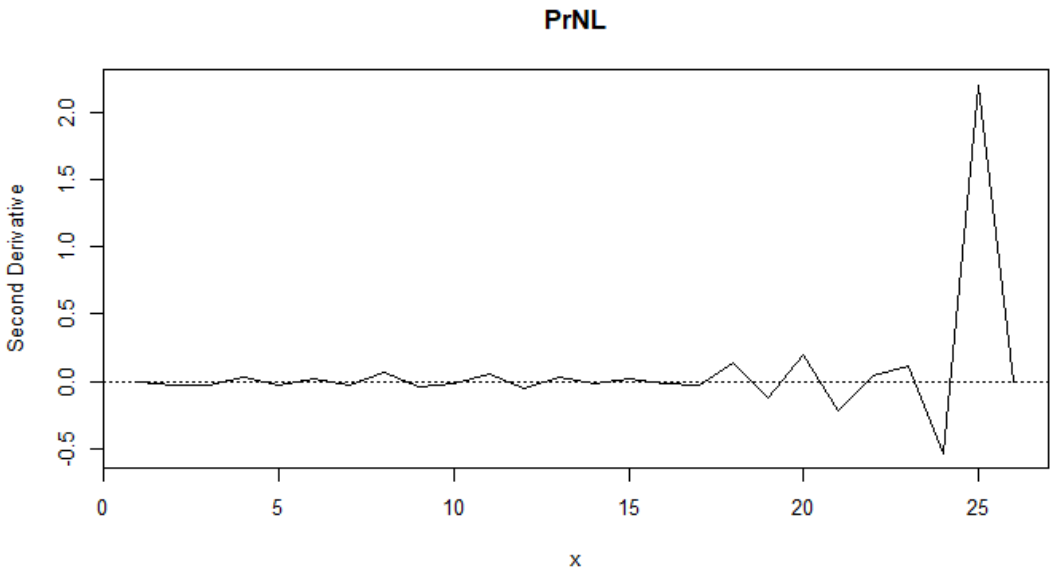

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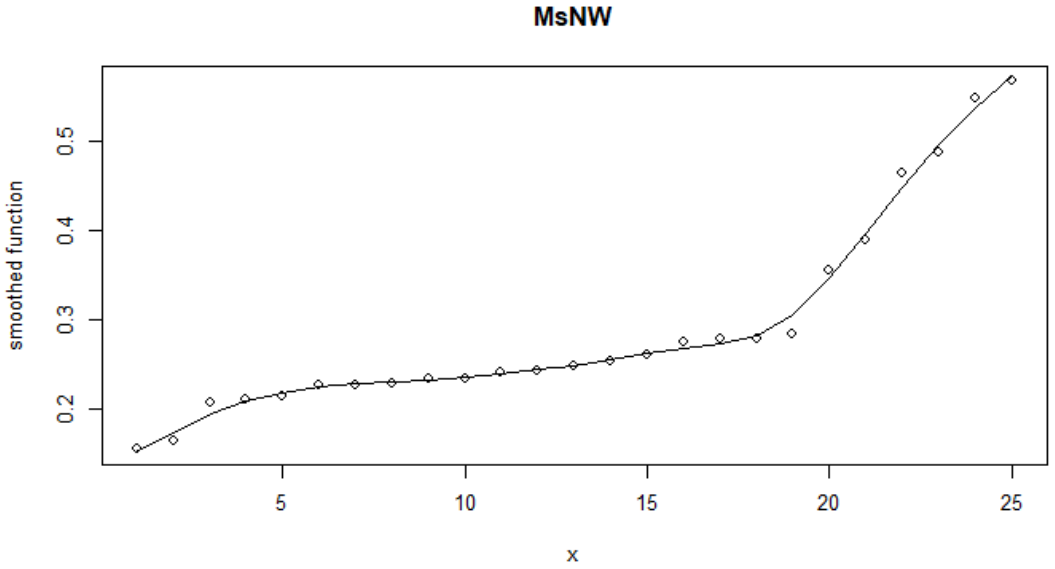

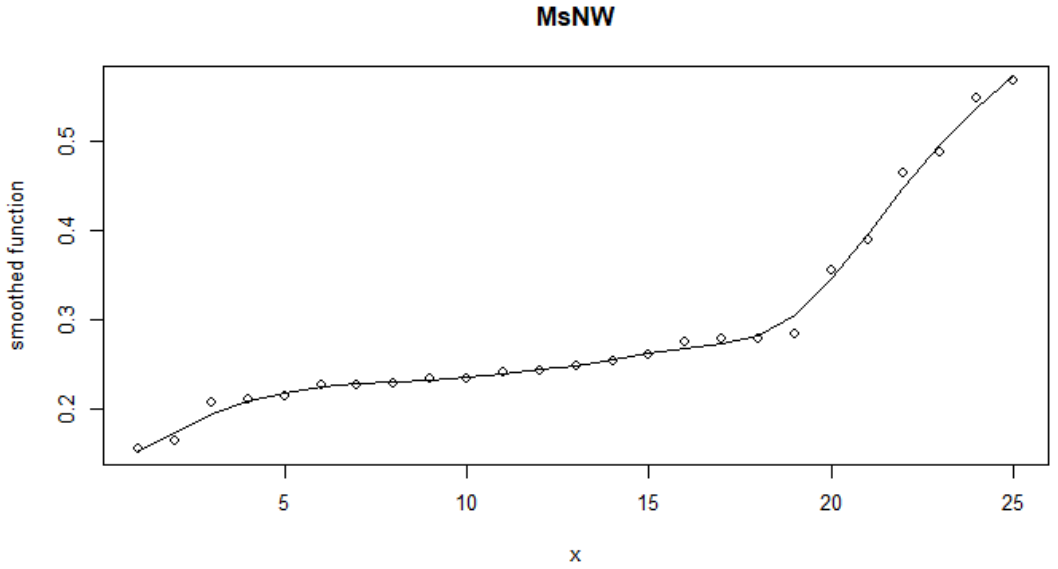

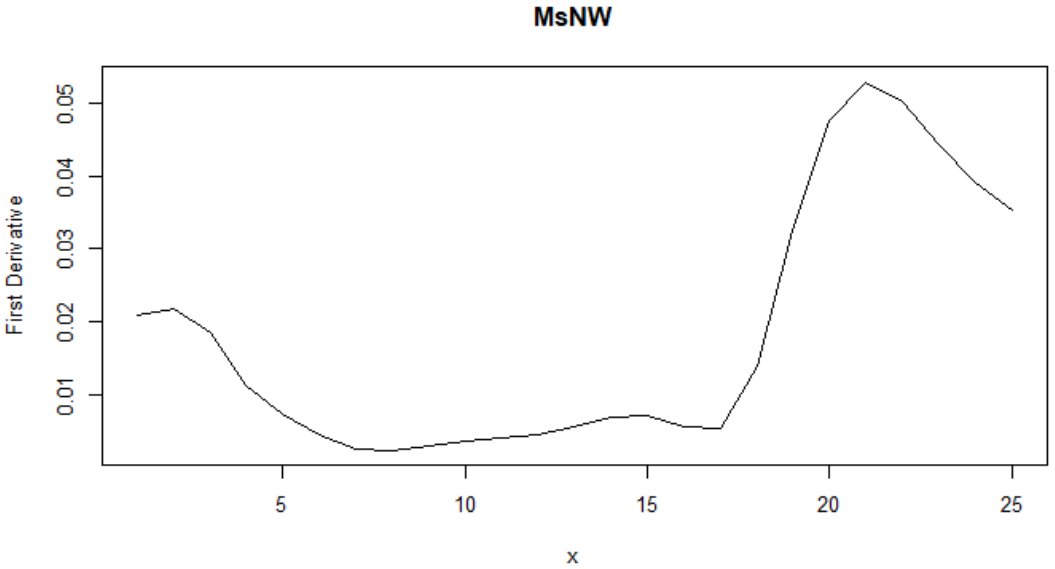

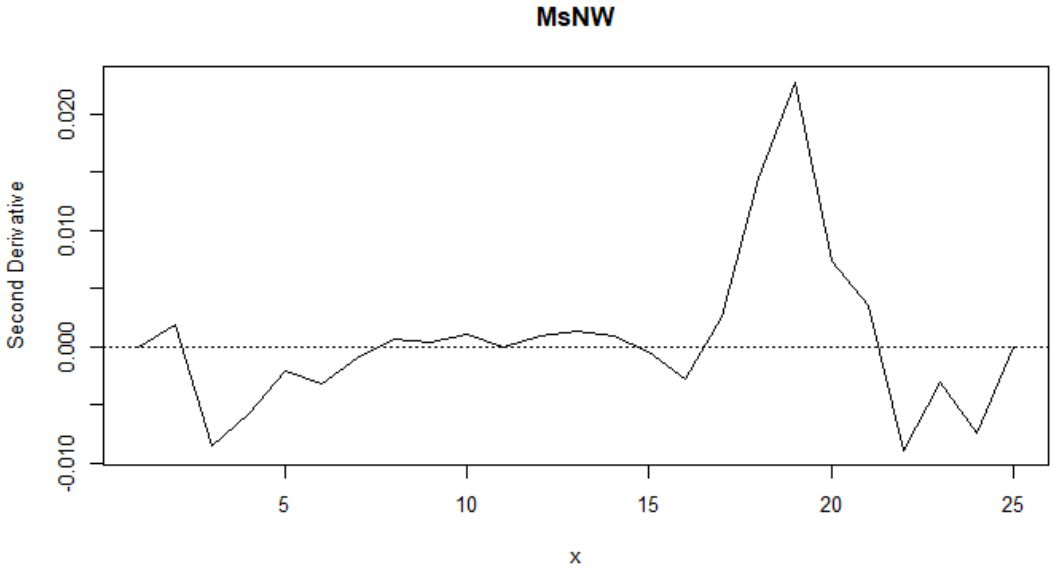

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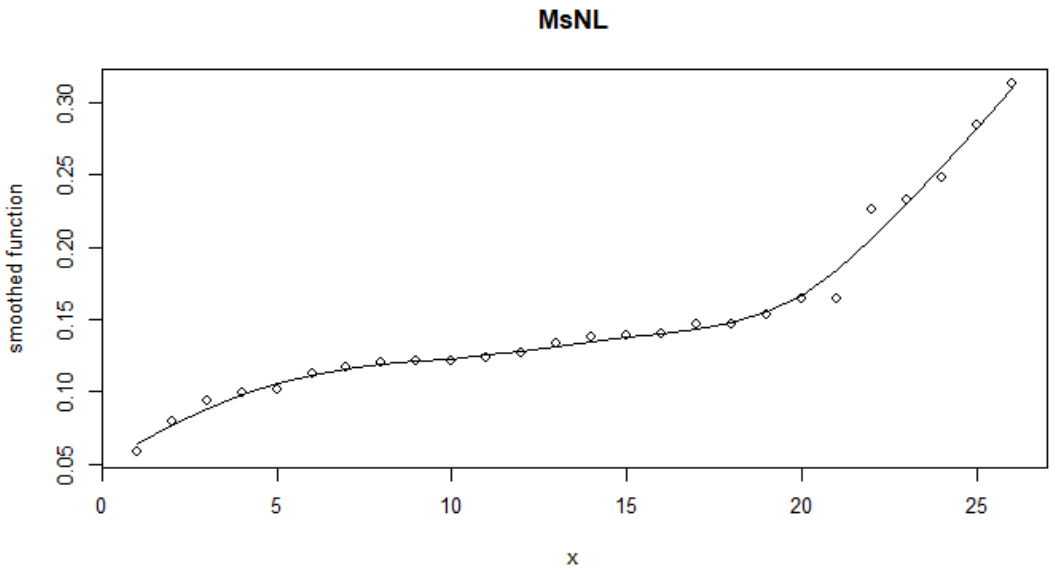

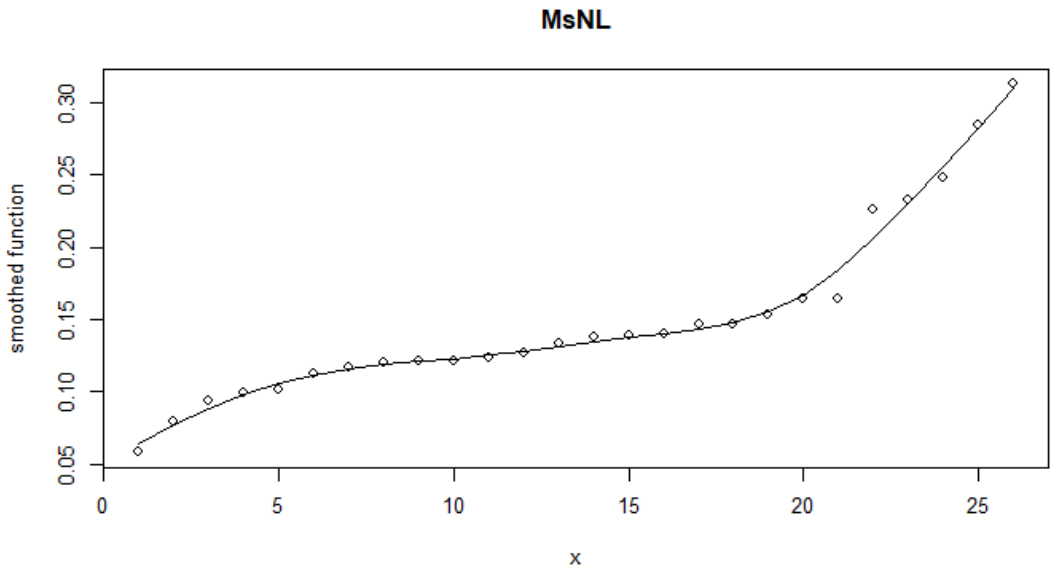

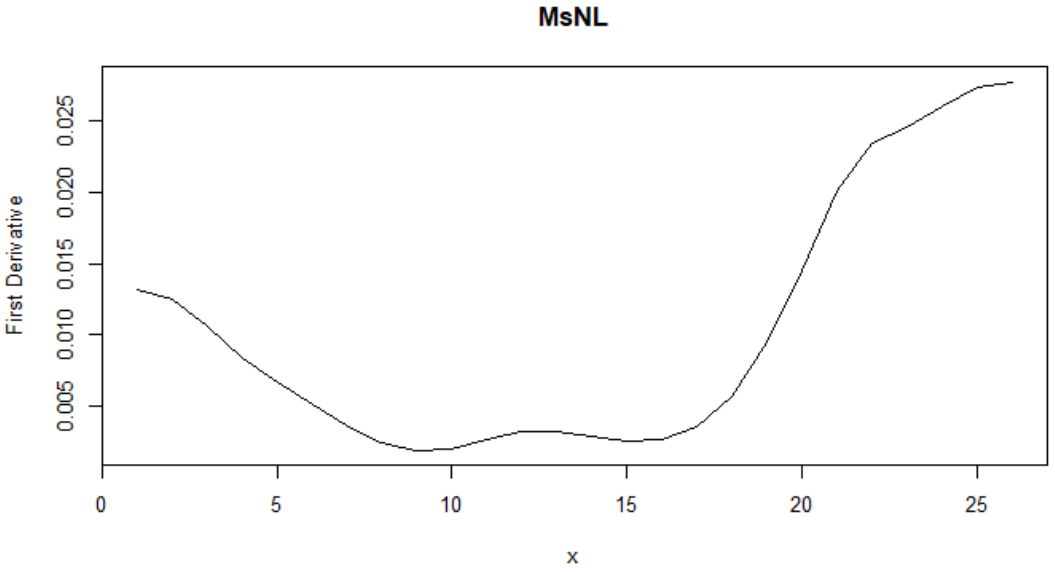

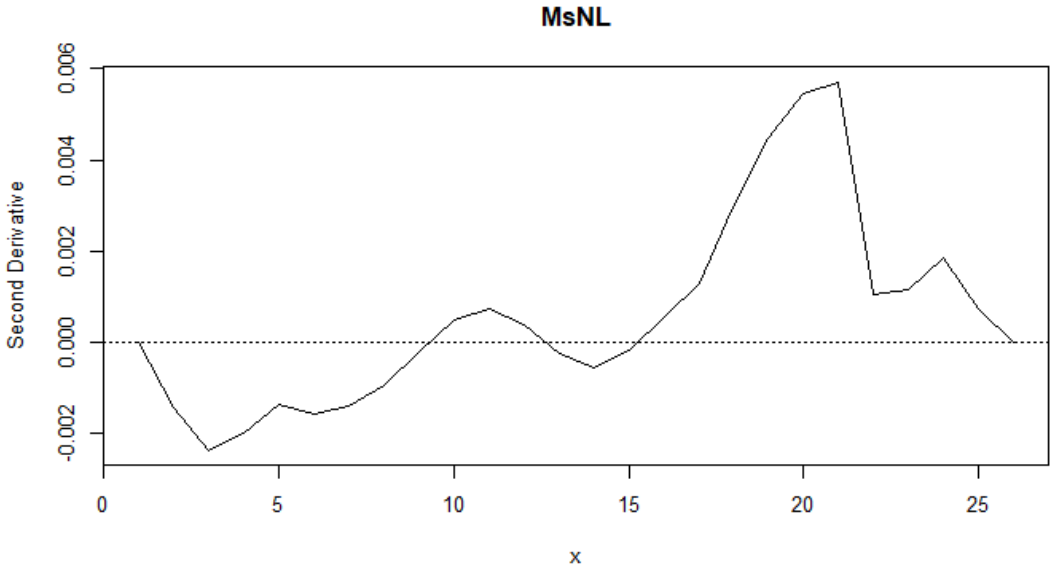

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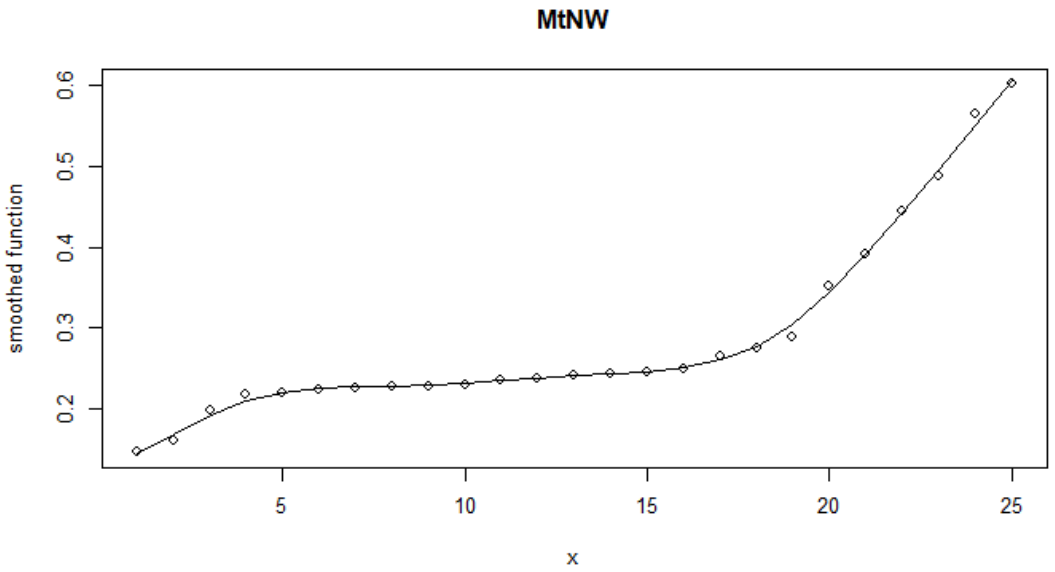

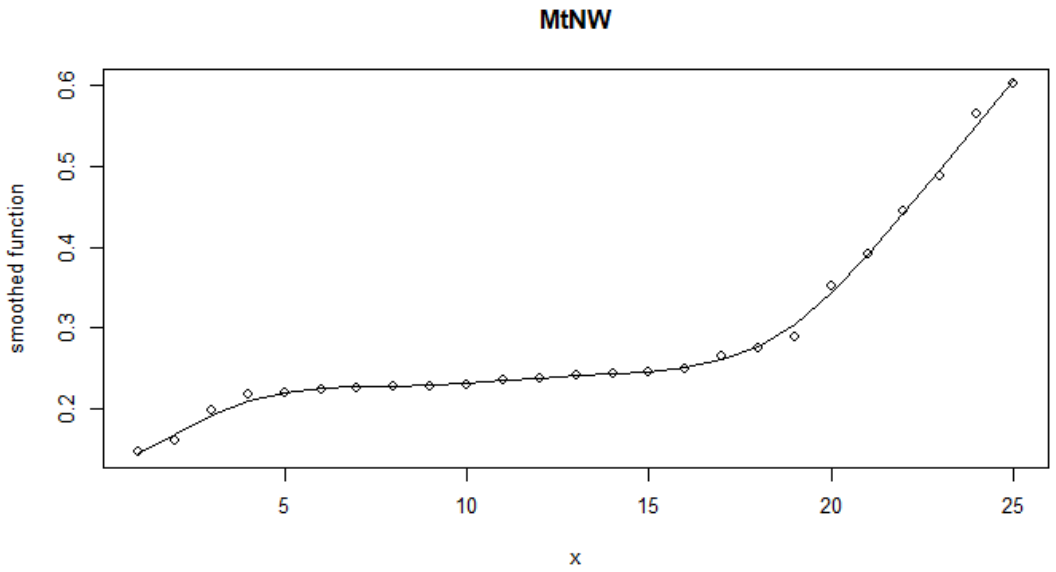

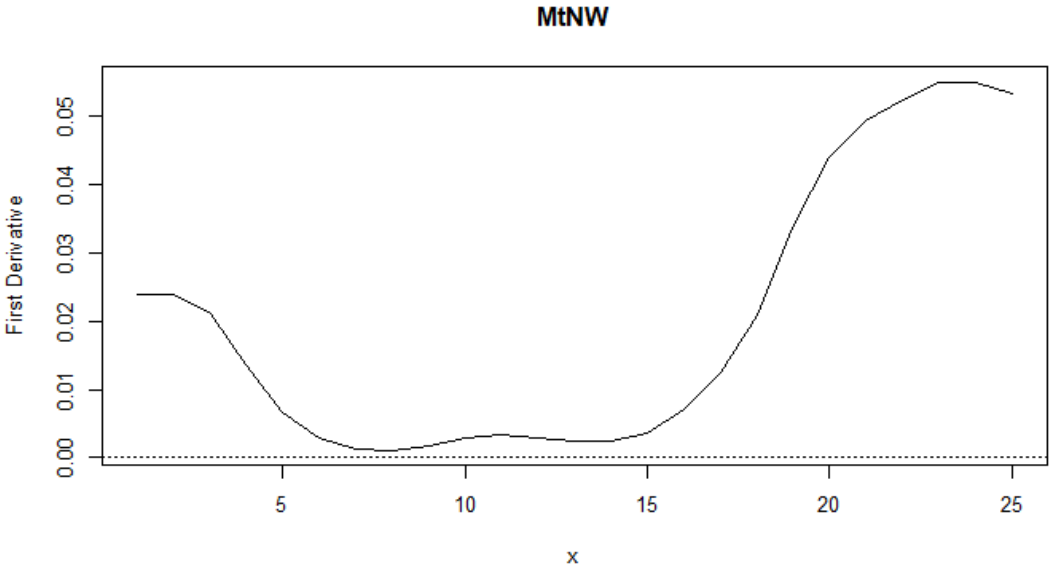

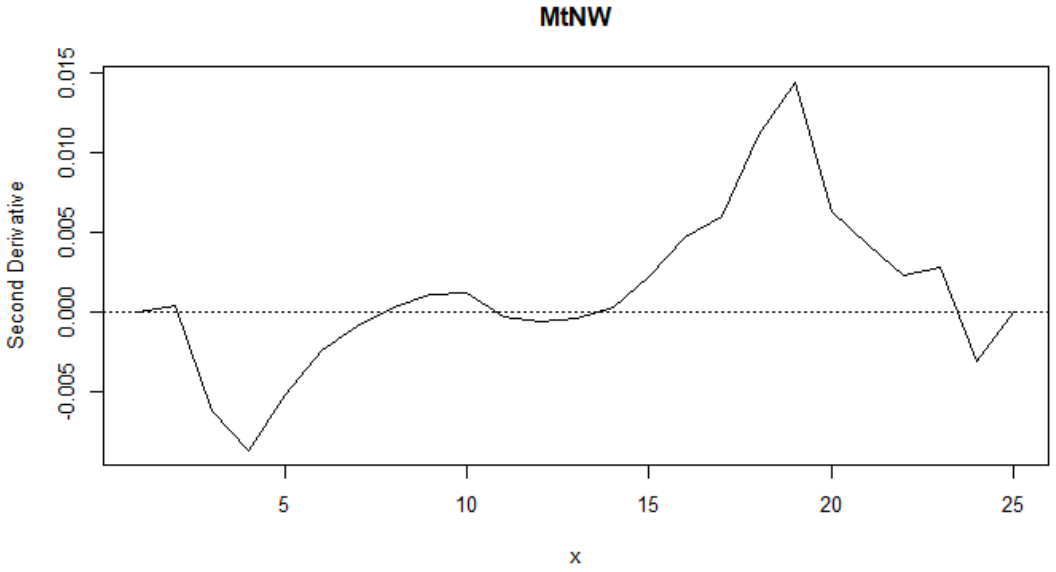

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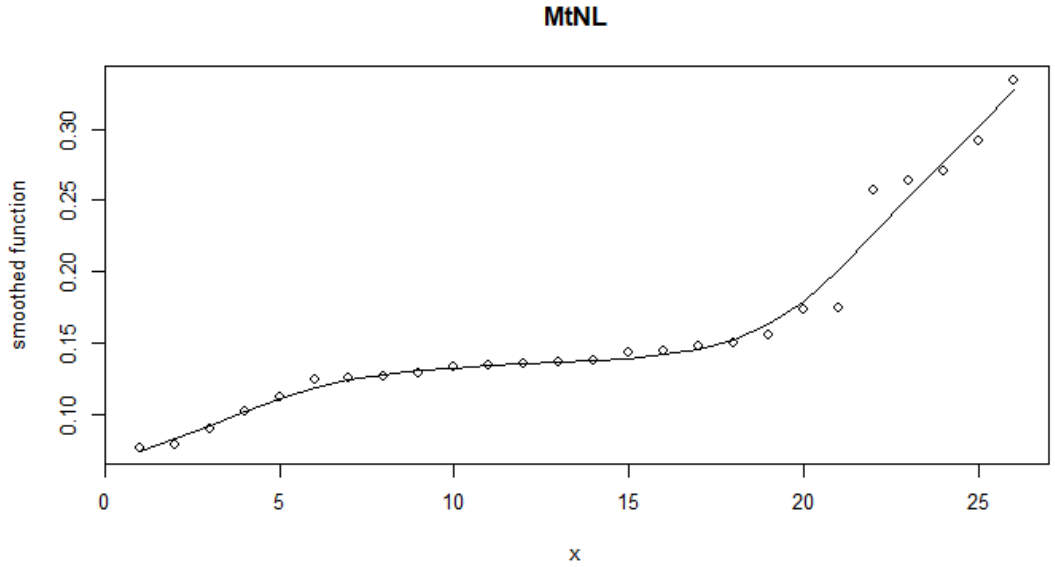

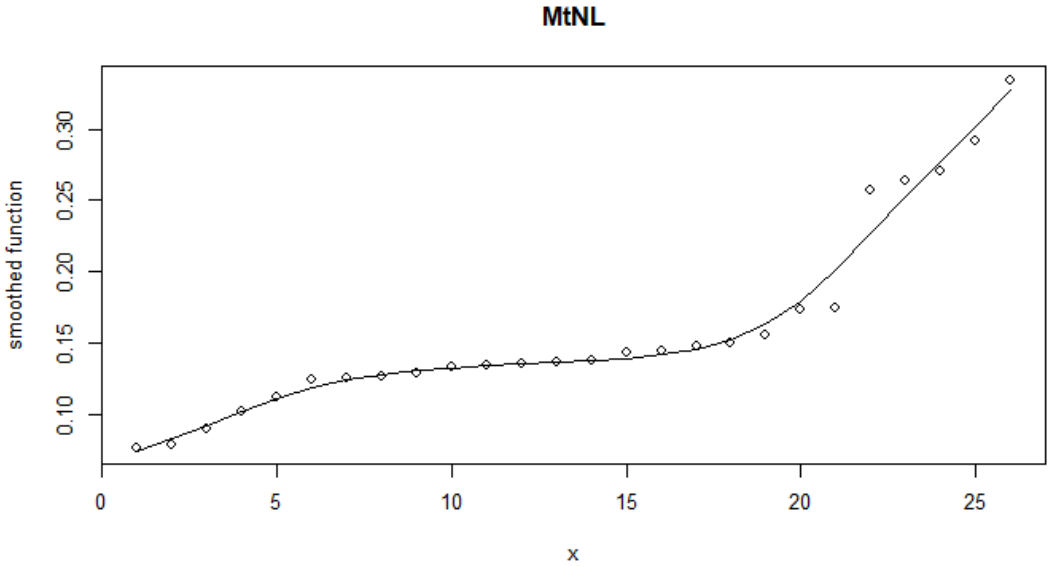

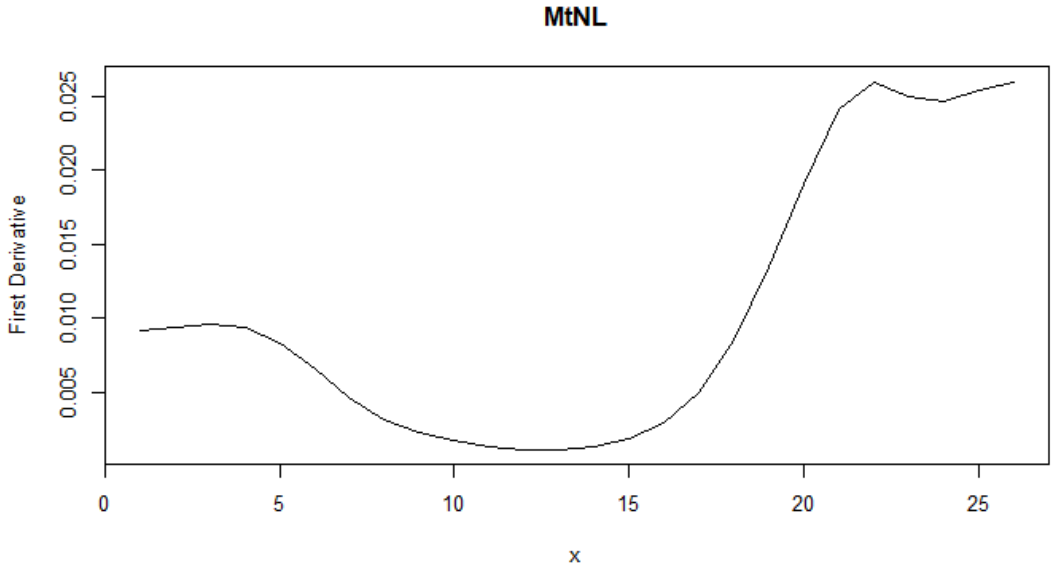

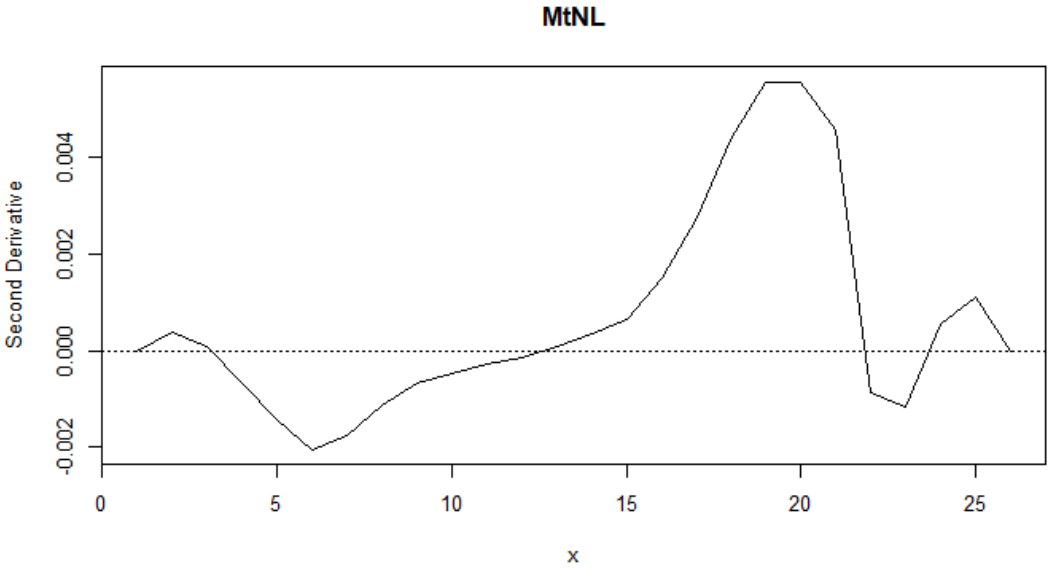

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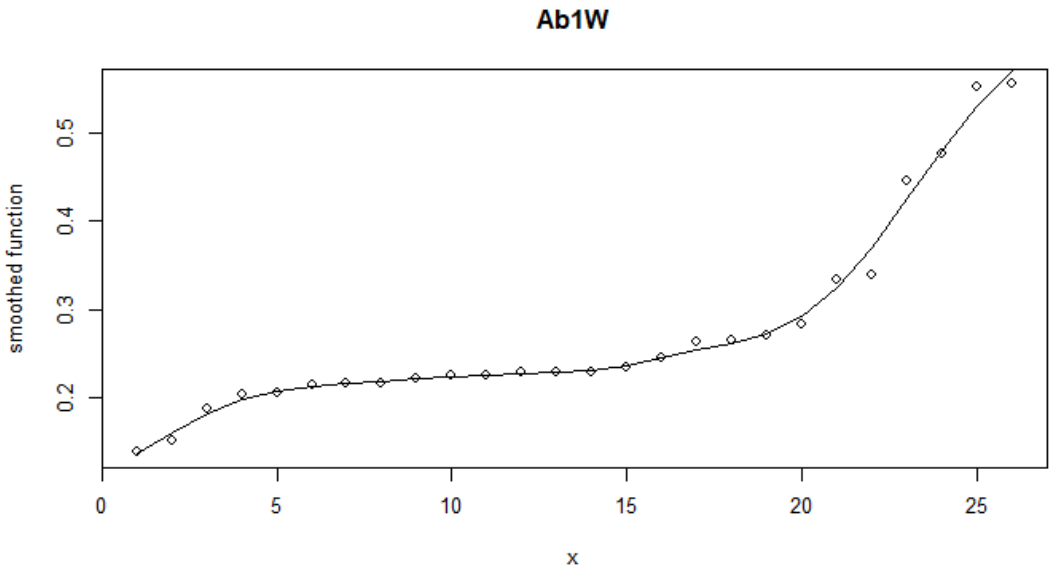

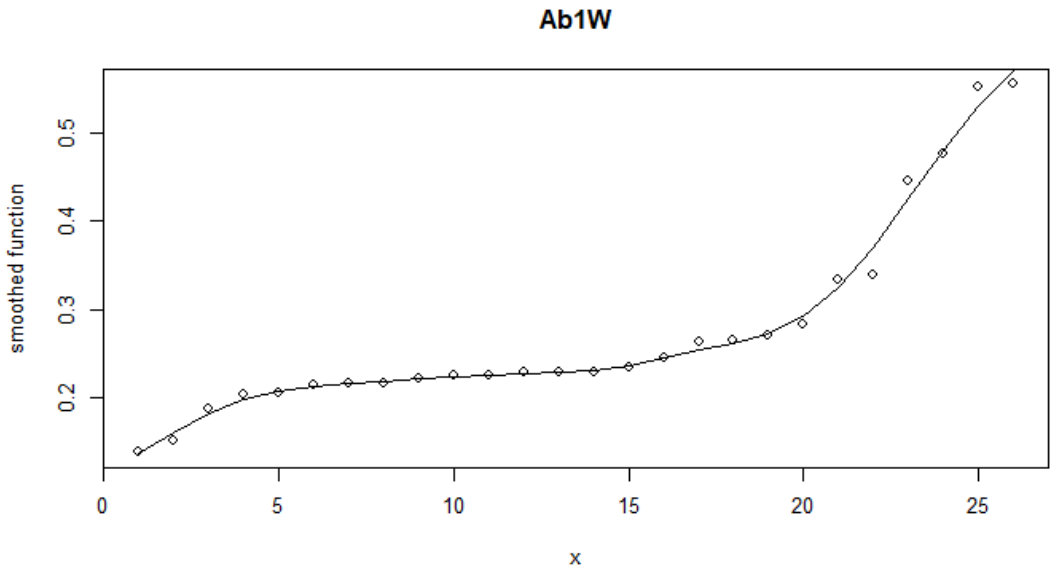

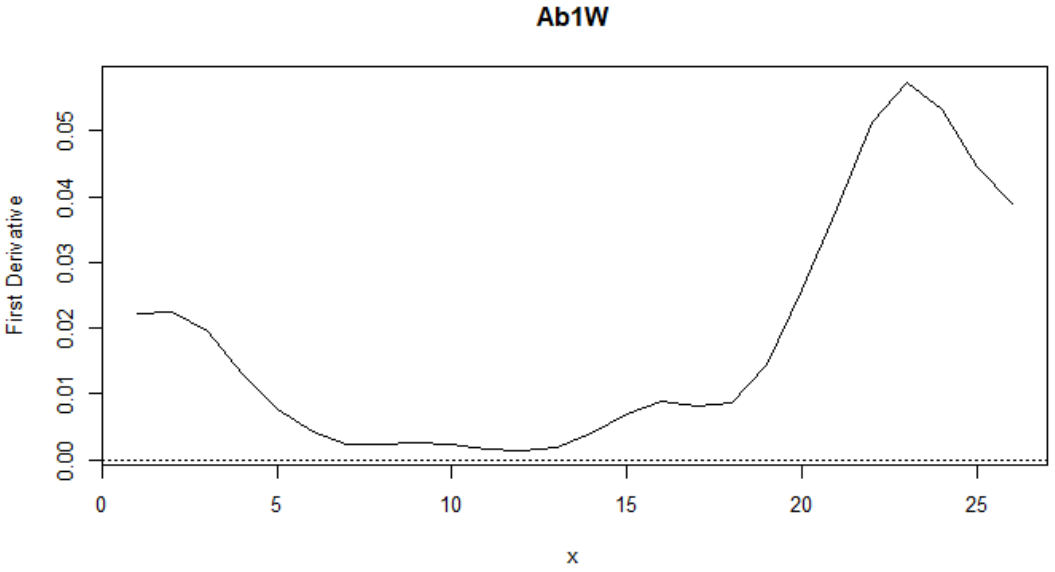

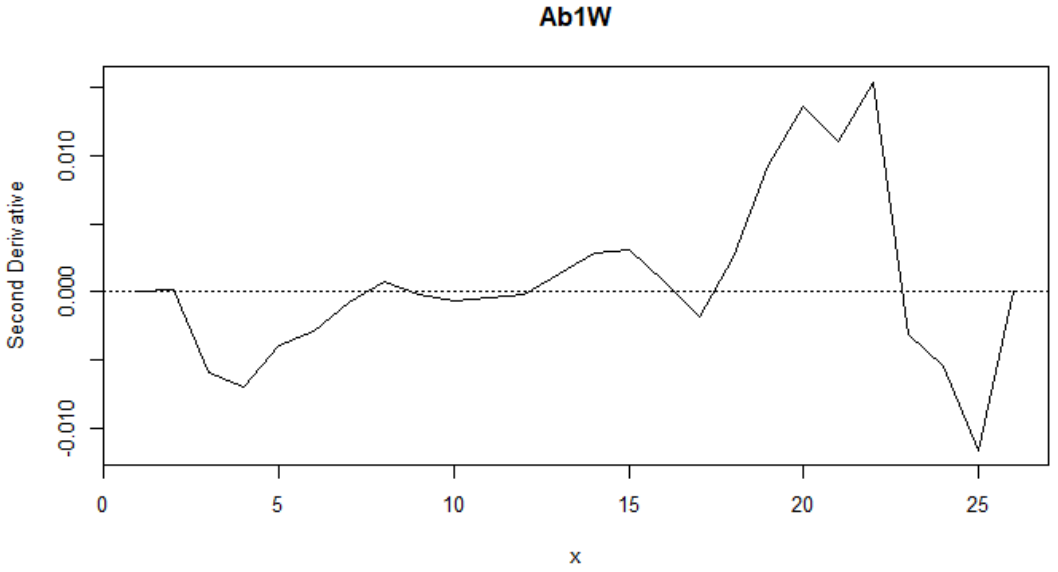

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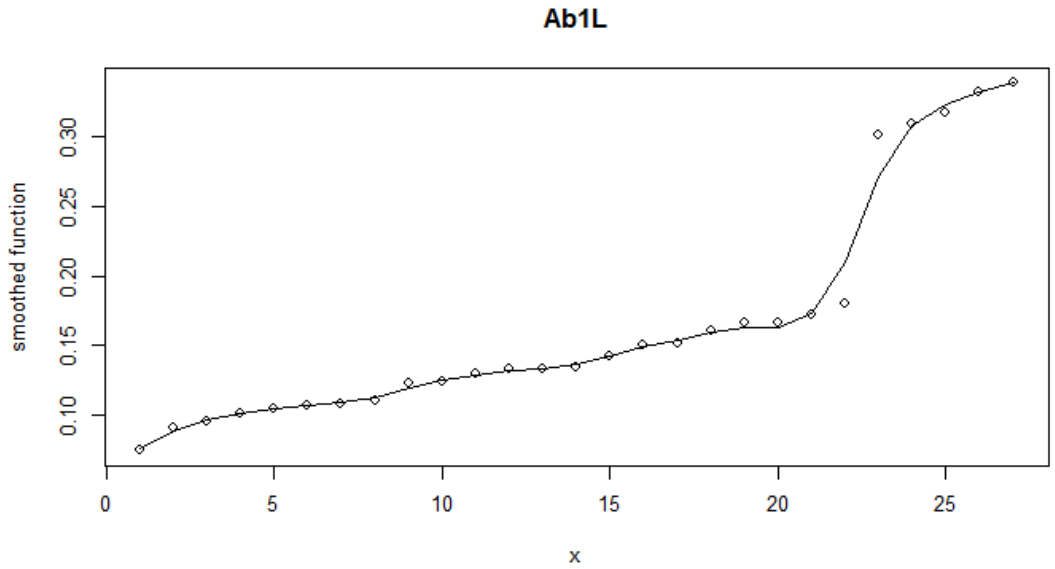

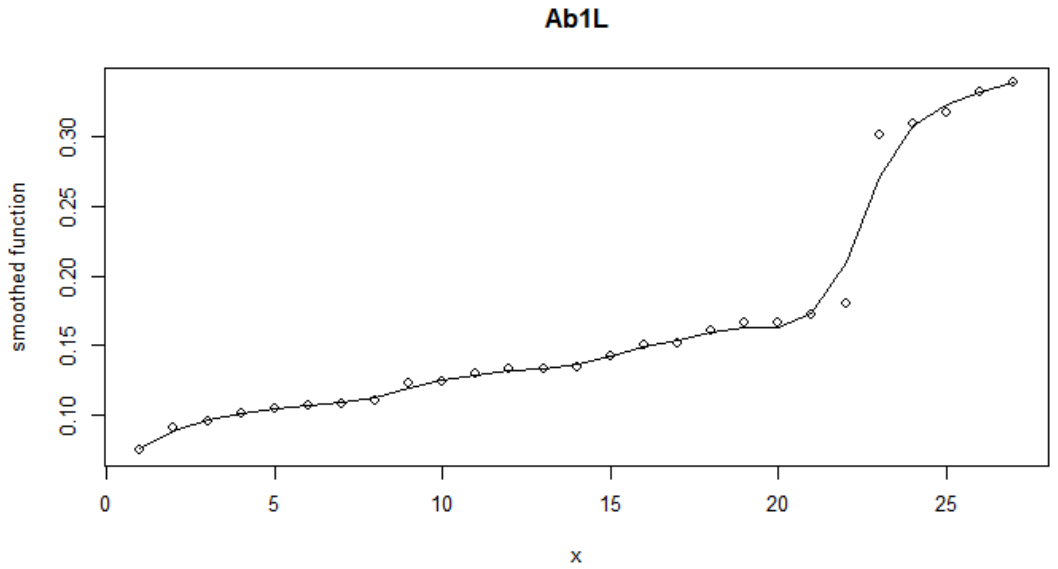

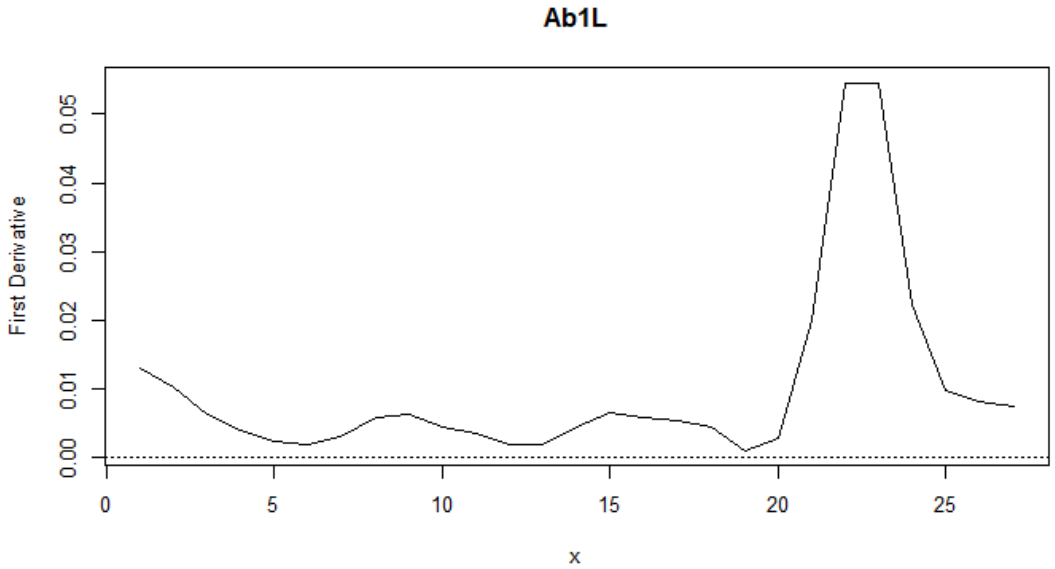

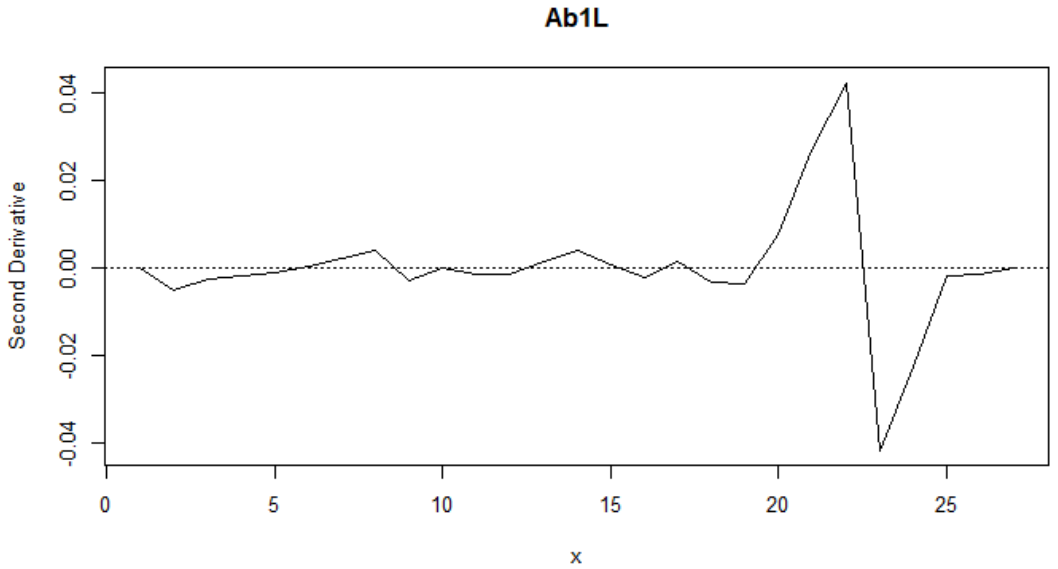

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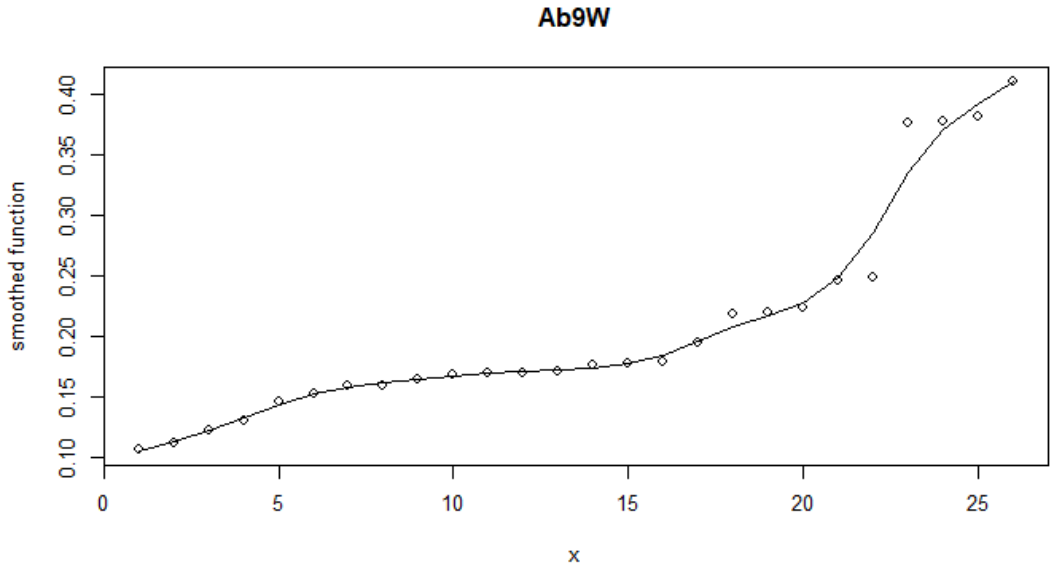

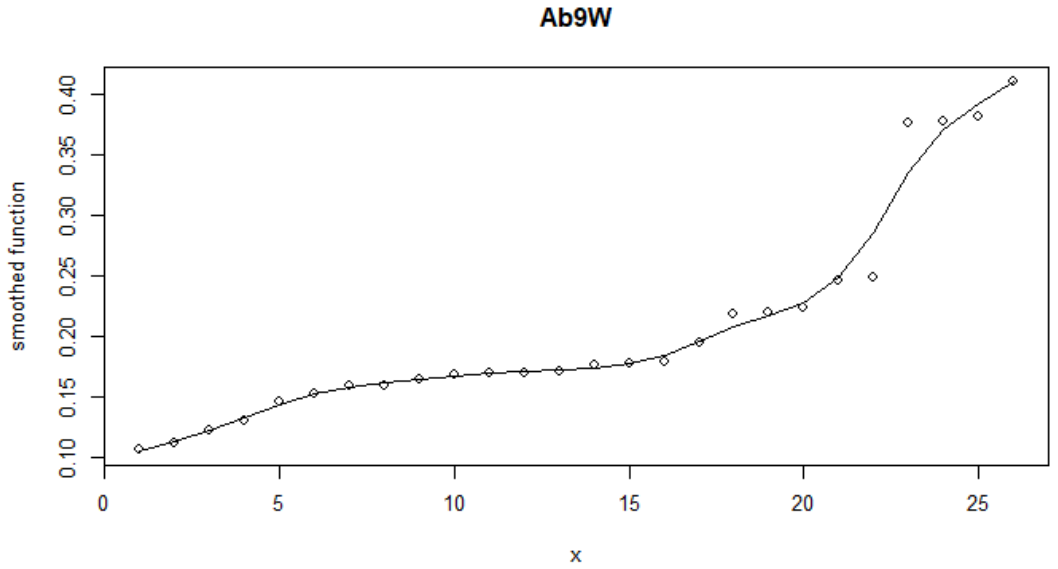

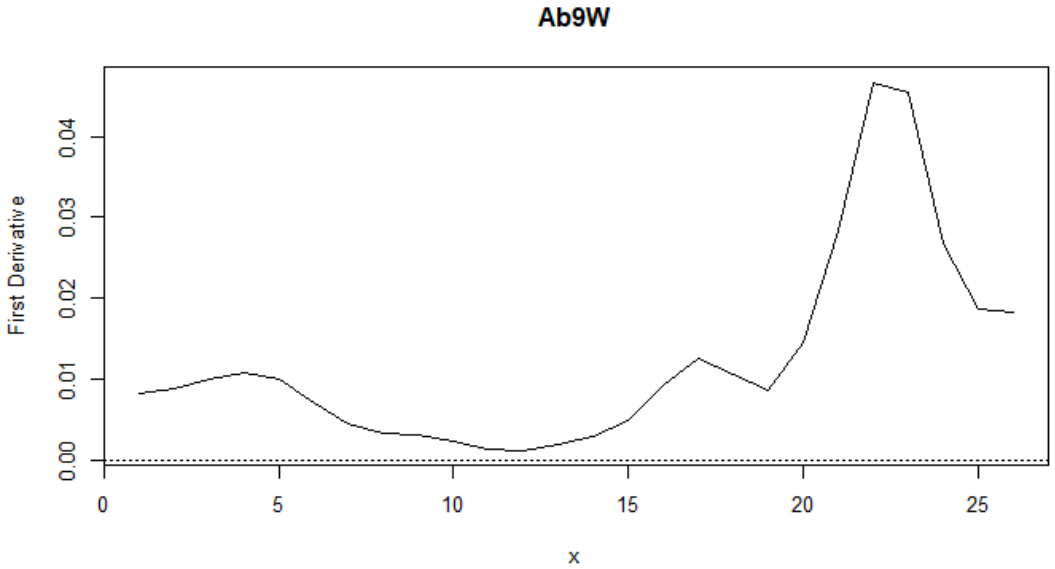

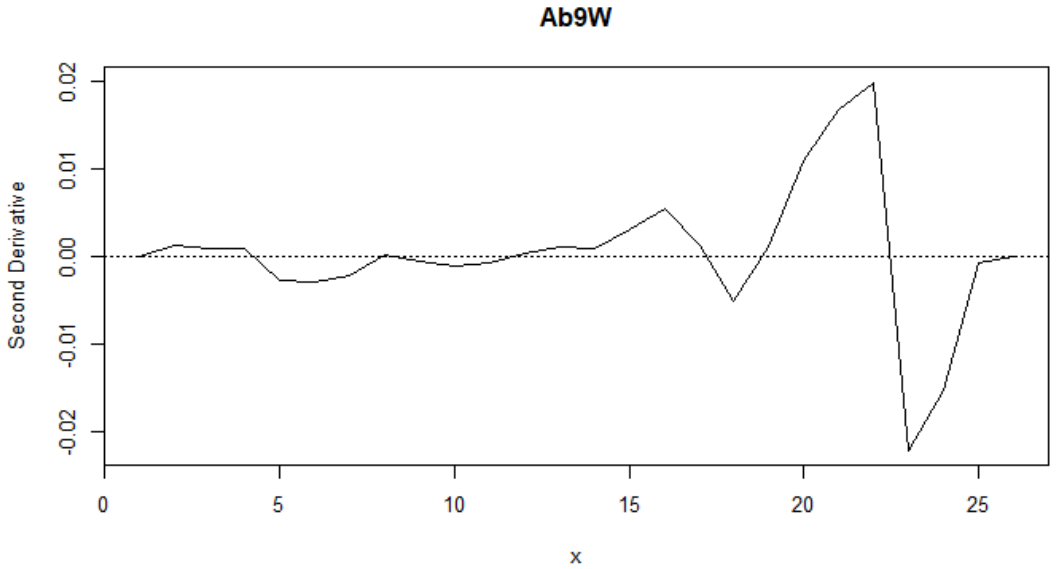

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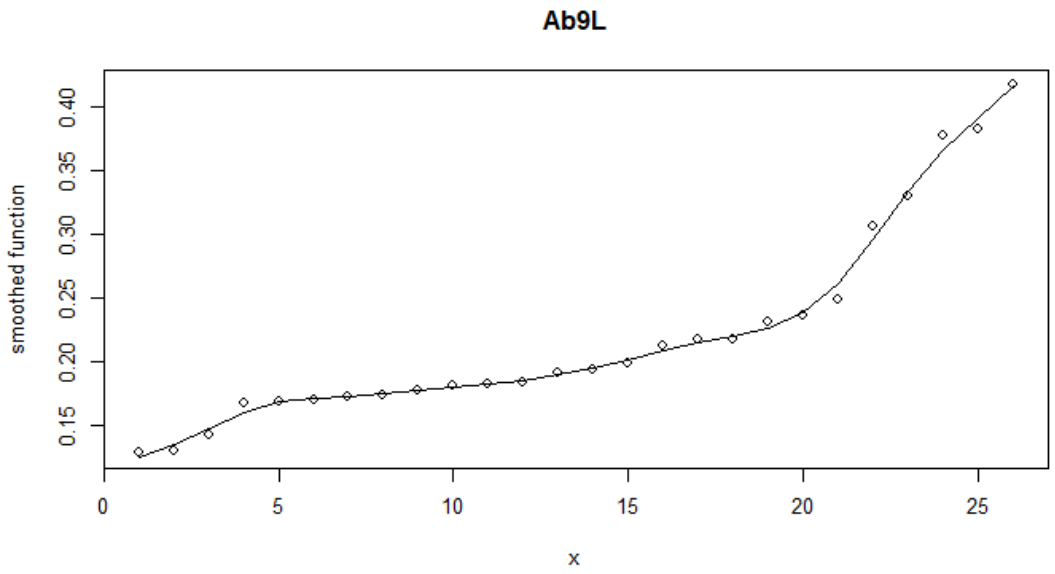

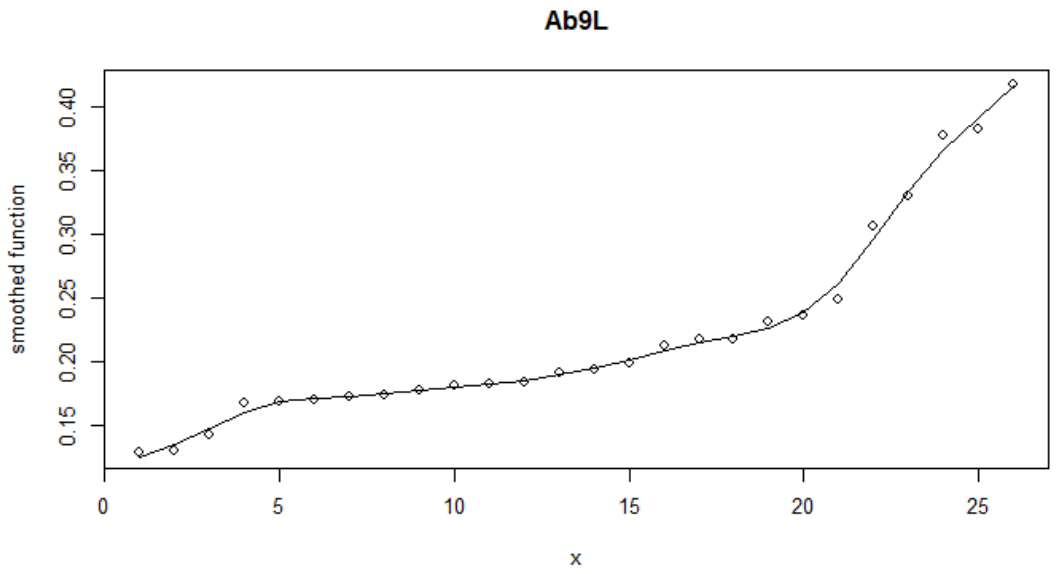

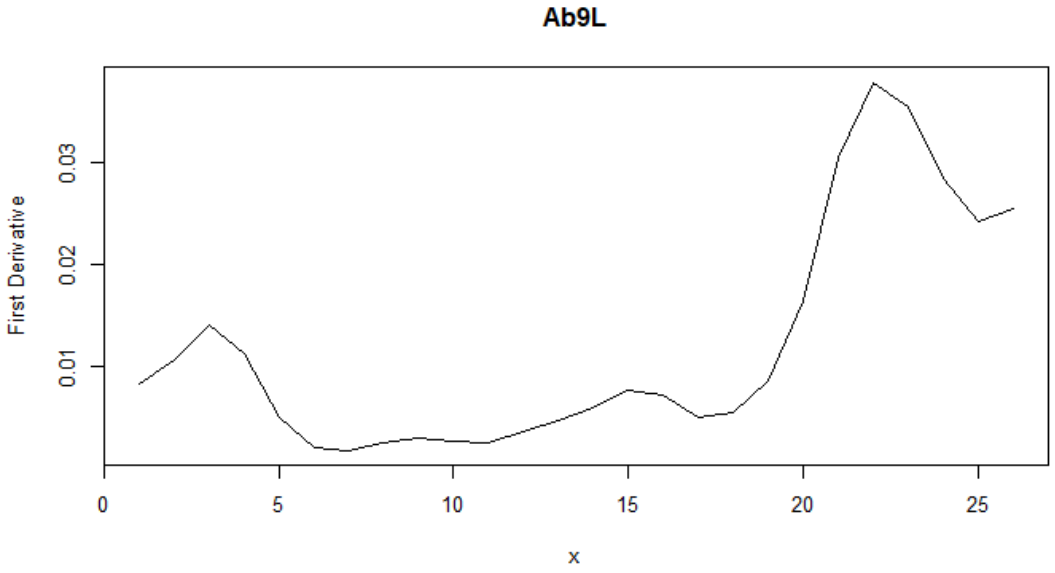

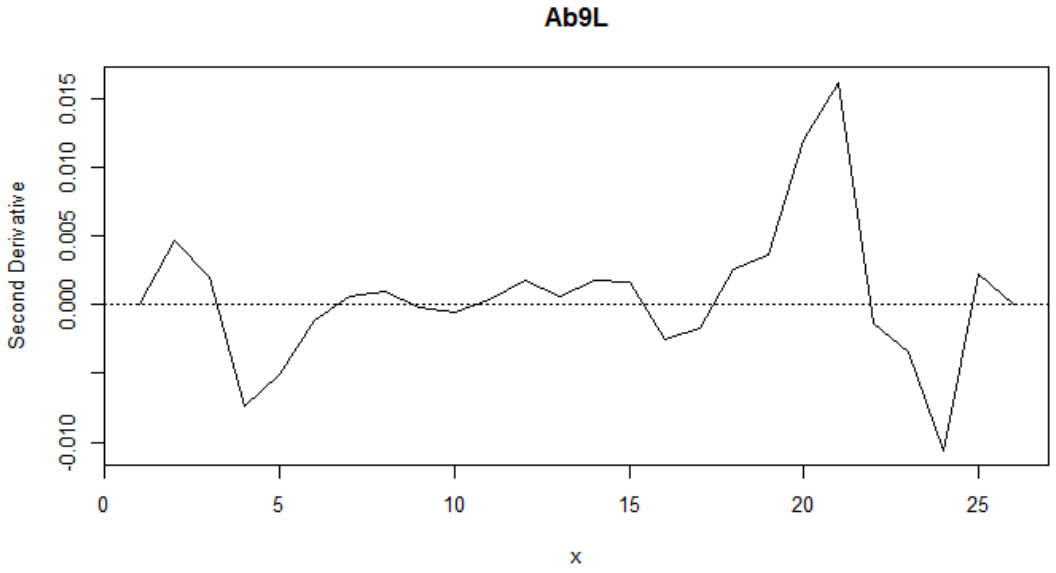

Eurycea Salamander Sedation via MS-222 (Tricaine methanesulfonate) Standard Operating Procedure

A. PURPOSE

The purpose of this standard operating procedure (SOP) is to describe the procedures used at San Marcos Aquatic Resources Center (SMARC) to sedate three *Eurycea* spp. Salamanders, the Comal, Texas blind, and San Marcos. Salamanders may need to be sedated for the purposes of performing diagnostic examinations, or, most used at SMARC, to mark individuals with Visible Implant Elastomer tags. Studies have not been conducted on the long term effects of repeated sedations in these species, so caution is exerted to not frivolously sedate organisms without purpose. However, no negative effects of sedation have been observed in the behavior or health of salamanders once they have been fully revived.

C. SAFETY PRECAUTIONS

- Refer to the Safety Data Sheets when using chemicals.
- Wear nitrile gloves while handling salamanders, especially those sedated via chemical methods.

D. EQUIPMENT AND MATERIAL REQUIRED

1. Approved chemical methods
    - Sodium pentobarbital (can only be administered by a veterinarian and is not discussed further in this SOP)
    - MS-222 (Tricaine methanesulfonate)
    - Sodium bicarbonate
    - Inhaled anesthetics (i.e. isoflurane) (only for air breathing amphibians)
2. Gram scale
3. Weigh boat
4. Metal spatula
5. Container for sedation water
6. Container for fresh recovery water
7. Gloves

E. PROCEDURES

1. **Sedation concentrations should be determined by the size and age class of the salamander. Guidelines are given here, but if you see negative effects, dosages should be adjusted—i.e., a salamander takes extra time to come out of or go into sedation.**

2. **For most hatchery use, MS-222 will be the easiest and most humane option. In aquatic *Eurycea* salamanders, it can be administered using a water bath immersion. This SOP will only discuss immersion. Consult the Fish Health Unit lead for specifics on other methods if needed.**
3. **For MS-222 water bath immersion:**
    - Determine the amount of MS-222 and bicarbonate needed based on the quantity of water being used. A dose of 0.4-0.5g/L (400-500 mg/L) has found to be effective for a water bath. This may vary by species. For reference a 2 g/L solution has been found to euthanize salamanders, so do not approach this level.
    - As a general rule of thumb, the ratio of MS-222 to bicarbonate should be 1:1. This can vary depending on water quality however.
        - Water containing MS-222 must be buffered with bicarbonate, as the MS-222 causes a significant drop in pH.
    - Using the gram scale and metal spatula, measure out the MS-222 and sodium bicarbonate into separate weigh boats. Then mix both into water swirling gently to fully dissolve.
        - Creating a mixture of these two to keep on hand is not recommended as the mixture becomes hydroscopic, reducing effectiveness. If you need to transport doses out to a field location, measure out the correct amount of each and keep in separate small packets. MS-222 is light sensitive so wrap those packets/vials with a light blocking barrier such as foil.
    - Add salamander to the MS-222 water mixture.
        - Animals may react (e.g., rapid swimming or splashing) to the mixture during the initial anesthetic phase but will eventually lose the ability to maintain proper orientation in the water column (i.e., assume a 'belly up' posture).
        - Monitor the salamander closely looking for signs of sedation—inability to right itself, decreased movement, no reaction to stimuli.
        - Once salamander is fully sedated (no reaction to stimuli, but heart still beating), it should be removed immediately from the sedation water bath.
        - Time to sedation varies by species and size of the salamander but generally happens within5 minutes.
        - Do not leave salamander too long in sedation bath as even a low dose could eventually cause euthanasia.
4. **Procedures**
    - Once the salamander is fully sedated, remove it from the water bath and perform the necessary procedure, such as sexing or tagging the salamander. Keep the skin moist by laying the salamander on a wet paper towel.
    - If salamander responds to stimuli immediately it can be placed back in sedation bath briefly.
5. **Revival**
    - Have a container of fresh water ready for salamander revival
        - If possible fresh flowing water in the container should be used.
    - Place salamander in fresh water and watch for signs of revival
        - Full revival takes 5-30 mins depending on the salamander, extent of sedation, extent of how long sedated etc.

    - You can also use a baster or pipette to gently push water over the gills to aid in recovery
    - If you are able, placing the animal on its feet rather than belly up may aid time to recovery.
    - If there are several salamanders that need to recover and you do not have flow-through water, change out recovery water frequently.
6. **Release**
    - We have not tested sedating, reviving, and releasing salamanders in the wild.
    - The FDA requires a 21-day withdrawal period after use of MS-222 for fish considered for human consumption
    - Make sure the salamander is fully recovered and has resumed normal behavior before releasing back into a tank.
    - Use best judgement and caution when releasing salamanders back into the wild. Some fish biologists use a rule of thumb of fish being fully recovered for 15-30 minutes before releasing back into the wild. Allow adequate time for recovery in field activity planning.

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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/890

Memorandum: April 4, 2018

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 18-15)

On November 28, 2017, 6 Fountain darters (*Etheostoma fonticula*) were submitted live from San Marcos ARC to the SNARRC. Another 3 Fountain darters were sampled in formalin by San Marcos and submitted directly to the Washington Animal Disease Diagnostic Laboratory for histopathological evaluation. The fish were originally collected from the wild from the Comal river on November 1 and 2 and moved into quarantine. They are on unfiltered flow through well water, with siphoning of tanks occurring at a minimum, weekly. Two weeks after being placed in quarantine, mortality numbers increased and affected fish appeared to have swollen gills with rapid operculations. Staff report that fish appear to be eating well otherwise.

Fish were euthanized with MS-222 and examined. Skin scrapes were negative for pathogens. Gill clips showed *Centrocestus* metacercariae which is a common finding. However, the numbers noted appeared unusually high in several fish. Internal examination was unremarkable. Whole body samples were collected for virology and kidneys were sampled for bacteriology. Additional squash preps of the intestines were performed and were negative for flagellates although occasional nematodes were noted in 2/5 fish examined.

Results:

Virology indicated that this group of fish was positive for Large Mouth Bass virus. Bacteriology cultures taken from kidneys onto BHIA media were negative for any bacterial isolation. Histopathology showed a severe diffuse meningitis and choroiditis in one fish. Granulomatous multifocal oophoritis with intralesional microsporidia was also noted. Another fish had an ulcerative dermatitis and myositis whereas the third fish only had mild splenic hyperplasia.

Final Diagnosis: LMBV. Microsporidia.

The finding of LMBV is not surprising given the history of these fish. These fish appear to harbor this virus subclinically. The histopathology results were striking but ultimately inconclusive. None of the fish had similar pathology, meaning no conclusive cause of illness was identified, although the finding of microsporidia may be a new finding in this species.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-15 for any follow-up correspondence.

cc: Ken Ostrand, San Marcos ARC

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/898

Memorandum: May 11, 2018

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 18-16)

On December 5, 2017, 51 Fountain darters (*Etheostoma fonticula*) were submitted in formalin directly from San Marcos ARC to the Washington Animal Disease Diagnostic Lab for histopathology. The fish were originally collected from the wild from the Comal River on November 1 and 2 and moved into quarantine. They were on unfiltered flow through well water, with siphoning of tanks occurring at a minimum, weekly. Two weeks after being placed in quarantine, mortality numbers increased and affected fish appeared to have swollen gills with rapid operculations. Staff reported that fish appeared to be eating well otherwise. Previous submissions for histopathology revealed non-specific findings so it was recommended to submit a larger number of fish for evaluation. Seven of the submitted fish had been euthanized and the remainder had been found dead in the tank and placed in formalin.

Results:

Histopathology was evaluated on a randomly selected sampling of ten of the submitted fish. Findings were mixed, with different fish showing different signs of illness including otitis interna with Myxobolus-type myxozoan spores, dermatitis and cellulitis, nephrocalcinosis, oophoritis, and several internal parasites including acanthocephalans, nematodes and trematodes.

Final Diagnosis: Undetermined

There were no common consistent findings among the fish evaluated. The observation of otitis interna in association with a myxozoan parasite in 1 fish provides a possible differential for some previously submitted fountain darters with unexplained histiocytic inflammation in the tissues of their head, but this is speculative. The other findings were likely not clinical significant and were found in only one or two fish and not representative of the population as a whole.

The lack of any consistent pathological findings makes other factors such as improper husbandry more likely. It is recommended that close attention be paid to any future collections of these fish and proper handling, transport, and quarantine of these fish be performed.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-16 for any follow-up correspondence.

cc: Ken Ostrand, San Marcos ARC
Dave Britton, San Marcos ARC

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/896

Memorandum: May 1, 2018

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 (SNARRC Case Number 18-17)

On December 6, 2017, one Texas blind salamander (*Eurycea rathbuni*) was submitted directly from San Marcos ARC to the Washington Animal Disease Diagnostic Laboratory for histopathological evaluation. This salamander was part of a wild collection made on December 1, 2017. At the time of collection, this individual had redness apparent on the rear limbs, discoloration around the abdomen, and was lethargic. It was placed in a tank by itself but was found dead the next morning. It was placed whole into formalin for histopathological evaluation.

Results:

Histopathology indicated a lack of adipose tissue with hepatocellular atrophy. Mild autolytic changes were seen throughout which may have masked subtle lesions.

Final Diagnosis: Undetermined

The cause of the discoloration noted grossly was not evident via histopathology. The salamander was in poor nutritional condition based on a lack of body fat stores and hepatocellular atrophy. This poor condition was likely a factor in the death but an underlying disease process was not identified.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-17 for any follow-up correspondence.

cc: Ken Ostrand, San Marcos ARC
Dave Britton, San Marcos ARC

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

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/886

Memorandum: March 16, 2018

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 18-22)

On February 2, 2018, SNARRC staff obtained 59 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. Eleven samples came from Salado Springs salamanders (*Eurycea chisholmensis*), ten samples came from San Marcos salamanders (*Eurycea nana*), ten samples came from Texas blind salamanders (*Eurycea rathbuni*), and 28 samples came from Comal springs salamanders (*Eurycea* sp). 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. Out of the 59 samples submitted, 33 (56%) were positive 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 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 can result in many of the previously negative animals to become positive. 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 18-22 for any follow-up correspondence.

Vial #	Date Swabbed	Species	Collection Location	Destination	Bd/Bsal results
373	10/4/2017	EN	Diversion	SMARC	+
374	10/6/2017	EN	Diversion	SMARC	+
375	10/31/2017	ER	Diversion	SMARC	+
376	11/7/2017	ESPP	COMAL	SMARC	+
377	11/7/2017	ESPP	COMAL	SMARC	-
378	11/7/2017	ESPP	COMAL	SMARC	+
379	11/7/2017	ESPP	COMAL	SMARC	-
380	11/7/2017	ESPP	COMAL	SMARC	+
381	11/7/2017	ESPP	COMAL	SMARC	+
382	11/7/2017	ESPP	COMAL	SMARC	-
383	11/7/2017	ESPP	COMAL	SMARC	-
384	11/7/2017	ESPP	COMAL	SMARC	-
385	11/7/2017	ESPP	COMAL	SMARC	+
386	11/7/2017	ESPP	COMAL	SMARC	-
387	11/7/2017	ESPP	COMAL	SMARC	+
388	11/7/2017	ESPP	COMAL	SMARC	-
389	11/7/2017	ESPP	COMAL	SMARC	+
390	11/7/2017	ESPP	COMAL	SMARC	-
391	11/7/2017	ESPP	COMAL	SMARC	-
392	11/7/2017	ESPP	COMAL	SMARC	-
393	11/7/2017	ESPP	COMAL	SMARC	+
394	11/7/2017	ESPP	COMAL	SMARC	+
395	11/7/2017	ESPP	COMAL	SMARC	-
396	11/7/2017	ESPP	COMAL	SMARC	+
397	11/7/2017	ESPP	COMAL	SMARC	+
398	11/7/2017	ESPP	COMAL	SMARC	+
399	11/7/2017	ESPP	COMAL	SMARC	+
400	11/7/2017	ESPP	COMAL	SMARC	+
401	11/7/2017	ESPP	COMAL	SMARC	+
402	11/9/2017	ER	Johnsons Well	SMARC	-
403	11/9/2017	EN	Diversion	SMARC	-
404	11/13/2017	ER	Primers Fissure	SMARC	+
405	11/13/2017	EN	Diversion	SMARC	+
406	11/15/2017	EN	Diversion	SMARC	+
407	11/17/2017	ER	Johnsons Well	SMARC	+
408	12/1/2017	ER	Diversion	SMARC	+
409	12/14/2017	EN	Diversion	SMARC	+
410	12/21/2017	EN	Diversion	SMARC	+
411	12/27/2017	EC	SALADO	SMARC	-
412	12/27/2017	EC	SALADO	SMARC	-
413	12/27/2017	EC	SALADO	SMARC	-
414	12/27/2017	EC	SALADO	SMARC	+
415	12/27/2017	EC	SALADO	SMARC	-
416	12/27/2017	EC	SALADO	SMARC	-

417	12/27/2017	EC	SALADO	N	SMARC	+
418	12/27/2017	EC	SALADO	N	SMARC	+
419	12/27/2017	EC	SALADO	N	SMARC	+
420	12/27/2017	EC	SALADO	N	SMARC	-
421	12/27/2017	EC	SALADO	N	SMARC	-
422	1/2/2018	EN	Diversion	N	SMARC	+
423	1/8/2018	EN	Diversion	N	SMARC	+
424	1/18/2018	EN	Diversion	Y	SMARC	+
425	1/19/2018	ER	Rattlesnake Cave	N	SMARC	+
426	1/23/2018	ER	Diversion	N	SMARC	-
427	1/23/2018	ER	Diversion	N	SMARC	-
428	1/23/2018	ER	Diversion	N	SMARC	-
429	1/23/2018	ER	Diversion	N	SMARC	-
430	1/23/2018	ESPP	Spring Island / SR3	N	SMARC	-
431	1/23/2018	ESPP	Spring Island / SR3	N	SMARC	-

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/888

Memorandum: March 19, 2018

To: Kenneth Ostrand, Project Leader, San Marcos Aquatic Resource Center
From: Ashlie Peterson, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final report for the fountain darters from the San Marcos River, TX (SNARRC Case Number 18-32).

On February 27, 2018, the Southwestern Fish Health Unit (SFHU) received 10 fountain darters (*Etheostoma fonticola*) from the San Marcos, TX. The receipt for donation stated that 10 fish were submitted 'San Marcos' fish. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU. Location of fish collection was recorded at latitude 29.87544497 N longitude -97.93192497 W in Hays County, Texas. The fish were examined for *Centrocestus formosanus* parasite enumeration. The final numbers are reported on the following page.

Screening for *Centrocestus formosanus* was conducted by examining the left gill arches of all 10 fish. *Centrocestus formosanus* was observed infecting these fish.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 18-32 for any follow-up correspondence.

cc: Linda Moon, San Marcos Aquatic Resource Center

FOD Parasite Data Sheet - Form P-03

Case History No. 18-32

Date examined: 2/27/18 Date Collected: 2/26/18

Collection site: 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)	501	541	256	165	526	183	223	168	352	169
Total Length (mm)	32	35	28	28	35	28	28	28	33	29

Centrocestus formosanus **cysts** **Number of cysts per arch (ie 3,2,1,1)**

Mature (left gills only)	L	6 5 2 0	0 0 0 0	1 0 0 0	0 0 0 0	0 0 0 0	0 0 1 2	0 0 0 0	0 1 0 0	0 0 0 0	0 0 0 0
Immature (left gills only)	L	0 1 0 0	0 1 1 0	0 1 0 0	1 2 3 5	0 0 0 0	0 0 0 1	1 0 0 0	0 0 0 0	1 1 4 0	0 0 0 0
Monogenea	L	0 0 0 0	0 0 0 0	0 0 0 0	0 2 1 1	1 0 0 0	0 0 0 0	0 0 0 1	0 0 0 0	0 0 1 0	0 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										

Examiner signature MR MB

Revised on 9/20/2017

FOD Parasite Data Sheet - Form P-03

Case History No. 18-33

Date examined: 2/27/18 Date Collected: 2/26/17

Collection site: 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)	127	426	293	334	151	450	429	423	203	163
Total Length (mm)	25	36	31	32	27	37	31	38	30	27

Centrocestus formosanus **cysts** **Number of cysts per arch (ie 3,2,1,1)**

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 1 0	2 3 7 2	1 4 2 4	0 3 6 2	1 0 0 0	7 11 2 5	8 7 6 4	1 7 5 1	2 3 0 1	1 2 1 2
Monogenea	L	0 0 0 0	0 1 0 0	0 0 0 1	0 0 0 0	0 1 0 0	0 0 1 0	0 0 0 0	0 0 1 0	0 0 0 0	0 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	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

Examiner signature MR

Revised on 9/20/2017

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/887

Memorandum: March 19, 2018

To: Kenneth Ostrand, Project Leader, San Marcos Aquatic Resource Center
From: Ashlie Peterson, Southwestern Fish Health Unit/Southwestern Native Aquatic Resources and Recovery Center

Subject: Final report for the fountain darters from the Comal River, TX (SNARRC Case Number 18-33).

On February 27, 2018, the Southwestern Fish Health Unit (SFHU) received 10 fountain darters (*Etheostoma fonticola*) from the Comal River, TX. The receipt for donation stated that 10 fish were submitted 'Comal' fish. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU. Location of fish collection was recorded at latitude 29.71068601 N longitude -98.12762003W in Hays County, Texas. The fish were examined for *Centrocestus formosanus* parasite enumeration. The final numbers are reported on the following page.

Screening for *Centrocestus formosanus* was conducted by examining the left gill arches of all 10 fish. *Centrocestus formosanus* was observed infecting these fish.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 18-33 for any follow-up correspondence.

cc: Linda Moon, San Marcos Aquatic Resource Center

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/897

Memorandum: May 2, 2018

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

Subject: Final Report for the San Marcos salamander (SNARRC Case Number 18-87)

On April 13, 2018, staff from Uvalde NFH euthanized a San Marcos salamander and fixed it in formalin. The salamander had been identified as thin and posturally on its back on April 5, 2018. Over time, it continued to worsen and no longer fled when disturbed. Due to the poor prognosis, it was euthanized. Records indicate that on 4/11/18, the temperature of the salamander tank was 20.5 C with a DO of 82.6%. The salamander was submitted to the Washington Animal Disease Diagnostic Lab for histopathology.

Results:

The most significant findings were extensive areas of hypereosinophilic and fragmented (indicating necrosis) skeletal myocytes with variable infiltrates of macrophages. In approximately 10% of the necrotic myocytes there were 3-4 micron ovoid to pyriform spores consistent with microsporidia. Similar spores were occasionally observed in otherwise normal myocytes. Microsporidia were also rarely noted in the pancreas and in a large ganglion adjacent to the brainstem.

Final Diagnosis: Microsporidial myositis

These histological findings are similar to those noted in a previous submission (SNARRC Case #18-82). The microsporidia also appears similar in appearance to those noted in the ovaries of affected San Marcos salamanders from the San Marcos ARC. Electron microscopy to better characterize the pathogen is underway. In attempts to better identify the species, any future histological submission should be in ethanol rather than formalin, which can preserve the DNA better. As this organism may be commonplace in this species, improvements in overall husbandry may be the best way to prevent the possible immunosuppression that may allow this organism to become widespread in the body.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-87 for any follow-up correspondence.

cc: Patricia Duncan, Uvalde NFH
Dave Britton, San Marcos ARC
Ken Ostrand, San Marcos ARC
Lindsay Campbell, San Marcos ARC

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/899

Memorandum: May 11, 2018

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

Subject: Final Report for the San Marcos salamander (SNARRC Case Number 18-88)

On May 1, 2018, staff from Uvalde NFH euthanized a San Marcos salamander and fixed it in formalin. The salamander had been identified as having an enlarged abdomen. Over time, it continued to worsen despite 2.5% salt treatments. Due to the poor prognosis, it was euthanized. Records indicate that on 4/25/18, the temperature of the salamander tank was 21.3 C with a DO of 110.7% and a TAN of 0.01. The salamander was submitted to the Washington Animal Disease Diagnostic Lab for histopathology.

Results:

Within approximately 10% of necrotic myocytes there are 3-4 micron in diameter refractile ovoid to pyriform spores that are strongly Fite's acid fast stain positive (microsporidia). Microsporidia is further note din the ovary. Overall, there is moderate lipid-depletion (atrophy) of adipocytes. Focally on the skin of one foot, there is mild orthokeratotic hyperkeratosis and keratinocytes have a few intracytoplasmic chytrid fungal thalli.

Final Diagnosis: Microsporidial myositis and oophoritis; Orthokeratotic hyperkeratosis in association with *Batrachochytrium dendrobatidis;* Coeolomic effusion

These histological findings are similar to those noted in previous submissions. Electron microscopy to better characterize the pathogen is underway. Infections with chytrid fungi have been observed in this species previously and appear to be incidental findings. The cause of the severe coelomic effusion noted was not clearly evident from the histological findings. The severe debilitation noted may have led to difficulty in maintaining osmotic balance or the declining nutritional condition may have led to hypoproteinemia and effusion. There was no evidence of mycobacteria as has been seen in salamanders from the San Marcos facility.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-88 for any follow-up correspondence.

cc: Patricia Duncan, Uvalde NFH
Dave Britton, San Marcos ARC
Ken Ostrand, San Marcos ARC
Lindsay Campbell, San Marcos ARC

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/902

Memorandum: June 13, 2018

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 18-90)

On May 2, 2018, SNARRC staff obtained 202 salamander skin swabs from 101 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 ten Texas blind salamanders (*Eurycea rathbuni*), and 91 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. All Texas blind salamanders were tested individually. The San Marcos salamander samples were tested in a 5-swab pooled sample except for a few that were accidentally tested individually.

Results:
All salamanders were negative for Bsal. One out of ten (10%) Texas blind salamanders was positive for Bd. 17/18 of the pooled samples (94.4%) of the San Marcos salamanders were positive for Bd. Individual and pooled 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 may likely result in many of the previously negative animals to become positive.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 18-90 for any follow-up correspondence.

cc: Ken Ostrand, San Marcos ARC
Dave Britton, San Marcos ARC

Salamander Swab Data Sheet

White and brown groupings indicate pooled samples. Blue highlighted samples were tested individually.

Vial #	Date	Collection Location	Species	TL (mm)	Lesions? (Y/N)	Bd	Bsal
432	2/7/2018	Johnsons Well	ER		N	-	-
433	2/7/2018	Johnsons Well	ER		N	-	-
434	3/8/2018	TXSTU Artesian	ER	75	N	+	-
435	3/20/2018	Spring Lake	EN	46	N	+	-
436	3/20/2018	Spring Lake	EN	56	N	+	-
437	3/20/2018	Spring Lake	EN	56	N	+	-
438	3/20/2018	Spring Lake	EN	54	N	+	-
439	3/20/2018	Spring Lake	EN	42	N	+	-
440	3/20/2018	Spring Lake	EN	57	N	+	-
441	3/20/2018	Spring Lake	EN	33	N	+	-
442	3/20/2018	Spring Lake	EN	40	N	+	-
443	3/20/2018	Spring Lake	EN	52	N	+	-
444	3/20/2018	Spring Lake	EN	33	N	+	-
445	3/20/2018	Spring Lake	EN	48	N	+	-
446	3/20/2018	Spring Lake	EN	34	N	+	-
447	3/20/2018	Spring Lake	EN	41	N	+	-
448	3/20/2018	Spring Lake	EN	58	N	+	-
449	3/20/2018	Spring Lake	EN	55	N	+	-
450	3/20/2018	Spring Lake	EN	60	N	+	-
451	3/20/2018	Spring Lake	EN	55	N	+	-
452	3/20/2018	Spring Lake	EN	56	N	+	-
453	3/20/2018	Spring Lake	EN	44	N	+	-
454	3/20/2018	Spring Lake	EN	45	N	+	-
455	3/20/2018	Spring Lake	EN	56	N	+	-
456	3/20/2018	Spring Lake	EN	50	N	+	-
457	3/20/2018	Spring Lake	EN	34	N	+	-
458	3/20/2018	Spring Lake	EN	60	N	+	-
459	3/20/2018	Spring Lake	EN	61	N	+	-
460	3/20/2018	Spring Lake	EN	39	N	+	-
461	3/20/2018	Spring Lake	EN	51	N	+	-
462	3/20/2018	Spring Lake	EN	37	N	+	-
463	3/20/2018	Spring Lake	EN	46	N	+	-
464	3/20/2018	Spring Lake	EN	48	N	+	-
465	3/20/2018	Spring Lake	EN	47	N	+	-
466	3/20/2018	Spring Lake	EN	51	N	+	-
467	3/20/2018	Spring Lake	EN	44	N	+	-
468	3/20/2018	Spring Lake	EN	46	N	+	-
469	3/20/2018	Spring Lake	EN	54	N	+	-
470	3/20/2018	Spring Lake	EN	50	N	+	-
471	3/20/2018	Spring Lake	EN	54	N	+	-
472	3/20/2018	Spring Lake	EN	59	N	+	-
473	3/20/2018	Spring Lake	EN	61	N	+	-
474	3/20/2018	Spring Lake	EN	54	N	+	-
475	3/20/2018	Spring Lake	EN	34	N	+	-
476	3/20/2018	Spring Lake	EN	55	N	+	-
477	3/20/2018	Spring Lake	EN	40	N	+	-
478	3/20/2018	Spring Lake	EN	51	N	+	-

479	3/20/2018	Spring Lake	EN	52	N	+	-
480	3/20/2018	Spring Lake	EN	50	N	+	-
481	3/20/2018	Spring Lake	EN	49	N	+	-
482	3/20/2018	Spring Lake	EN	29	N	+	-
483	3/20/2018	Spring Lake	EN	30	N	+	-
484	3/20/2018	Spring Lake	EN	43	N	+	-
485	3/20/2018	Spring Lake	EN	33	N	+	-
486	3/20/2018	Spring Lake	EN	60	N	+	-
487	3/20/2018	Spring Lake	EN	57	N	+	-
488	3/20/2018	Spring Lake	EN	40	N	+	-
489	3/20/2018	Spring Lake	EN	36	N	+	-
490	3/20/2018	Spring Lake	EN	55	N	+	-
491	3/20/2018	Spring Lake	EN	57	N	+	-
492	3/20/2018	Spring Lake	EN	58	N	+	-
493	3/20/2018	Spring Lake	EN	44	N	+	-
494	3/20/2018	Spring Lake	EN	43	N	+	-
495	3/20/2018	Spring Lake	EN	38	N	+	-
496	3/20/2018	Spring Lake	EN	44	N	+	-
497	3/20/2018	Spring Lake	EN	39	N	+	-
498	3/20/2018	Spring Lake	EN	50	N	+	-
499	3/20/2018	Spring Lake	EN	54	N	+	-
500	3/20/2018	Spring Lake	EN	56	N	+	-
501	3/20/2018	Spring Lake	EN	55	N	+	-
502	3/20/2018	Spring Lake	EN	58	N	+	-
503	3/20/2018	Spring Lake	EN	50	N	+	-
504	3/20/2018	Spring Lake	EN	40	N	+	-
505	3/20/2018	Spring Lake	EN	45	N	+	-
506	3/20/2018	Spring Lake	EN	47	N	+	-
507	3/20/2018	Spring Lake	EN	58	N	+	-
508	3/20/2018	Spring Lake	EN	53	N	+	-
509	3/20/2018	Spring Lake	EN	45	N	+	-
510	3/20/2018	Spring Lake	EN	46	N	+	-
511	3/20/2018	Spring Lake	EN	48	N	+	-
512	3/20/2018	Spring Lake	EN	57	N	+	-
513	3/20/2018	Spring Lake	EN	50	N	+	-
514	3/20/2018	Spring Lake	EN	53	N	+	-
515	3/20/2018	Spring Lake	EN	45	N	+	-
516	3/30/2018	Diversion	EN	50	N	+	-
517	4/11/2018	Rattlesnake Cave	ER	70	N	-	-
518	4/11/2018	Rattlesnake Cave	ER	74	N	-	-
519	4/11/2018	Rattlesnake Cave	ER	78	N	-	-
520	4/11/2018	Diversion	EN	46	N	-	-
521	4/11/2018	Diversion	EN	48	N	-	-
522	4/11/2018	Diversion	EN	44	N	-	-
523	4/11/2018	Diversion	EN	31	N	-	-
524	4/11/2018	Diversion	EN	29	N	-	-
525	4/11/2018	Diversion	EN	30	N	-	-
526	4/11/2018	Diversion	EN	26	N	-	-
527	4/11/2018	Diversion	EN	25	N	-	-
528	4/11/2018	Diversion	ER	39	N	-	-
529	4/11/2018	Rattkesnake Well	ER	34	N	-	-
530	4/11/2018	Diversion	EN	20	N	-	-

with 531 and 532

531	4/11/2018	Rattlesnake Cave	ER	68	N	-	-	with 518 and 519
532	4/11/2018	Rattlesnake Well	ER	67	N	-	-	

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Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/900

Memorandum: May 15, 2018

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

Subject: Final Report for the San Marcos salamander (SNARRC Case Number 18-91)

On May 8, 2018, staff from Uvalde NFH euthanized a San Marcos salamander and fixed it in formalin. The salamander had been identified as having abnormal behavior. The animal was placed in an isolation tank. Over time, it continued to worsen despite 2.5% salt treatments. Due to the poor prognosis, it was euthanized. Records indicate that on 5/2/18, the temperature of the salamander tank was 22.2 C with a DO of 100.9%. The salamander was submitted to the Washington Animal Disease Diagnostic Lab for histopathology.

Results:

Within approximately 20% of necrotic myocytes there are intrasarcoplasmic sporophorous vesicles containing microsporidian spores or free microsporidian spores, which are strongly positive with a Fite's acid stain. In other areas, skeletal myocytes are thin with large central nuclei or mitotic figures (regeneration) and are surrounded by edema. Microsporidian spores were also noted in the testes and in the kidney.

Final Diagnosis: Microsporidial myositis; Testicular and renal microsporidiosis

These histological findings are similar to those noted in previous submissions. However, histologically, this animal was the most severely affected thus far. Electron microscopy to better characterize the pathogen is underway. There was no evidence of mycobacteria as has been seen in salamanders from the San Marcos facility. Future animals should be fixed and submitted in ethanol to better characterize the molecular identification of the pathogen.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-91 for any follow-up correspondence.

cc: Patricia Duncan, Uvalde NFH
Dave Britton, San Marcos ARC
Ken Ostrand, San Marcos ARC
Lindsay Campbell, San Marcos ARC

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/903

Memorandum: June 4, 2018

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

Subject: Final Report for the San Marcos salamander (SNARRC Case Number 18-96)

On May 25, 2018, staff from Uvalde NFH euthanized a San Marcos salamander and fixed it in ethanol. The salamander had been identified as having abnormal posturing and being lethargic. Due to the poor prognosis and previous history at this facility with microsporidia, it was euthanized. Records indicate that on 5/23/18, the temperature of the salamander tank was 22.1 C with a DO of 119.4%. The salamander was submitted directly to the Washington Animal Disease Diagnostic Lab for histopathology.

Results:

Microsporidial spores were noted multifocally and extensively in the skeletal muscle and ovary and an interstitial aggregate noted in the kidney. A cross section of a nematode parasite was noted in the small intestine.

Final Diagnosis: Microsporidial myositis and oophoritis; Rare microsporidial sporophorous vesicles in the kidney; Aphasmid-type intestinal nematodiasis (probable *Amphibiocapillaria* sp)

These histological findings are similar to those noted in previous submissions. There was no evidence of mycobacteria as has been seen in salamanders from the San Marcos facility. The single intestinal nematode noted was not associated with a host response and is likely an incidental finding. Follow up electron microscopy will be performed to further characterize the microsporidian.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-96 for any follow-up correspondence.

cc: Patricia Duncan, Uvalde NFH
Dave Britton, San Marcos ARC
Ken Ostrand, San Marcos ARC
Lindsay Campbell, San Marcos ARC

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/905

Memorandum: June 25, 2018

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

Subject: Final Report for the San Marcos salamander (SNARRC Case Number 18-100)

On June 13, 2018, staff from Uvalde NFH found a dead San Marcos salamander and fixed it in ethanol. The salamander had been identified as being slightly discolored in the morning and was found dead in the afternoon. The body appeared bloated and the tail area was yellow and the body was covered in pale and dark blotches. Records indicate that on 6/13/18, the temperature of the salamander tank was 22.8 C with a DO of 42.9%. The salamander was submitted directly to the Washington Animal Disease Diagnostic Lab for histopathology.

Results:

In the skeletal muscle there are small multifocal areas of skeletal myocyte loss and/or degeneration and necrosis. In areas with necrosis and fragmented myofibers, there are sporophorous vacuoles containing microsporidial-type spores as well as infiltrates of low numbers of macrophages. The testes also contain aggregates of microsporidial spores.

Final Diagnosis: Mild multifocal microsporidial myositis; Testicular microsporidiosis

As in previous submissions, this animal had evidence of microsporidia. However, overall, the lesions are much less extensive than in previous submissions making cause of death a bit unclear. As in previous submissions, the husbandry of these animals is concerning. This is the second submission with an unacceptably low DO level and could be causing stress in these animals that, coupled with the microsporidia, is leading to their death. The cause of the low DO levels must be evaluated and corrected to ensure that it does not continue.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-100 for any follow-up correspondence.

cc: Patricia Duncan, Uvalde NFH
Dave Britton, San Marcos ARC
Ken Ostrand, San Marcos ARC
Lindsay Campbell, San Marcos ARC

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/904

Memorandum: June 11, 2018

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

Subject: Final Report for the San Marcos salamander (SNARRC Case Number 18-102)

On June 2, 2018, staff from Uvalde NFH euthanized a San Marcos salamander and fixed it in ethanol. The salamander had been identified with a white blotch on the skin the day prior. On June 2, the salamander looked bloated and was lethargic. Due to the poor prognosis and previous history at this facility with microsporidia, it was euthanized. Records indicate that on 5/30/18, the temperature of the salamander tank was 21.7 C with a DO of 52.6%. The salamander was submitted directly to the Washington Animal Disease Diagnostic Lab for histopathology.

Results:

In the skin, multifocal parakeratotic hyperkeratosis was seen on the body and legs/feet. In some of these areas there are small to moderate numbers of chytrid-type fungal thalli. In other areas, there is loss of the epidermis and replacement by dense bands of Gram-negative bacilli. The bacteria extend ito the superficial portions of the underlying skeletal muscle and are occasionally associated with highly degenerative leukocytes. A cross section of a nematode parasite was noted in the small intestine. A Fite's acid-fast stain is negative for microsporidia.

Final Diagnosis: Parakeratotic hyperkeratosis with intralesional chytrid-type fungal thalli (probable *Batrachochytrium dendrobatidis*); Moderate ulcerative dermatitis and bronchitis with intralesional Gram-negative bacilli; Aphasmid-type intestinal nematodiasis (probable *Amphibiocapillaria* sp)

Unlike previous submissions, there was no evidence of microsporidia in this salamander. The skin of this species is usually non-keratinizing except for the feet and sometimes portions of the legs. In this individual there are fewer characteristic Leydig cells that are replaced by a keratinizing epithelium on multiple segments of the body. In some of these areas, keratinocytes contain chytrid fungal organisms and this is the first time that the WADDL pathologist (Dr. Allan Pessier a chytrid

expert) has observed chytrids in this species or the related Barton Spring Salamander at a location other than the feet or distal leg. The chytrids may be the inciting cause of the hyperkeratosis or may have opportunistically colonized the body after some other primary cause of skin injury/irritation. In addition to chytridiomycosis there was an ulcerative dermatitis with intralesional Gram-negative bacteria. These could be either primary or secondary pathogens. The single intestinal nematode noted was not associated with a host response and is likely an incidental finding.

Due to the ubiquitous nature of chytrid in this species, there are no treatment recommendations at this time for the chytrid. However, it is recommended to continue working on improving maintaining good husbandry for this species. For instance, the DO level reported on 5/30/18 is quite low and is concerning. As mentioned previously, continuous poor water quality issues can cause a wide variety of health issues.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-102 for any follow-up correspondence.

cc: Patricia Duncan, Uvalde NFH
Dave Britton, San Marcos ARC
Ken Ostrand, San Marcos ARC
Lindsay Campbell, San Marcos ARC

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/910 July 26, 2018
Memorandum

To: Kenneth Ostrand and Lindsay Campbell, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 18-103).

On June 19, 2018, Southwestern Fish Health Unit (SFHU) staff received 60 fountain darters (*Etheostoma fonticola*) from the San Marcos River (GNIS ID: 1375972), Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for fish health testing. San Marcos ARC staff recorded collection of 25 fish from latitude 29.8941° and longitude -97.9299, 21 fish from latitude 29.8900° and longitude –97.9340°, and 15 fish from latitude 29.8754° and longitude -97.9319°. One location was recorded for reporting purposes for the NWFHS: latitude 29.8900° and longitude -97.9340° in Hays County, Texas. We received one less fish than reported in the collection information from San Marcos ARC.

Assays and examinations for the sampled fish included virology and parasitology. Viruses screened for included those listed as NWFHS targeted pathogens as well as any other viruses that may be detected in cell culture. Sixty fish were screened for viruses. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC. External examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of the left side set of gills for 10 fish. Testing was performed per the NWFHS Laboratory Procedures Manual (edition 5.0) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 2 of 10 fish examined. Virology testing resulted in detection of aquareovirus – a non-target virus regarded as benign – in our CHSE cell line and later confirmed by PCR. The NWFHS database report and parasite data sheets that contain the specific number and type of parasites isolated from each fish are attached to the end of this memo report.

If you have any questions about test methodology or results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 18-103 for all follow up correspondence.

cc: Martha Keller, DVM, MS, Southwestern Fish Health Unit/ Southwestern Native Aquatic Resources and Recovery Center

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
DX18-103	SAN MARCOS RIVER	TX	6/12/18	KENNETH OSTRAND

Latitude 29.8900 **Longitude** -97.934

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	0	LMBV-288F	0	0	0	--
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

Aquareovirus - a non-target virus regarded as benign - was isolated on CHSE cells and confirmed by PCR.

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 Fish Health Unit/SNARRC, U.S. Fish and Wildlife Service PO Box 219 7116 Hatchery Rd Dexter, NM 88230	Jason Woodland

FOD Parasite Data Sheet - Form P-03

Case History No. 18-103

Date examined: 6-19-18 Date Collected: 6/12/18, 6/14/18, & 6/15/18

Collection site: 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)	194	153	81	361	310	65	362	214	119	317
Total Length (mm)	28	26	21	32	32	18	34	29	23	34

Centrocestus formosanus **cysts** **Number of cysts per arch (ie 3,2,1,1)**

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	1,0,4,1	0,0,0,0	0,0,0,0	0,3,1,2	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	0,0,1,0	0,0,0,0	0,0,1,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,2,0	2,1,2,1	0,1,0,0
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	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

Examiner signature

Revised on 9/20/2017

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/913

Memorandum: July 31, 2018

To: Rachel Wirick, 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 18-104)

On June 22, 2018, staff from Uvalde NFH collected 6 Fountain Darters (*Etheostoma fonticola*) and fixed them in formalin. The Fountain Darters had been stable until they were found dead in their tanks. The only significant history event was that the water source well had been switched on June 10 after a station-wide power failure. On the day prior to discovering the mortalities (June 21), staff measuring water flows may have inadvertently caused stagnant water to enter the tanks. Records indicate that on 6/22/18, the temperature of the two affected tanks were 23.1 and 22.4C with a DO of 6.75 and 7.99 respectively. The fish were submitted directly to the Washington Animal Disease Diagnostic Lab for histopathology.

Results:

In the gills and opercular cartilage, multifocally, trematode metacercaria are noted to be encysted. In the stomach, metazoan parasites are noted to be encysted within the gastric musculature. Significant autolysis was noted in all tissues.

Final Diagnosis: Trematodiasis; Autolysis

The significant autolysis may have prevented identification of any significant lesions. The history notes that these fish were found dead in the tank. It is recommended that unless time of death is known to less than a few hours, no further fish should be submitted for histopathology as the high rate of autolysis limits proper evaluation. The encysted trematode larvae are likely *Centrocestus*, which is a common finding in this species and appears to be clinically insignificant. The metazoan parasites noted in the stomach did not seem to be associated with any inflammation and were also likely clinically insignificant. However, as mentioned previously, the severe autolysis precluded further

evaluation. If the cause of death was due to a water quality issue, it is unlikely that could be identified through histopathology.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-104 for any follow-up correspondence.

cc: Patricia Duncan, Uvalde NFH
Mark Yost, Uvalde NFH
Dave Britton, San Marcos ARC
Ken Ostrand, San Marcos ARC
Lindsay Campbell, San Marcos ARC

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/908

Memorandum: July 16, 2018

To: Lindsay Campbell, 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 San Marcos salamanders (SNARRC Case Number 18-106)

On June 29, 2018, seven San Marcos salamanders (*Eurycea nana*) were submitted in ethanol directly to the Washington Animal Disease Diagnostic lab for histopathological evaluation. The salamanders were originally collected from the wild from Spring Lake and the San Marcos river. These salamanders were noted to have an increase in mortalities with visible lesions/ulcers widely disseminated. Several salamanders appeared to be missing limbs. One gravid female had ruptured her abdomen and was euthanized. The remaining animals had been found dead in their tanks. The most recent water quality data taken on 6/26/18 indicated a DO of 5.72mg/L and a temp of 21.8C.

Results:

Multifocally, acid-fast bacteria were noted on the serosal surfaces, especially around the ovary, oviducts, and kidneys, but also noted in the liver. Epidermal loss and necrosis in the skin and legs was associated with chytrid fungal thalli and Oomycete-type hyphae as well as short-rod bacteria in one salamander. A few asphasmid-type nematodes were noted in the intestines. In the ovaries and testes, microsporidia was noted. Microsporidia was also seen in the muscle tissue.

Final Diagnosis: Mycobacterial coelomitis; Mycobacterial oophoritis; Ovarian and testicular microsporidiosis; Hyperkeratosis with chytrid and saprolegniasis; Intestinal nematodiasis

The cause of this mortality event is attributed to coelomic mycobacteriosis as previously observed in salamanders from the San Marcos facility. The lesions in most of the animals were relatively acute and could be related to recent stress or overwhelming exposure to environmental mycobacteria. Several individuals in this batch of animals had grossly visible elastomer identification injections and in one salamander, acid-fast bacteria surrounded the capsule of a presumed elastomer implant. The role of the implant to the mycobacteriosis is uncertain. The severe erosive lesions described on the

legs are attributed to saprolegniasis in one salamander and short rod bacteria in another. Both can occur secondary to skin trauma, stress, water quality changes or high environmental organic loads. Gonadal microsporidiosis was observed in three salamanders and in the skeletal muscle of one salamander. This is interpreted as an incidental finding in these cases. The intestinal nematodiasis is likewise interpreted as an incidental finding.

As previously noted, mycobacteriosis is a persistent and chronic pathogen and there is currently no treatment. Any additional animals added to systems with mycobacteria are likely to become infected. Given the severity of the lesions and the lack of comparable lesions in animals from Uvalde, this appears to be a facility problem rather than a ubiquitous issue such as the microsporidia. Serious consideration should be given to completely eradicating all animals from the affected system(s) and restarting the program.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-106 for any follow-up correspondence.

cc: Lindsay Campbell, Dave Britton, Ken Ostrand, San Marcos ARC

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/909

Memorandum: July 16, 2018

To: Rachel Wirick, Uvalde NFH
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 18-108)

On June 28, 2018, staff from Uvalde NFH euthanized three San Marcos salamander and fixed them ethanol. The salamanders had been identified as doing poorly with one having a white patch on the skin and one showing and inability to maintain its position in the water column. Due to the poor prognosis and previous history at this facility with microsporidia, they were euthanized. Records indicate that on 6/27/18, the temperature of the salamander tank was 21.8 C with a DO of 60.3%. The salamanders were submitted directly to the Washington Animal Disease Diagnostic Lab for histopathology.

Results:

In the skin of one salamander, there was focal disruption and necrosis of the ventral epidermis with intralesional slender light brown pigmented fungal hyphae (phaeohyphomycosis). The skeletal muscle of all animals showed degeneration and necrosis with small to moderate numbers of sporophorous vesicles containing microsporidal spores. In other areas, skeletal myocytes are atrophied or lost. In the testis/ovary, sporophorous vesicles containing microsporidial spores are noted.

Final Diagnosis: Microsporidial myositis; Testicular/ovarian microsporidiosis; Mycotic dermatitis (phaeohyphomycosis)

Clinical signs are once again attributable to the microsporidial myositis previously seen from other salamanders at this facility. One animal did have a peracute fungal dermatitis with a pigmented fungus (phaeohyphomycosis). The dermatitis was likely secondary due to the debilitation however.

Although the DO level reported is higher than previous submissions, it is still much too low and continues to likely be a factor in the debilitation of these salamanders. As mentioned previously, continuous poor water quality issues can cause a wide variety of health issues.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-108 for any follow-up correspondence.

cc: Patricia Duncan, Uvalde NFH
Mark Yost, Uvalde NFH
Dave Britton, San Marcos ARC
Ken Ostrand, San Marcos ARC
Lindsay Campbell, San Marcos ARC

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/911

Memorandum: July 27, 2018

To: Lindsay Campbell, 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 San Marcos salamanders (SNARRC Case Number 18-109)

On July 9, 2018, five San Marcos salamanders (*Eurycea nana*) were submitted in ethanol directly to the Washington Animal Disease Diagnostic lab for histopathological evaluation. The salamanders were originally collected from the wild from Spring Lake and the San Marcos River. These salamanders were noted to have an increase in mortalities with visible lesions/ulcers widely disseminated. Several salamanders appeared to be missing limbs. The only reported changes in the hatchery were the addition of enrichment items into the tanks and the possibility of elevated gasses in the water due to the switching of wells. The most recent water quality data taken on 7/3/18 indicated a DO of 5.75 mg/L and a temp of 21.6C.

Results:

The histologic findings were similar to previous submissions with mycobacteriosis in the coelomic cavity and around aspects of the reproductive tract. One animal had a very marked mycobacterial salpingitis. Two of the five animals had microsporidial myositis, one being clinically significant and the other animal being relatively mild. The salamander with a burst abdomen appears to be multifactorial related to follicular degeneration from microsporidiosis combined with regional inflammation due to mycobacteriosis. Elastomer was noted in one animal partially deposited next to the kidney. Orthokeratotic hyperkeratosis with chytrid-type fungal thalli noted on distal limbs of two animals.

Final Diagnosis: Microsporidial myositis (2 animals); Microsporidial oophoritis (3 animals); Mycobacterial coelomitis (4 animals); Mycobacterial salpingitis (1 animal); Mycobacterial oophoritis (1 animal); Chytrid hyperkeratosis (2 animals)

As seen in previous submission, mycobacteriosis appears to the primary cause of illness in these animals. While microsporidia is present, its clinical significance is less clear. As previously noted, mycobacteriosis is a persistent and chronic pathogen and there is currently no treatment. Its pattern centered in the reproductive tract could indicate an ascending infection from the environment. Any additional animals added to systems with mycobacteria are likely to become infected. It is recommended that any similar clinical presenting animals be humanely euthanized. It is also recommended that consideration be given to performing future elastomer tagging more distally on the tail to prevent accidental injection into the kidneys of future animals.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-109 for any follow-up correspondence.

cc: Lindsay Campbell, Dave Britton, Ken Ostrand, San Marcos ARC

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/918

Memorandum: September 24, 2018

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 18-113)

On August 1, 2018, SNARRC staff obtained 62 salamander skin swabs taken from 31 salamanders, from San Marcos ARC for chytrid testing. These swabs came from wild salamanders that are currently being held at San Marcos ARC. Samples came from 10 Texas blind salamanders (*Eurycea rathbuni*), 13 Comal Springs salamanders (*Eurycea* spp), and 8 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. All Texas blind salamanders were tested pooled initially and if a positive was detected, were then tested individually. The remaining salamander samples were tested in 3-5-swab pooled samples. Samples tested together are color coded on the attached data sheet.

Results:
All salamanders were negative for Bsal. One out of ten (10%) Texas blind salamanders was positive for Bd. 4/4 of the pooled samples (100%) of the San Marcos salamanders were positive for Bd. Individual and pooled 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. Findings continue to indicate a much lower prevalence of Bd in the Texas Blind salamanders compared to other species. Any future movements of these animals should be done with care, as group housing may result in many of the previously negative animals to become positive.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 18-113 for any follow-up correspondence.

cc: Ken Ostrand, San Marcos ARC
Dave Britton, San Marcos ARC

Swab #	Date	Collection Location	Species	TL (mm)	Lesions Y/N	Bd	Bsal
533	5/9/2018	Johnsons Well	ER	62*	N	-	-
534	5/9/2018	Johnsons Well	ER	73	N	-	-
535	5/18/2018	Primers Fissure	ER	71	N	-	-
536	5/25/2018	Diversion	ER	59	N	+	-
537	6/5/2018	Diversion	ER	-	N	-	-
538	6/5/2018	Diversion	EN	-	N	+	-
539	6/20/2018	Spring Island	ESPP	59	N	+	-
540	6/20/2018	Spring Island	ESPP	47	N	+	-
541	6/20/2018	Spring Island	ESPP	49	N	+	-
542	6/20/2018	Spring Island	ESPP	49	N	+	-
543	6/20/2018	Spring Island	ESPP	54	N	+	-
544	6/20/2018	Spring Island	ESPP	49	N	+	-
545	6/20/2018	Spring Island	ESPP	45	N	+	-
546	6/20/2018	Spring Island	ESPP	58	N	+	-
547	6/20/2018	Spring Island	ESPP	45	N	+	-
548	6/20/2018	Spring Island	ESPP	50	N	+	-
549	6/20/2018	Spring Island	ESPP	54	N	+	-
550	6/20/2018	Spring Island	ESPP	45	N	+	-
551	6/21/2018	Spring Island	ESPP	54	N	+	-
552	6/22/2018	Diversion	EN	45	N	+	-
553	6/22/2018	Diversion	ER	54	N	-	-
554	6/29/2018	Diversion	ER	55	N	-	-
555	7/23/2018	Diversion	ER	75	N	-	-
556	7/23/2018	Diversion	ER	49	N	-	-
557	7/23/2018	Diversion	EN	37	N	+	-
558	7/23/2018	Diversion	EN	40	N	-	-
559	7/23/2018	Diversion	EN	40	N	-	-
560	7/23/2018	Diversion	EN	41	N	-	-
561	7/23/2018	Diversion	EN	36	N	-	-
562	7/23/2018	Diversion	EN	48	N*	-	-
563	7/26/2018	Diversion	ER	43	N	-	-

Samples pooled together are color coded. Samples 533-537 were tested pooled and then again individually.

United States Department of the Interior
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Southwestern Fish Health Unit
P.O. Box 219, 7116 Hatchery Road
Dexter, New Mexico 88230

In Reply Refer To:
FWS/R2/FR-SFHU/921

Memorandum: October 2, 2018

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 18-117)

On August 21, 2018, SNARRC staff received ten Fountain Darters (*Etheostoma fonticola*) from San Marcos Aquatic Resource Center. Although all were submitted live, one died en route. The Fountain Darters were submitted due to San Marcos experiencing an increase in mortalities starting on 8/13/18. The mortalities were occurring in both refugia and quarantine systems and this included the known LMBV+ fish. The only clinical signs noted were that affected fish were pale and lethargic and had a tendency to swim to the surface when bothered, but then fell sideways back down afterwards. A 2% salt treatment was administered for one hour on 8/13/18 to the quarantine fish, primarily because mortalities were found with fungal growth on them. No records of water quality was submitted from the facility. On arrival at the Fish Health unit, the fish were euthanized and examined. Six fish were sampled for virology, bacteriology and parasitology and three fish were fixed in Z-fix. All clinical testing was conducted per the American Fisheries Society-Fish Health Section Bluebook and standard SFHU protocols. The fixed fish were submitted to the Washington Animal Disease Diagnostic Lab for histopathology.

Results:

External examinations and scrapes revealed no external pathogens. Virology, bacteriology and parasitology testing was negative for any pathogens. Histologically, mild chronic steatitis was noted, along with a mild dermatitis. Metacercaria were noted encysted in tissue, but the tissue did not show an associated inflammatory or degenerative response.

Final Diagnosis: Steatitis; Dermatitis; Metaceracariasis

The chronic low-level steatitis and dermatitis is suggestive of chronic low-level debilitation, but no definitive cause is elucidated from histology. The encysted trematode larvae are likely *Centrocestus*,

which is a common finding in this species and appears to be clinically insignificant. No encysted metacercariae were noted in the gills. In general, no cause of death was elucidated. However, given the wide-spread nature of the affected fish, a water quality issue cannot be ruled out. No water quality parameters were reported for these particular fish. In talking with staff, low-level voltage could also be a concern. Recommendation is to closely evaluate all parameters and evaluate the response by the fish to any changes made.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please refer to case number 18-117 for any follow-up correspondence.

cc: Dave Britton, San Marcos ARC
Ken Ostrand, San Marcos ARC

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/927

Memorandum: November 29, 2018

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

Subject: Final Report for the wild salamanders (SNARRC Case Number 18-124)

On September 27th, 2018, SNARRC staff obtained 200 salamander skin swabs from 100 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 100 San Marcos salamanders (*Eurycea nana*). 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. The San Marcos salamander samples were tested in a 5-swab pooled sample.

Results:
All salamanders were negative for Bsal, and 95% (19/20) of the pooled samples were positive for Bd. Pooled 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 this positive specie. Historically, this specie has not shown any clinical signs related to the fungus. However, any future movements of these animals should be done with care, as group housing may likely result in many of the previously negative animals to become positive.

If you have any further questions or concerns, please do not hesitate to contact the SFHU staff. Please reference case number 18-124 for any follow-up correspondence.

cc: Ken Ostrand, San Marcos ARC
Dave Britton, San Marcos ARC

Salamander Swab Data sheet

Colors used to differentiate pooled samples

Vial	Date	Bd	Bsal
587	9/26/2018	+	-
588	9/26/2018	+	-
589	9/26/2018	+	-
590	9/26/2018	+	-
591	9/26/2018	+	-
592	9/26/2018	+	-
593	9/26/2018	+	-
594	9/26/2018	+	-
595	9/26/2018	+	-
596	9/26/2018	+	-
597	9/26/2018	+	-
598	9/26/2018	+	-
599	9/26/2018	+	-
600	9/26/2018	+	-
601	9/26/2018	+	-
602	9/26/2018	+	-
603	9/26/2018	+	-
604	9/26/2018	+	-
605	9/26/2018	+	-
606	9/26/2018	+	-
607	9/26/2018	+	-
608	9/26/2018	+	-
609	9/26/2018	+	-
610	9/26/2018	+	-
611	9/26/2018	+	-
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614	9/26/2018	+	-
615	9/26/2018	+	-
616	9/26/2018	+	-
617	9/26/2018	+	-
618	9/26/2018	+	-
619	9/26/2018	+	-
620	9/26/2018	+	-
621	9/26/2018	+	-
622	9/26/2018	+	-
623	9/26/2018	+	-
624	9/26/2018	+	-
625	9/26/2018	+	-
626	9/26/2018	+	-
627	9/26/2018	+	-
628	9/26/2018	+	-
629	9/26/2018	+	-
630	9/26/2018	+	-
631	9/26/2018	+	-
632	9/26/2018	+	-
633	9/26/2018	+	-
634	9/26/2018	+	-

635	9/26/2018	+	-
636	9/26/2018	+	-
637	9/26/2018	-	-
638	9/26/2018	-	-
639	9/26/2018	-	-
640	9/26/2018	-	-
641	9/26/2018	-	-
642	9/26/2018	+	-
643	9/26/2018	+	-
644	9/26/2018	+	-
645	9/26/2018	+	-
646	9/26/2018	+	-
647	9/26/2018	+	-
648	9/26/2018	+	-
649	9/26/2018	+	-
650	9/26/2018	+	-
651	9/26/2018	+	-
652	9/26/2018	+	-
653	9/26/2018	+	-
654	9/26/2018	+	-
655	9/26/2018	+	-
656	9/26/2018	+	-
657	9/26/2018	+	-
658	9/26/2018	+	-
659	9/26/2018	+	-
660	9/26/2018	+	-
661	9/26/2018	+	-
662	9/26/2018	+	-
663	9/26/2018	+	-
664	9/26/2018	+	-
665	9/26/2018	+	-
666	9/26/2018	+	-
667	9/26/2018	+	-
668	9/26/2018	+	-
669	9/26/2018	+	-
670	9/26/2018	+	-
671	9/26/2018	+	-
672	9/26/2018	+	-
673	9/26/2018	+	-
674	9/26/2018	+	-
675	9/26/2018	+	-
676	9/26/2018	+	-
677	9/26/2018	+	-
678	9/26/2018	+	-
679	9/26/2018	+	-
680	9/26/2018	+	-
681	9/26/2018	+	-
682	9/26/2018	+	-
683	9/26/2018	+	-
684	9/26/2018	+	-
685	9/26/2018	+	-
686	9/26/2018	+	-

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/929 December 10, 2018
Memorandum

To: Kenneth Ostrand and Lindsay Campbell, San Marcos Aquatic Resource Center

From: Jason Woodland, Southwestern Fish Health Unit/SNARRC

Subject: National Wild Fish Health Survey (NWFHS) report memo for fish collected from the San Marcos River, Texas (Case Number 19-08).

On November 6, 2018, Southwestern Fish Health Unit (SFHU) staff received 60 fountain darters (*Etheostoma fonticola*) from the San Marcos River (GNIS ID: 1375972), Texas. These fish were collected by staff at the San Marcos ARC and shipped live to the SFHU laboratory for fish health testing. San Marcos ARC staff recorded collection of 25 fish from latitude 29.7129° and longitude -98.1375, 20 fish from latitude 29.8900° and longitude –97.9340°, and 15 fish from latitude 29.8726° and longitude -97.9318°. One location was recorded for reporting purposes for the NWFHS: latitude 29.7129° and longitude -98.1375° in Hays County, Texas. We received one less fish than reported in the collection information from San Marcos ARC.

Assays and examinations for the sampled fish included virology and parasitology. Viruses screened for included those listed as NWFHS targeted pathogens as well as any other viruses that may be detected in cell culture. Sixty fish were screened for viruses. Screening for parasites was conducted as part of an ongoing parasite enumeration study with San Marcos ARC. External examinations by gross observation and microscopy were completed by SFHU staff. Screening for *Centrocestus formosanus* was conducted by examination of the left side set of gills for 10 fish. Testing was performed per the American Fisheries Society- Fish Health Section Blue Book (2016 edition) and standard SFHU protocols.

Results:
Centrocestus formosanus was observed in 2 of 10 fish examined. No viruses were detected in cell culture. The parasite data sheets that contain the specific number and type of parasites isolated from each fish are attached to the end of this memo report.

If you have any questions about test methodology or results, or if the SFHU can be of additional assistance, please do not hesitate to contact Southwestern Fish Health Unit staff. Please reference case history number 19-08 for all follow up correspondence.

cc: Dave Hampton, Southwestern Fish Health Unit/ Southwestern Native Aquatic Resources and Recovery Center

FOD Parasite Data Sheet - Form P-03

Case History No. 19-08

Date examined: 11/06/2018 Date Collected: 10/29-31/2018

Collection site: San Marcos River

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5	Fish #6	Fish #7	Fish #8	Fish #9	Fish #10
Weight (mg)	151	164	222	227	266	202	422	200	419	187
Total Length (mm)	28	29	30	30	33	30	35	29	37	30

Centrocestus formosanus **cysts** **Number of cysts per arch (ie 3,2,1,1)**

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	0,3,2,0	0,0,0,0	0,0,0,0	2,1,1,0	0,0,0,0	0,0,0,0
Monogenea	L	0	1,1,2,0	0	0,0,0,1	0,0,1,0	0,1,0,0	0	0	0	0,0,2,0
Myxobolus sp.	L	0	0	0	0	0	0	0	0	0	0
Other	L	0	0	0	0	0	0	0	0	0	0

Examiner signature Jim Bundy

Revised on 9/20/2017