

Final Report to the Share with Wildlife Program
For Contract No. 05-516.0000.0082
“Development of an Immunological Approach to
Determining Host Fishes of the Texas Hornshell (*Popenaias popeii*)”

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Background

The Texas hornshell, *Popenaias popeii*, is one of eight freshwater mussel species native to New Mexico, and the only one extant within the state (Lang 2001). Once found throughout the Rio Grande basin in New Mexico, Texas, and Mexico, the U.S. range of this species is now restricted to the Black River in Eddy County, New Mexico (Lang 2001), and a stretch of the lower Rio Grande in Texas (Robert Howells, TX Parks and Wildlife Dept., pers. comm.). Due to its reduced range and declining populations, the Texas hornshell is considered “endangered” under the New Mexico Wildlife Conservation Act and is a Priority 2 candidate for federal listing under the Endangered Species Act.

Freshwater mussels have a complex life history. Adult males release sperm that are filtered from the water column by females. Fertilization leads to production of larvae called glochidia that are brooded within the female gills. At the appropriate time in development, the glochidia are released and become obligate parasites on the gills of fishes (or very rarely, other freshwater vertebrates). After feeding on the host for several days-to-weeks, the glochidia transform into juvenile mussels and drop off of the host and settle in benthic habitats where they grow to adulthood. Because of the obligate nature of its relationship with its hosts, population viability of freshwater mussels is inextricably linked to viability of host fishes. In the case of the Texas hornshell, 23 of 30 fish species tested in laboratory trials were found to be suitable hosts (Gordon 2000). However in a natural setting, these so-called “physiological” hosts will only come into contact with glochidia if they are in proximity to a gravid female mussel.

Objectives

The objectives of this project are to:

1. Verify the presence of an immunological response to infection by Texas hornshell glochidia in fish species that are likely hosts for this mussel.

2. Collect blood samples from fishes located in proximity of Texas hornshell populations to determine which fishes serve as ecological hosts and to determine the proportion of individuals infected within these populations of host species.
3. Identify fishes infected with glochidia of the Texas hornshell.

Objective 1. Identification of host immunological response

1. Laboratory Experiment 1 (spring 2005)

We infested 5 channel catfish (*Ictalurus punctatus*), 2 carp (*Cyprinus carpio*) and 1 bullhead (*Ameiurus* sp.) with glochidia in aquaria at Bitter Lake National Wildlife Refuge during spring 2005. Glochidia were removed from the demibranchs of a female *P. popeii* and pipetted over the gills of the fish. We retained 5 channel catfish and 1 carp as an experimental control. Fish were bled 8 days post-infestation, and the serum was separated from the whole blood sample after coagulation of the cellular components, and preserved at -80°C. Gills were removed from two fish that died during the experiment; these were cleared with 2% w/v potassium hydroxide and examined for encysted glochidia under a dissecting microscope (Fig. 1). Surviving fish were released to their point of capture.

Very few glochidia (≤ 3) were recovered from the gills of infected fish that died during the experiment and no sloughed nor transformed juveniles were recovered from the experimental tanks. We therefore believe that the infestations were not normal and may have been complicated by application of glochidia to surfaces that do not normally come into contact with the mussels or perhaps a pathogen, immune response or some other circumstance interfered with these infestations. This has been observed in the past with *P. popeii* (B. Lang pers. comm.) and other mussel species (D. Neves pers. comm.).

One female *P. popeii* held in captivity was observed releasing glochidia (Fig. 2-A). Viability of these glochidia was tested by exposing them to a saline solution daily after release. Most glochidia were responsive and classified as viable for the first three days after release, although some were viable up to five days after release.

2. Laboratory Experiment 2 (spring 2006)

We developed and submitted a plan to Dexter National Fish Hatchery to experimentally infest fish with glochidia in May and June 2006 (attached as Appendix 1) and, in conjunction with Brian Lang, we obtained naïve fishes for this experiment from outside of the range of *P. popeii* in the Black River. A total of 15 fish (5 *Moxostoma congestum*, 5 *Ictalurus punctatus*, and 5 *Lepomis* spp.) were exposed to glochidia via direct pipetting of glochidia to the face, gills and opercula, and by further exposing fish in an

aerated bath of glochidia. This allowed glochidia to come into contact with the external tissues and may have better mimicked normal routes of infestation. Approximately 3 pipettes of glochidia were applied to each fish, with subsamples preserved for enumeration and estimation of number of glochidia exposed to each fish. Infected fish were placed in individual aquaria with false bottoms. All material recovered from tanks were sieved to preserve all particles with a size similar to glochidia, i.e. >180 μm (similar to Lang 2002). Samples were concentrated by settling and preserved in formalin. All fish were bled after about 14 days. Additionally, 5 fish of each species were maintained as controls and blood samples were taken for use as negative controls in laboratory tests.

Similar to the previous year, we observed natural releases of glochidia in captivity. With slightly increased flow, due to a different aeration system, we observed suspension of the mucus mass containing the glochidia in a net-like “conglutinate” mass (Fig. 2-B). As far as we know, this is the first evidence of a mechanism of infestation in *P. popeii* and may explain the pattern of infestation that we have observed in field studies. Based on the shape of the conglutinate, i.e. a net, it seems likely that glochidia will be delivered to the surface of fishes, rather than gills (the typical location of infestation of other mussels). Such routes of infestation are known for some mussel species such as *Strophitus undulatus*, which produces a net-like conglutinate (Watters 2002). Similarly, species of *Anodonta* have been known to infect their hosts on the outer surfaces of their bodies (Jansen and Hanson 1991), in contrast to the many mussel species known to infest gill tissues.

3. Laboratory and Immunological Procedures

General procedures for observing antibody/antigen reactions followed methods of O’Connell and Neves (1999) and other sources (J. Stevenson, Miami University; M. O’Connell, University of New Orleans; pers. comm.). We screened these procedures using 152 samples run against antibodies and developed a standard operating procedure (attached as Appendix 2) consistent with O’Connell and Neves (1999).

Equivocal results required that we continue to revise the procedures used to detect antibody formation in infested fishes. Through additional discussions with experts (M. O’Connell) and review of newer literature, such as Dodd *et al.* (2006), we reconsidered our techniques. During the spring of 2008, we attempted a suite of methodological modifications for the detection of antibody production against *P. popeii* antigens by fishes. We modified the original procedure, by varying staining and washing techniques. We varied the wash, stain, and destain periods, but still saw no evidence of antibody-antigen interaction on positive control samples from either experimental infestations nor in blood collected from wild, infested fishes.

Objectives 2 and 3: field sampling

1. Field Sampling 1 (spring/summer 2005)

We collected 30 fish from the Black River in 2005, but observed only one infestation by glochidia: on the face and opercula of a blue sucker (*Cycleptus elongatus*). Blood samples were taken from the caudal fins of 15 fishes. Sampling sites are plotted in Figure 3.

Glochidia were collected from 3 females for use in immunological tests. Gills from these females were excised and glochidia were removed from them by mechanical destruction of the gill tissue. The females were preserved and two are retained with Brian Lang at NMDGF; one female is archived at Miami University.

2. Field Sampling 2 (spring/summer 2006)

Using a variety of fishing techniques (i.e., hoop and trammel nets, seining, electroshocking), we collected 299 fishes representing 14 species (Figure 4-a), from the Black River between May 8 and June 15. We measured total and standard lengths, and weight for almost all fishes and tagged 161 individuals. Fishes were not tagged if tagging was likely to represent a substantial threat to their health. Glochidia were observed on 20 individuals from 6 species, and 7 additional fish (including 2 additional species) may have been infested. Glochidia on fishes were observed throughout the sampling period. River carpsucker (*Carpiodes carpio*) and gray redhorse (*Moxostoma congestum*) were most frequently infested. Eight of 10 river carpsucker (80%) and eight of 64 (12.5%; with one more possibly infected) gray redhorse were infested (see Fig. 4-A). All other species of fishes were either infested at very low rates (< 10% and never more than 1 individual and/or 2 possible individuals), or were rarely caught. We obtained blood samples from 215 fish. All blood samples are currently archived at -80° C at Miami University. Small fishes such as minnows were preserved and examined in the lab.

5. Field Sampling 3 (spring/summer 2007)

Fish sampling, using similar techniques and methods described above, throughout the inhabited range of *P. popeii* was continued during the summer of 2007. We observed 447 fishes, representing 15 species, from the 14 km stretch of the Black River between May 26 and June 20. Glochidia were observed on 18 individuals from four species, and three additional gizzard shad may have been infested (Fig. 4-B). Because it was difficult to determine whether cysts and other bumps on the skin of shad were glochidia, we preserved these individuals and collected blood samples. Although it is unlikely that shad are hosts, because they would be susceptible to secondary infection and when feeding in benthic areas are typically in open water, we examined individuals with skin conditions similar in appearance to glochidial infestation to ensure accurate reporting of infestation. Glochidia on fishes were observed throughout the sampling period. River carpsucker (*Carpiodes carpio*) and gray redhorse (*Moxostoma congestum*) were most frequently infested. Eight of 29 river carpsucker (30%) and five of 118 (4.0%) gray redhorse were infested. Additionally, it is possible that a gizzard shad and a single bass were infested, but infestation could not be confirmed. We obtained blood samples from over 300 fishes. All blood serum samples are archived at -80° C at Miami University. Small fishes such as minnows were preserved separately and were not counted in the totals above, but were examined for presence of glochidia.

6. Overall analysis of field samples 2005-2007

Field sampling locations are plotted in Figure 3. We tested the entire dataset for differences in infestation rates between species, based on the results from field surveys.

We used a chi-square contingency table to test for differences in infestation rates between species, using data from the entire project ($\chi^2 = 144.240$, $df=17$, $p < 0.001$). Many potential hosts are fairly rare in the current range of *P. popeii*, therefore we were unable to preserve a large number of individuals to examine in the laboratory for infestation intensity (number of glochidia attached to a given host). However, in the samples that could be enumerated, the difference in infestation intensity between host species was large (Fig. 5). We used a graphical approach to examine the value of different host species toward recruitment of mussels (Fig. 6). As abundance, prevalence, and intensity of infestations on hosts increase, the importance of that host species to the recruitment of mussels increases. The most important hosts will have values that lie in the far, upper, right corner of the three-dimensional plot. Hosts that have values in other parts of the plot will be limited in the proportion of individuals infested (low prevalence), the number of glochidia per infestation (low intensity), and/or the number of fish present (low abundance).

For example, a parasite might take advantage of an abundant species of host and infest a large proportion of them (i.e. attain high prevalence), despite being unable to infest individuals of that host species at high intensities. This would plot in the far, lower, right corner and could lead to production of a large number of offspring in the next generation. Alternatively, a parasite could efficiently infest (attaining high prevalence) hosts that occur in low numbers (low abundance), but carry large numbers of the parasite (high intensity). In this case, the host would plot at the far, upper, left corner. The outcomes of these scenarios may be equivalent in terms of number of mussels recruited.

In the Black River, fishes with the highest infestation intensity and prevalence were catostomids (sucker family), namely *C. carpio*, *C. elongatus*, and *M. congestum*. However, none of these species occurred in high numbers compared to other species (Fig. 6). Because these catostomids likely contributed the most to recruitment of *P. popeii*, we believe that they represent ecological hosts, a distinct subset of fishes that are exploited more efficiently than other physiological hosts (Lang 2001). All other host species likely represent insignificant contributions to the recruitment of *P. popeii*.

Infestation rate within species did not differ between individual sampling sites (*C. carpio* $\chi^2 = 23.84$, $df = 15$, $p = 0.068$; *C. elongatus* $\chi^2 = 4.87$, $df = 3$, $p = 0.18$; *M. congestum* $\chi^2 = 37.00$, $df = 33$, $p = 0.29$), nor did infestation rates differ between sites, when species were pooled ($\chi^2 = 58.21$, $df = 44$, $p = 0.061$). Some of these tests approached significance and might exhibit real differences which are obscured by small sample size. Pooling sites nearby one another or by habitat type may change these results. However, the proportion of fishes encountered that were ecological hosts differed by site ($\chi^2 = 145.00$, $df = 44$, $p < 0.001$), with ecological hosts having greatest frequency at sites scattered along the sampled stretch of the river.

These ecological hosts are generally benthic-feeding, highly vagile catostomids. There was no effect of fish size (*C. carpio*, $\chi^2=0.5304$, $df=1$, $p=0.467$; *C. elongatus*, $\chi^2 = 0.107$, $df = 1$, $p = 0.743$; *M. congestum*, $\chi^2 = 0.146$, $p=0.703$). Additionally, the length-weight relationship between infested and uninfested fishes did not differ within species (*C. carpio* $F = 1.399$, $df = 1$, $p=0.245$; *C. elongatus* $F = 1.85$, $df = 1$, $p = 0.25$; *M. congestum* $F = 0.377$, $df = 1$, $p = 0.540$; Fig. 7), suggesting that fish body condition did not differ between infested and uninfested fishes. When pooled together, infested fishes were larger compared to other fishes in the survey ($F = 67.38$, $df = 729$, $p < 0.001$). Ecological hosts had larger average body size than the other species sampled ($F = 527.63$, $df = 1,739$, $p < 0.0001$).

Although several species became infested at low prevalence and intensity, only a few species were infested consistently and at high prevalence and/or intensity. While these species are not the most abundant in our surveys, they represent the largest potential contribution to recruitment of *P. popeii* of all the fishes surveyed. If the number of glochidia infested on these fishes reflects the number of transformed juveniles, then these results demonstrate a clear disparity among fish species' potential to contribute to recruitment of juvenile mussels. These three species have subterminal mouths, are benthic feeding and may process large quantities of material from the bottoms of rivers (Pflieger 1997). It is likely that use of these environments predisposes them to incidental contact with mussels and their glochidia. The pattern of external and, typically, facial infestation sites suggests that a passive mechanism may be responsible. The feeding motions of these fishes are likely to increase their exposure to the net-like masses produced by *P. popeii*. Smaller-bodied fishes, e.g. *Lepomis* spp. and *Cyprinella lutriensis*, were all infested on the gills. This discrepancy in the location of attached glochidia suggests that smaller and more pelagic fishes are more likely to become infested by ingesting drifting glochidia, which would lead to much lower infection intensity.

Using large-bodied hosts may have important ecological and evolutionary consequences for *P. popeii*, because large-bodied hosts offer opportunities for parasite species to ameliorate environmental uncertainty. Large-bodied hosts may represent a more valuable resource than small-bodied hosts because of the larger surface area for glochidia attachment. This may be especially important in mussel species that have few opportunities to deliver their offspring to appropriate hosts. Because the ecological hosts of *P. popeii* are not the most abundant fishes in the river, the mussel may be trading off high host prevalence for the more predictable resource offered to them by large-bodied hosts which are more likely to survive long enough for glochidia to transform into juveniles. These large-bodied hosts are also likely to better tolerate high infestation intensity. Desert rivers are notoriously unpredictable with both short, high-intensity, scouring floods and periods of low-flow or complete drying. Large fishes are often more vagile and may provide better opportunities for recolonization where local mussel populations have been extirpated. Although *P. popeii* likely used mainstem

habitats, use of large-bodied hosts might allow them to exploit smaller tributaries, or reestablish mainstem sites after catastrophic events.

Conclusions

We were unable to develop an effective method for measuring immunological response of host fishes to infestation by Texas hornshell. Reasons for this negative result might include: weak response of fishes to glochidial attachment due short attachment periods, or known methods (i.e. O'Connell and Neves 1999) being unsuitable for this mussel-host system. We will maintain the blood samples at -80°C in case a refined method is developed.

We feel, however, that our field approach allowed us to identify most accurately the ecologically relevant hosts of *P. popeii*: river carpsucker, blue sucker, and gray redhorse. A total of more than 700 fishes were observed in the wild, but few were observed to have encysted glochidia and, of these, the overwhelming majority were large-bodied, benthic feeding catostomids. Glochidia of *P. popeii* appear to attach largely to the face and opercula of fish hosts. This is consistent with our observations of the shape of released glochidial masses and habitat use patterns and feeding ecology of this suite of catostomids.

Catfishes were occasionally observed to have cysts that were superficially similar to those caused by glochidial attachment, but these did not appear to be glochidia when viewed with a microscope. Few other species appear to be consistently or intensely exploited by *P. popeii*. Interestingly, while early lab studies (Gordon 2000) indicate a broad range of physiologically relevant host fishes, our field data point to only a few of those species as being ecologically relevant.

Literature Cited

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Appendix 1:

STUDY PLAN
U. S. Fish and Wildlife Service
Dexter National Fish Hatchery and Technology Center

Study Number: DX-06-001

Title: Development of an Immunological Approach to Determine Host Fishes of the Texas Hornshell (*Popenaias popeii*)

Principal Investigators: Brian Lang, New Mexico Department of Game and Fish, and Todd Levine, Miami University, Oxford, Ohio

Co-Invest./Cooperators: Dexter National Fish Hatchery and Technology Center, Dexter, NM 88230

Background and Justification:

The Texas hornshell, *Popenaias popeii*, is one of eight freshwater mussel species native to New Mexico, and it is the only one currently extant within the state. Once found throughout the Rio Grande basin in New Mexico, Texas, and Mexico, this species is now restricted to the Black River in Eddy County, New Mexico, and the lower Rio Grandenear Laredo, Texas. Due to its reduced range and declining populations, the Texas hornshell is considered “endangered” by the state of New Mexico and is a Priority 2 candidate for federal listing under the Endangered Species Act.

Freshwater mussels have a complex life history. Adult males release sperm that are filtered from the water column by females. Fertilization leads to production of larvae called glochidia that are brooded within the female gills. At the appropriate time in development, the glochidia are released and become obligate parasites on the gills of fishes (or very rarely, other freshwater vertebrates). Because of the obligate nature of its relationship with its hosts, population viability of freshwater mussels is inextricably linked to viability of host fishes.

Objectives:

We propose to develop and test a method for determining ecological hosts of the Texas hornshell. Because infection of host fishes by glochidia can trigger an immune response in fishes (O’Connell and Neves 1999), we will use immunological techniques to determine which fishes in the Black River are serving as, or have previously served as,

hosts for the Texas hornshell.

Our objective is to:

Verify the presence of an immunological response to infection by Texas hornshell glochidia in fish species that are likely hosts for this mussel.

Material and Methods:

We will use an immunological approach to determine the ecological hosts for the Texas hornshell. The “immunological responses” that we will measure are antibody-antigen reactions between antibodies contained in host fish blood serum and antigen proteins present in ground tissues of glochidia.

We will conduct laboratory experiments to demonstrate the presence of an antigen-antibody reaction in several species of fishes infected with glochidia of the Texas hornshell. Target species will include 1) bullhead (*Ameiurus* sp.), 2) gray redhorse (*Moxostoma congestum*), 3) channel catfish (*Ictalurus punctatus*) and 4) sunfish (*Lepomis* sp.). These species are from three separate families and represent a broad taxonomic range.

We will obtain naïve (never infected) fish from outside the Black River watershed and bring them to Dexter National Fish Hatchery and Technology Center, where the experiments will be conducted.

A bank of 29 gallon tanks will be set up, and walled off from the rest of the facility with heavy gauge black plastic on 2x4 frames. This will serve two functions, containment and privacy for wild specimens acclimating to culture conditions. Entry to the study area will be limited only to personnel actively involved in the study.

Standard water quality parameters will be monitored and recorded daily. These should include at a minimum: dissolved oxygen, nitrates, nitrites, ammonia, pH, temperature, and conductivity.

Four gravid female Texas hornshell will be collected and maintained in the lab for collection of viable glochidia. All glochidia remaining after fish inoculations will be flash frozen in liquid nitrogen. Up to **10** fish of each species will be infected with glochidia following Gordon and Layzer (1993) method as described in Lang (2001). This group of fishes will constitute the treatment group. Up to 10 additional members of each fish species will be held under identical conditions, but will not be exposed to glochidia. This group will serve as the control fishes.

Fish will be maintained until glochidia transform into juveniles and detach from their hosts (approximately 6-14 days post-infection). Once glochidia have detached, blood will be drawn from each fish (experimental and control groups) for future analysis at Miami University, Oxford, OH.

Containment protocols:

This study will be conducted using the *Guidelines for the Use of Fishes in Research* (Nickum et al. 2004) for containment protocols and acclimation to laboratory conditions.

Specifically 1) "Prior to bringing fish into a laboratory, facilities and plans should be in place to ensure that the fish cannot escape from the facility, especially species not native to the watershed, and that the introduced fishes can be isolated physically from fishes already present.

2) Each holding unit should have its own set of nets and other equipment. Facilities and equipment used for previous studies should be disinfected prior to use in new studies, typically with a chlorinated disinfectant (300 ppm). If the introduced fishes may carry disease agents, especially pathogens or parasites that are not endemic to the area, quarantine-level facilities should be used.

3) Effluents from units used to hold newly introduced fishes should, at a minimum, pass through screens with openings sufficiently small to retain any escaped fish, followed by mechanical grinding devices and, in turn, chemical or other treatment sufficient to kill all pathogens and parasites that can be expected to be present."

This policy shall be stepped down to daily standard operating procedures for the study location at Dexter to:

Transportation to facility

Vehicle Shipments:

Exterior of vehicle should be disinfected with a 200-ppm solution of Quaternary Ammonium Compound in the designated area. The solution should be liberally applied with a garden sprayer on all external surfaces and be allowed to sit for 15 minutes before moving about the station. Operators should use eye protection and protective gloves.

Vehicle should be moved immediately to the quarantine area. All

acclimation water should be released into the underground containment tank for disinfection. When fish are acclimated to ambient conditions they should be netted from the transport tank and placed into the culture vessel. Care should be taken to avoid spilling and dripping water from the transport tank.

When fish are removed from the tanks chlorine should be added to a concentration of 300-ppm. Walls of the tank should be scrubbed with a brush and held for one hour. Water from the tanks should be discharged into the underground containment tank. Safety glasses and protective clothing should be worn when handling the chlorine solution. All equipment, boots and nets should be cleaned and disinfected. The ground between the truck and the holding facility should be disinfected with a 200ppm solution of Quaternary Ammonium Compound.

Hands should be washed thoroughly after handling the fish and shoes disinfected in a footbath before moving to other parts of the hatchery.

Box Shipments:

Animals should be delivered to the quarantine area for acclimation. Bags are floated in the culture vessel until acclimation is completed. Fish are removed from the bags with as little water as possible. The remaining water in the bag is poured down the drain to the underground containment tank for disinfection. The bag should be placed in a bucket and sprayed down with a 300 ppm chlorine solution or autoclaved. Hands should be disinfected before moving away from the quarantine area.

Facility protocols:

1. Hands and arms should be washed with a disinfectant soap upon entering and leaving the study area. Hands and arms should be washed before moving from one tank to another during daily cleaning operations.
2. The study area will have a footbath containing a 200-ppm Quaternary Ammonium Compound solution. Staff should step in the solution when entering and leaving the facility. Footbath solution should be maintained at an adequate level and be changed daily. To prevent damage to personal clothing, rubber boots may be worn when entering the study area. Boots should be sprayed with 200-ppm Quaternary Ammonium Compound and stored at the entrance to the study area.

3. Prior to entering the study area, a labcoat or other protective clothing (raingear or coveralls) should be worn. A locker will be provided for storage of protective wear outside the entrance to the study area. Protective clothing should be removed and stored when leaving the area.
4. Records should be updated daily. Any problems or conditions out of the ordinary should be brought to the attention of the principal investigator AND the fish health representative. No treatments or alterations should be made without written direction of the fish health representative.
5. Any mortality that may occur during the course of the study period should be documented and immediately transported in a sealed zip lock bag to the fish health unit for evaluation. Moribund fish shall remain in the system and brought to the attention of the fish health representative to determine the disposition of the animal. Any animals to be removed shall be euthanized in the facility and transported as outlined above.
6. Materials taken into the quarantine facility may not be removed from the facility for any reason unless there has been adequate disinfection. Adequate disinfection can only take place when animals have been removed from the facility and the entire facility can undergo decontamination. For this reason it is necessary that only essential items be brought into the quarantine area.
7. Refuse generated within the facility should be sealed in biohazard bags and brought to the fish health center to be autoclaved.
8. The study facility will be dismantled and sterilized upon study completion by Game and Fish personnel, with assistance from center staff.

Acclimation to Laboratory Conditions

“Fish should be given time to acclimate to new environments, feeds, and routine activities before being used in studies. Slow acclimation to change often is critical. It is not uncommon for fish to exhibit acute health problems 48 to 72 hours following transfer. A commonly used acclimation period is 2–4 weeks.” For this reason, NMDGF personnel will attempt to bring in sufficient study specimens at least a month prior to

onset of study so that fish can be treated for stress and disease, and acclimated to a feeding regime before the study. This is particularly important to this study, as the immunological response to glochidial attachment is unknown, and any stress response could mask the desired unknown.

Disposition of study animals: All study animals will be euthanized with the exception of the gray redhorse. If a suitable location such as a public aquarium is identified, these fish may be donated to a public facility. No fish should be released to the wild after the study concludes.

Schedule: Sampling for study specimens will begin in May, so study facility will be operational by May 1, 2006 to allow the biofilters and system to be tested prior to bringing in fish. Study will begin in May 2006 and terminate by July 2006.

Estimated Costs:

Supplies:

setup of the containment area and the study tanks:	\$400
Labcoats, boots, chemicals for decontamination	\$200

Labor:

Setup (\$15.00/hourly/40 hours)	\$600
Daily maintenance and water quality (\$15/1.5 hours/15 days)	\$400

Utilities for 1.5 months	\$600
Miscellaneous related costs	\$300

<i>Total estimated cost to facility:</i>	<i>\$2500</i>
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Intended Method of Information Dissemination:

Technical information bulletin, in conjunction with NMDGF to be completed by September 30, 2006.

Reference Literature:

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Appendix 2: Antibody Detection

Glochidia preparation

Materials:

Lab notebook
Glochidia (frozen)
Secure container for counting glochidia
1.5 mL microcentrifuge tube
Blue Sharpie
Volumetric pipette
TBS (Tris-buffered saline)

Procedure:

- 1) Remove glochidia from the freezer and monitor them as they thaw. Work with them as soon as they are thawed.
- 2) Place glochidia on the plankton net sieve and rinse with distilled water and then TBS.
- 3) Rinse the glochidia out of the plankton net sieve and into a Petri dish.
- 4) Begin counting the glochidia by using the suction tubes and the microscope, transferring glochidia to a 1.5 mL microcentrifuge tube.
- 5) Dilute glochidia to 1 glochidium per microliter of TBS by adding TBS with a volumetric pipette.
- 6) Use pestle to grind the glochidia, by pressing down and twisting handle.
- 7) Label glochidia vial with date, number of glochidia and identify which TBS solution was used; record these data in the lab notebook as well.
- 8) Store in refrigerator for maximum of 1 week, preferably use the same day as preparation.

Ouchterlony Double Diffusion Method

Materials:

Lab notebook
1 or more small Petri dishes
Balance
Disposable weigh boat
50 mL flask
0.25 g Seachem Agarose

Known antigen solution (e.g. Ovalbumin)

Known antibody solution (e.g. serum against albumin-AntiOvalbumin)

Samples:

Prepared glochidia

Fish serum

Disposable transfer pipet

Pipet (20 uL)

Yellow pipet tips

Buffer (current buffer TBS - Tris-buffered saline)

Procedure:

- 1) Record the samples that you are planning to use and the controls and their location on the gel and in the lab notebook.
- 2) Weigh 0.25 g of agarose in a weigh boat and place in flask by folding the weigh boat in half and placing tip into flask and tapping boat.
- 3) Add 20 mL of buffer solution to flask.
- 4) Cover flask with plastic wrap (leave, or make, a small hole) and microwave for 50 sec.
- 5) Allow the gel to cool to room temperature (you may run the flask under tap water to cool it faster).
- 6) Pour the gel into the smaller of the 2 parts of the Petri dish.
- 7) Allow the gel to set up until it is slightly whitish and solid.
- 8) Squeeze the bulb of the pipet and insert pipet tip into the gel.
- 9) Turn the pipet back and forth about $\frac{1}{4}$ - $\frac{1}{2}$ turn in the gel and release bulb.
- 10) Remove the pipet and repeat to make enough wells in the gel.
- 11) Turn the dish over and label all wells.
- 12) Transfer 20 uL to each pair of wells, filling one with antigen (Ovalbumin or glochidia) and the other with test solution (antiOvalbumin or fish serum); make sure that each well containing antigen is next to a well containing test solution.
- 13) Carefully place the dish in the 37° C incubator (24 hours) or on a shelf (somewhere safe and unlikely to be disturbed, 72 hrs)
- 14) Observe for banding between antigen and antibody wells. Bands are half-moon shaped and are an opaque, white color.

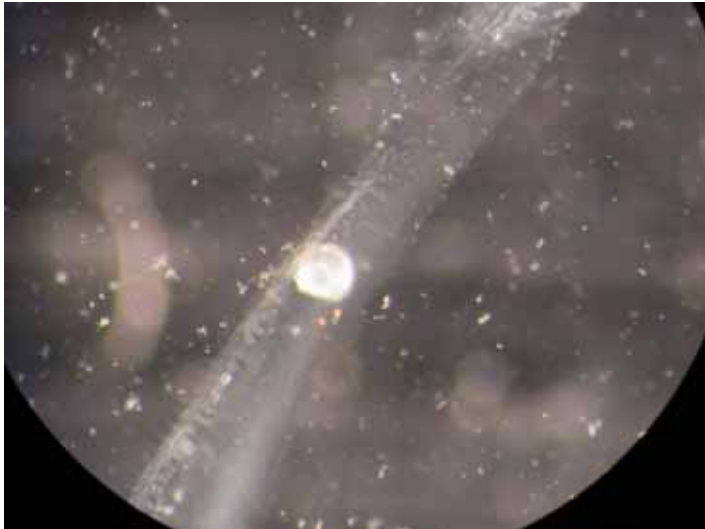


Figure 1. Microscopic view of a mussel gill with encysted glochidium from the first laboratory infestation. Gill was cleared with KOH.

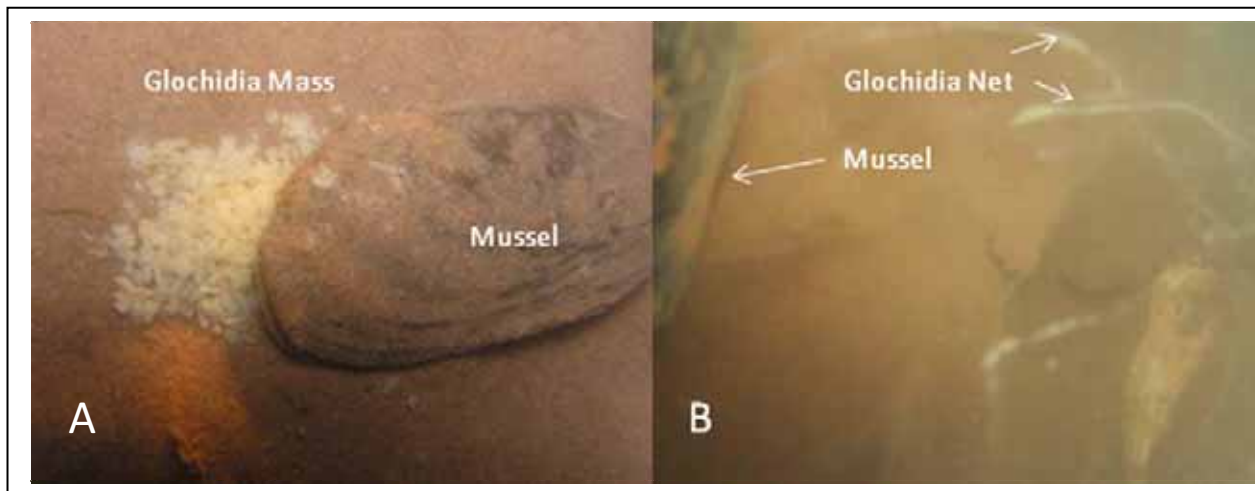


Figure 2-A. First glochidial mass observed in captivity
Figure 2-B. Example of glochidial mass observed in 2006

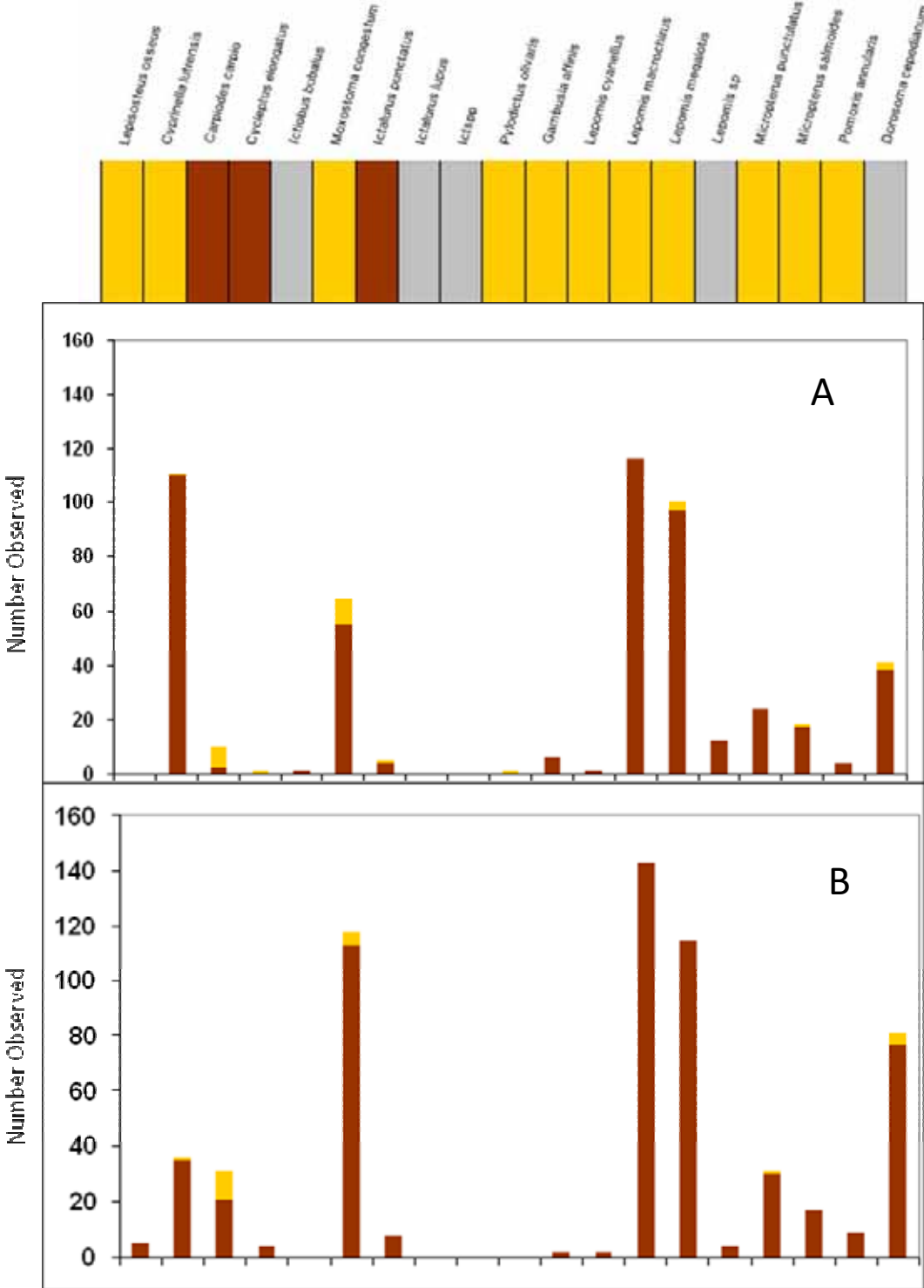


Figure 4-A. Top panel - physiological host/non-host determinations (Gordon 2000), Middle panel - 2006 fish data
Figure 4-B. Lower panel - 2007 fish data

Red = nonhost species; yellow = host species; gray = not tested by Gordon (2000)

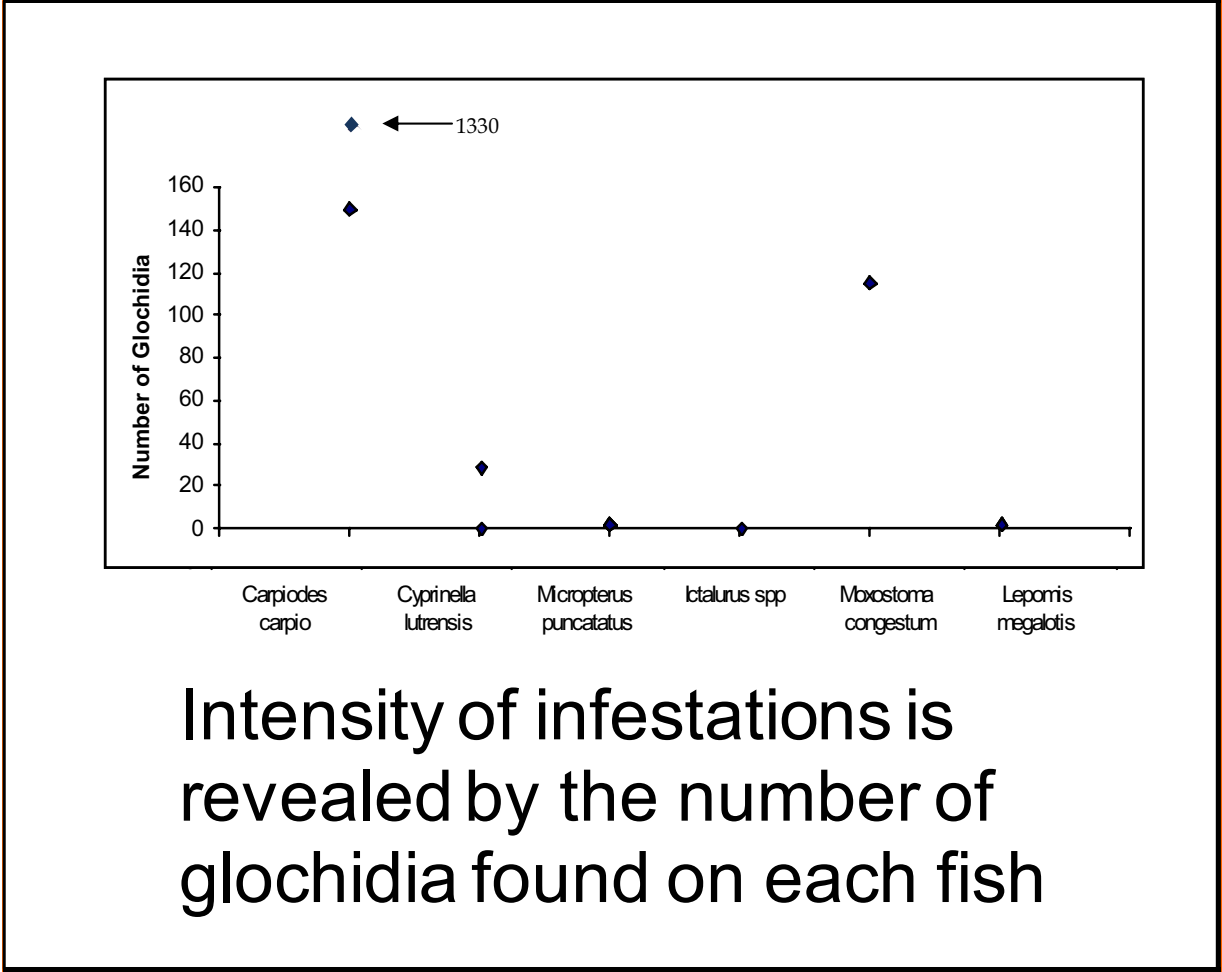


Figure 5. Infestation intensity differed between species.

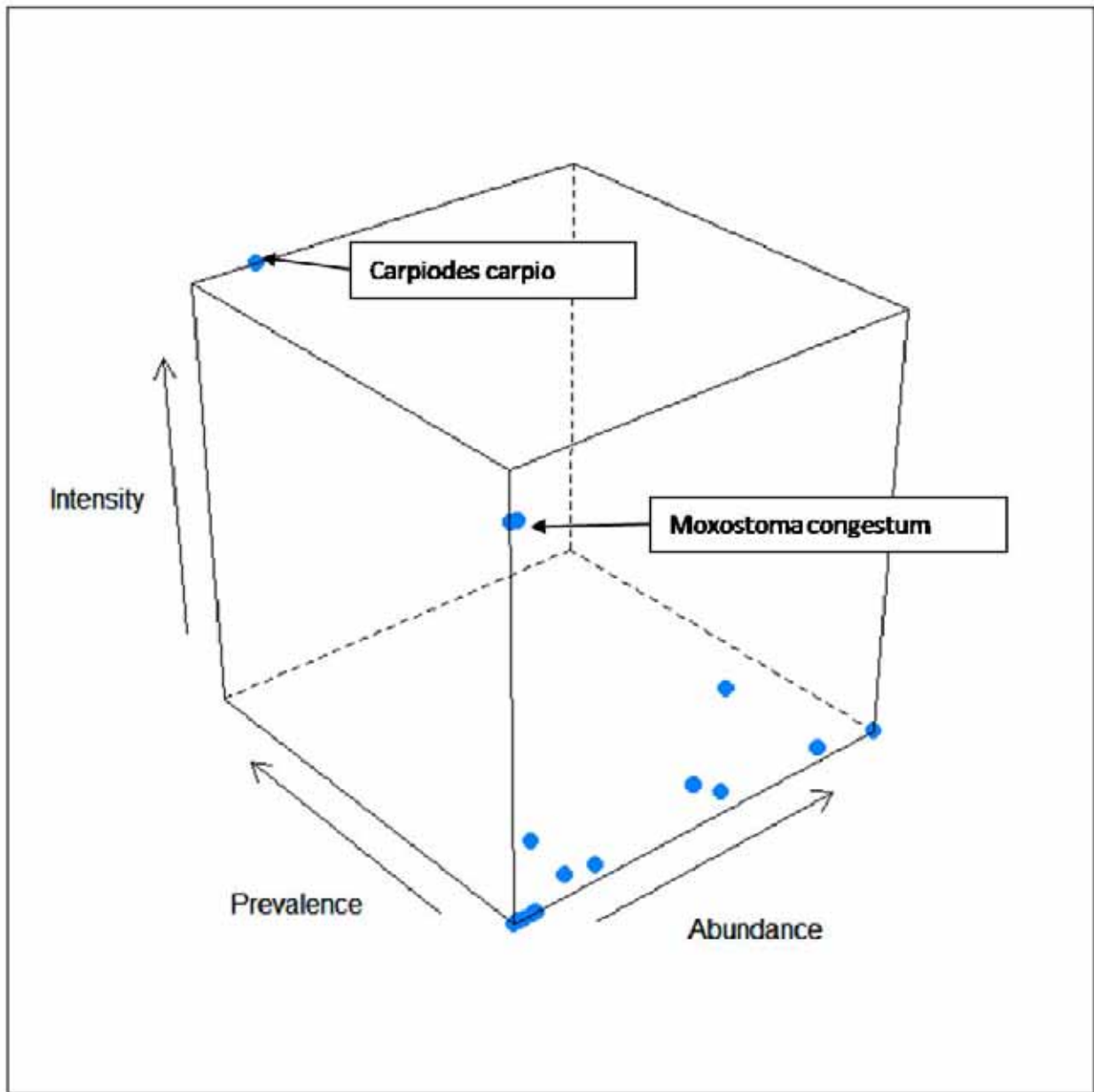


Figure 6. Data from all species encountered in field sampling with values averaged across years. Because blue sucker were returned live to the Black River, infestation intensity was not estimated.

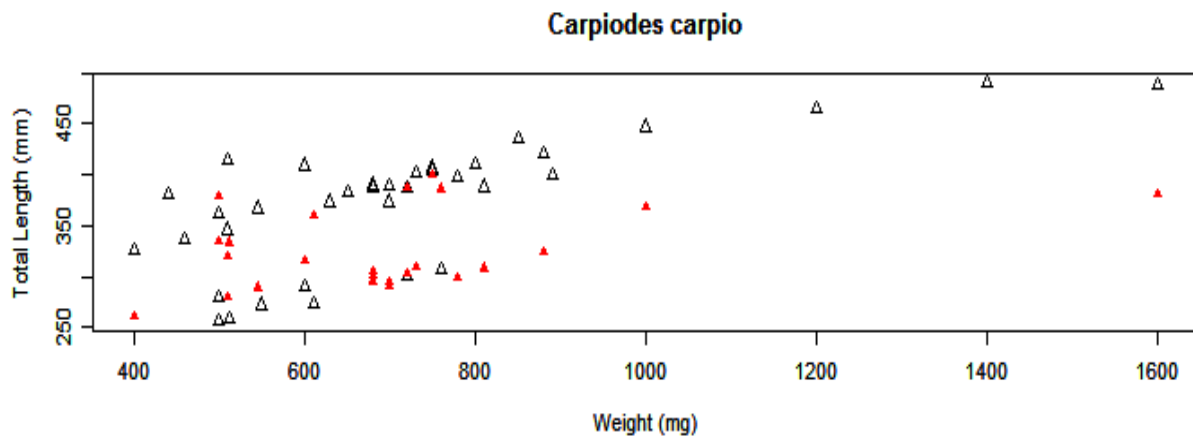
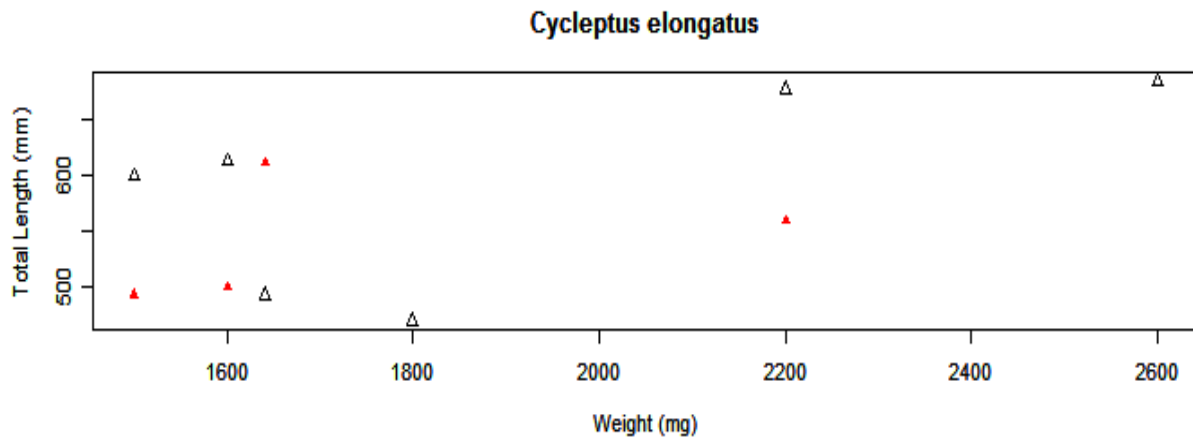
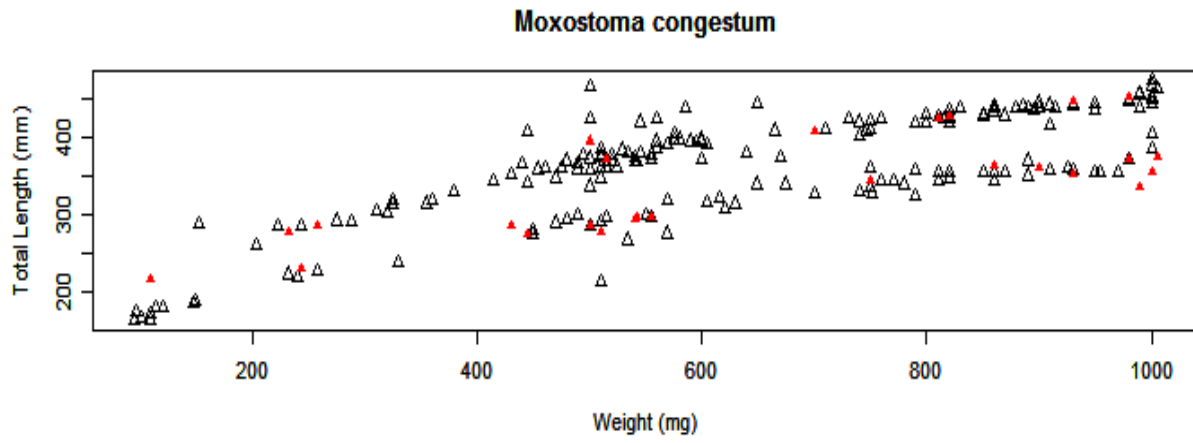


Figure 7. Length and weight of infested (red triangles) and uninfested (open triangles) fishes from the three ecological host species. The interspersing of the open and red triangles indicates no difference in condition of infested and uninfested fish.