Final Report- HWY-2009-16
Propagation and Culture of Federally Listed Freshwater Mussel Species

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16- Abstract

Road and related crossing construction can markedly alter stream habitat and adversely affect resident native flora. The National Native Mussel Conservation Committee has recognized artificial propagation and culture as an important potential management tool for sustaining remaining freshwater mussel populations and has called for additional propagation research to help conserve and restore this faunal group. While not a substitute for habitat preservation, this technology can help bypass some of the bottlenecks in natural population growth and colonization of new habitats. These studies were initiated to build on prior efforts focused on the captive propagation of four endangered and threatened species of freshwater mussels native to North Carolina. Specific objectives included: 1) Determining the required host fish species of two federally endangered mussel species in North Carolina, the Tar River Spinymussel (*Elliptio steinstansana*), and the Dwarf Wedgemussel (*Alasmidonta heterodon*), 2) Attempting to propagate and culture federally endangered freshwater mussels in North Carolina, and 3) Conducting laboratory experiments to determine the effects of temperature on the growth and survival of mussel species propagated. Initial efforts prompted the addition of studies focused on the role of shredding insects in providing important particles of nutritional value to juvenile mussels. Fantail darters (*Etheostoma flabellare*) were identified as efficient hosts supporting the metamorphosis of *Alasmidonta heterodon*. The mottled sculpin (*Cottus bairdi*) was the most efficient fish host for rearing *Alasmidonta raveneliana* and white shiners (*Luxilus albeolus*) proved the most efficient for *Elliptio steinstansana.* Specific management protocols were devised for improving fish collection, transport, quarantine and holding and preventing predation of recently metamorphosed juvenile mussels. A preferred initial temperature of 13^{o}C transitioning to a grow out temperature of 16^{o}C was identified for *Lasmigona decorata*, and subtrate and aquaria holding preferences for each species were identified. Insufficient knowledge about the nutritional requirements of recently metamorphosed juvenile mussels was identified as a prominent factor limiting propagation success and juvenile growth. The culture and use of the byproducts of shredding insects, and in particular *Tipula* sp., when they reprocess leaves was identified as a potentially nutrient rich resource for enhancing the diets of some species of juvenile mussels in captivity. Taken together these studies have markedly advanced our knowledge of factors that contribute to the successful captive propagation of freshwater mussels.

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Executive Summary

The goal of this project was to improve propagation and culture techniques for four federally endangered species in North Carolina: the dwarf wedgemussel (*Alasmidonta heterodon*), Appalachian elktoe (*Alasmidonta raveneliana*), Carolina heelsplitter (*Lasmigona decorata*), and Tar River spinymussel (*Elliptio steinstansana*). The initial knowledge base and research objectives varied for each species.

Alasmidonta heterodon needed additional host determination studies, and we conducted trials in both 2009 and 2010. While several species facilitated transformation, we determined that fantail darters (*Etheostoma flabellare*) were the most efficient of those tested. We maintained survival of juvenile *A. heterodon* up to one year, and growth was comparable with other species previously cultured in the laboratory.

With *Alasmidonta raveneliana*, we attempted to determine which fish host was most efficient at producing juveniles from a number of known fish hosts. While the mottled sculpin (*Cottus bairdi*) faired poorly in captivity, it was the most prolific host for juvenile mussel production. Juveniles survived to 1 month but were not seen alive after the initial 30-day observation period.

We had determined the best hosts for *Lasmigona decorata* in previous research but sought to refine grow out techniques and evaluate the effects of different temperatures on juvenile growth and survival. While proliferation of Chironomidae in the test chambers inhibited growth and survival, we were able to draw some conclusions from that experiment. We suggest future attempts to culture the species hold fish infested with glochidia at 13°C and start their grow out at that same temperature. The grow out temperature could then slowly be raised to 16°C. We monitored individuals propagated in 2007 for the duration of the project in the hatchery setting. While growth was slower than expected at the Marion Conservation Aquaculture Center (CAC), survival was over 90%, and they reach adulthood and spawned in the fall of 2009. We also conducted additional research into the use of serotonin for extracting glochidia from gravid adults. Two conglutinate producing mussel species (*Ptychobranchus subtentum* and *Strophitus undulatus*) were immersed in varying concentrations of fluoxetine or serotonin to assess those chemicals' effect on glochidial release and viability. Serotonin at concentrations of 20-40 mg/l proved most effective in inducing glochidial release in those species without affecting viability. We held *P. subtentum* in the hatchery for 7 months following the experiment and saw very little mortality and no differences between treatments and the control group. We saw inconsistent results with *L. decorata* when they were immersed in serotonin. Some individuals responded well and released virtually their entire brood in 24 hours, others released very little. Some mortality was seen following exposure, but other exposures seemed to cause no negative effect. The mechanism of glochidial release of *L. decorata* appeared different than the two other species tested. This may have accounted for some of the differences observed in the effectiveness of serotonin.

With *Elliptio steinstansana*, we compared the effectiveness of various fish hosts and found that the white shiner (*Luxilus albeolus*) was the most effective of those tested.

We had complete mortality of all juveniles propagated in 2009 due to an infestation of predacious flatworms in the culture chambers. In 2010, we took several measures to eliminate flatworms from the culture environment, and those were successful. Still, survival in downwellers was around only 10% after 2 months. There was no survival of newly transformed juveniles after 2 months in sediment in recirculating systems, but mussels older than 2 months faired well when cultured in sediment. We saw no obvious differences in growth and survival rate between sediment types. Mussels propagated in 2008 and moved to the hatchery in 2009 all survived and grew rapidly, reaching sizes almost as large as the wild caught adult broodstock.

Additional studies focused on improving our understanding of freshwater mussel nutrition, and improving the diets of captive reared mussels. These studies were the focus of a masters degree student's thesis work. The studies documented that shredding insects, and particularly *Tipula,* can provide small particles for juveniles mussels. Juvenile mussels held in tanks with *Tipula* and access to the fine particulate organic matter they generate displayed greater growth rates than animals that were not held in the same tanks as *Tipula*. Follow-up studies are needed to assess the nutrient value of the enhanced particle load, and how *Tipula* can be used to contribute to the feeding of juvenile freshwater mussels reared in captivity for eventual release and population augmentation.

The body of work conducted has help refine techniques for the captive propagation of the endangered freshwater mussel species that were studied. Effective host species for supporting glochidial transformation of larval mussels have been identified or confirmed, and collection, transport, housing and quarantine protocols for host fish have been revised. Procedures accommodating the release of glochidia have been documented, and more effective larval attachment procedures have been devised. Environmental controls have been effectively implemented to reduce predation and reduce juvenile mortality, and substrate requirements of some species have been more clearly defined. Clear targets for additional work have also been identified. Additional work is needed to improve the overall nutritional health of freshwater mussels held in captivity and in particular the diets of recently transformed juveniles. The work with *Tipula* needs to continue and explore their potential captive culture so they can be used to produce nutrient rich infusions for feeding juvenile mussels. Species-specific dietary requirements need to be studied to improve the survival and growth of recently transformed juveniles, and maintenance diets for older juveniles need to be defined. Protocols for inducing reproductive activity need do be devised so adult spawning can be induced and less dependent on costly field surveys to collect the few remaining adults animals. An additional mussel propagation facility in the Piedmont is needed to support the propagation of Piedmont and Coastal Plain species. Nonlethal techniques for assessing the health of mussels need further development to ensure individual animals can be studied without having their health impaired. Taken together, this body of work has yielded some vital information supporting the continued development of techniques for the captive propagation of freshwater mussels. However a substantial amount of work remains before the successes achieved propagating some prior species can be achieved with the endangered species that were the subject of these studies.

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INTRODUCTION AND BACKGROUND

North Carolina has one of the fastest growing populations in the United States, and the North Carolina Department of Transportation (NCDOT) is tasked with providing necessary infrastructure for the state's growing communities. As development spreads across the landscape the construction of crossing structures for roads can adversely alter stream habitat, and riparian vegetative cover. Cumulative changes from urban sprawl can reduce water infiltration, and stream flow. The Endangered Species Act requires protection of habitat surrounding federally listed species. Construction and permitting is often restricted or slowed by these environmental regulations. Increasingly the concern surrounds one of six federally endangered freshwater mussel species living in North Carolina. Once listed, for a species to be considered for removal from the endangered species list, new populations must be established. Biologists have explored artificial propagation and culture of freshwater mussels as one method for establishing new populations and augmenting remaining populations before they are listed. The National Native Mussel Conservation Committee (NNMCC 1998) has recognized artificial propagation and culture as an important potential management tool and has called for additional propagation research to help conserve and restore this faunal group. While not a substitute for habitat preservation, this technology can help bypass some of the bottlenecks in natural population growth and colonization of new habitats.

Freshwater mussels have a unique lifecycle that requires their larvae (called glochidia) to attach to the gills or fins of a host fish to metamorphose into the next life stage (the juvenile stage). Often this relationship is quite species-specific with a particular species of mussel requiring a particular species of fish or small group of closely related fish. Because of the relatively sessile nature of mussels and their complete dependence upon a host fish, natural reproduction becomes less likely as populations dwindle. This downward spiral occurs because females are less likely to encounter males, and those females that do successfully spawn are less likely to encounter a fish and infect it with their glochidia. Artificial intervention through propagation and culture may be the only way to bypass this bottleneck and achieve recovery with some species.

In 2004, NCDOT made an initial investment in artificial propagation and culture at our laboratory at North Carolina State University College of Veterinary Medicine (CVM) that is now showing great promise. Initial propagation protocols developed in existing propagation facilities (Mussel Barn a-6) established with support from the NC Biotechnology Center, College of Veterinary Medicine, and the US Fish and Wildlife Service were refined. Nine species were propagated, and grow out raceways were established at a state fish hatchery (Table Rock, a-7) in Morganton, NC. These raceway facilities enabled good growth and survival of a variety of mussel species initially propagated in the CVM Mussel Barn that were then transferred to the hatchery. In 2007, we began experimentation with use of a pond at the NCWRC Marion Fish Hatchery for mussel grow out. This water source also provided good growth and survival for multiple species. The facility at Marion (a-8) was expanded, and a building renovated to support mussel propagation and grow out, which expanded grow-out capacity to 4-5 times what was available at the time. This space, along with our existing space at Table Rock has

supported the growth of juvenile mussels propagated in the Mussel Barn. These hatchery facilities not only provided additional space for the work that was conducted during this project but also provided redundancy to prevent catastrophic loss of propagated mussels.

Figure a-1. Two-year-old Eastern Lampmussels (*Lampsilis radiata*)

Figure a-2. 17-month-old Creepers (*Strophitus undulatus*)

Figure a-3. Eastern Pondmussels (*Ligumia nasuta*) 20 mm in only 4 months.

Figure a-4. Two-year-old Eastern Creekshells (*Villosa delumbis*) are among eight species that we have propagated that have matured and spawned at the hatchery.

Because of the success demonstrated in the first research grant from the NCDOT, the NC Wildlife Resources Commission (NCWRC) provided monetary support for our propagation efforts in fiscal years 2007 and 2008. Since that time, mussels propagated during the grant from NCDOT have been released into the wild. Additionally, we determined the required host fish species for the Carolina Heelsplitter (*Lasmigona decorata* - a federally endangered species) and had relatively good early success in maintaining growth and survival of those juveniles in the laboratory (Fig. a-5). The US Fish and Wildlife Service and NCWRC have both been actively supporting continued propagation efforts and have provided funds complementing those provided by NCDOT. This unique conservation partnership reflects the best of what can be accomplished when agencies and academia work together to address conservation and societal issues.

Figure a-5. Eight-month-old Carolina Heelsplitters (*Lasmigona decorata*) propagated and cultured in the NCSU Freshwater Mussel Propagation Facility (Mean length = 5 mm).

The majority of propagation and culture research by our laboratory and others has been done with mussels from the subfamily Lampsilinae (Jones et al. 2005, Barnhart 2006, Eads et al 2006). The survival and growth of the Carolina Heelsplitter in our laboratory represents not only success with a federally endangered species but progress in an entirely new subfamily of freshwater mussels (the Anodontinae) – a subfamily with different habits and life requirements. We have now successfully cultured three Anodontinae species in captivity, the Carolina Heelsplitter (*Lasmigona decorata*), the Creeper (*Strophitus undulatus*), and the Slippershell Mussel (*Alasmidonta viridis*). These studies were initiated to further refine culture propagation techniques and attempt to translate these initial successes for the propagation of North Carolina's other endangered species in the same subfamily – the Dwarf Wedgemussel (*Alasmidonta heterodon*) and the Appalachian Elktoe (*Alasmidonta raveneliana*).

In prior studies we have recognized that juvenile mussels grow more rapidly when provided with a natural water source. The majority of laboratories culturing freshwater mussels feed newly transformed juveniles only cultured or commercially obtained algae. Little is known about freshwater mussel nutrition and diet preferences (Nichols and Garling 2000) and nutritional factors may be contributing to the poor growth of some species in propagation laboratories. As filter feeders, they ingest whatever is in the water column, and reject what they don't send to the stomach as pseudofeces. The mussel's gills have been shown to help sort out food particles from non-food particles, but even particles that are sent to the stomach are not necessarily digested and used for energy. Mussels consume various types of algae, but recent studies have shown some mussels get their nutrition from bacteria in their natural environment (Nichols and Garling 2000). Bacteria may be the main food resource for some mussels. Juvenile rainbow mussels (*Villosa iris*) have been shown by Yeager and coworkers to eat primarily bacteria (1994). Bacteria in streams that are available to mussels for food are found attached to the fine particulate organic matter (FPOM) floating in the water column. As seen in marine

bivalves, Nichols and Garling (2000) suggest that as freshwater mussels filter feed, they strip the bacteria off of FPOM detritus (Prirur *et al* 1990). Fine particulate organic matter may play an important role in freshwater mussel nutrition.

Fine particular organic matter detritus is created by the break-up of leaves into small particles as the leaves decompose and the process is faster with the help of shredding invertebrates. Aquatic insect shredders accelerate the detritus breakdown process by increasing surface area for microbial colonization, which further decomposes the material and releases nutrients (McDiffett 1970). The bacterial rich FPOM created by shredding insects may be providing an important food source for freshwater mussels. But algae-based captive propagation diets for freshwater mussels may be lacking the rich bacterial-provided nutrients that are present in streams and the native natural water sources fed in Marion and other hatcheries. We conducted a series of experiments to determine the types and quality of FPOM being generated by shredding insects and the potential nutritional value it may have for the captive propagation of freshwater mussels.

Definition of Need

Freshwater mussels are filter feeders that play a vital role in stream ecosystems and benefit humans by removing algae, bacteria, detritus, and even fine sediment from North Carolina's surface waters. There are six federally endangered mussel species known to be living in North Carolina. Many of the other approximately 60 species in the state are in various stages of decline with some approaching critical levels of endangerment. Mussel populations have been impacted by a wide range of human activities (Bogan 1993). Freshwater mussels are indicators of water quality and environmental health, and the loss of freshwater mussels from streams reflects a sufficient decline in water quality, stream hydrology or habitat to adversely effect mussel survival or propagation.

The ongoing drought is taking a further toll on mussel populations and has increased the need to further propagation and culture efforts in the state. Although the broad distribution of freshwater mussels and their abundance once enabled populations to recover from periods of extended drought, habitat changes, and declining populations have made these species less resilient. Many headwater streams where our rare mussel species (e.g. *Lasmigona decorata*) occur are drying up, and mussels are dying. While they have some ability to burrow into the substrate and survive a dry stream bed for a time, this adaptation has its limits. Many are unable to completely bury, and those that do, must eventually have water for respiration, feeding, and removal of wastes. Because of human-induced declines in water and habitat quality over the last century, many mussel populations that were once found throughout entire watersheds are now restricted to a few protected headwater streams. Mussels that once occupied larger rivers could re-populate headwater streams that were decimated by extreme droughts. Now, many of those populations in larger river channels do not exist in the state, and restoration of many isolated headwater populations will not occur naturally when normal water levels return. Particularly susceptible are the federally endangered Carolina Heelsplitter, Dwarf Wedgemussel, and Tar River Spinymussel (*Elliptio steinstansana*). Other imperiled species, not yet federally listed are also being affected. Artificial means, such as propagation and culture will be vital in restoring these populations once normal water levels return to the smaller streams.

Propagation and culture of freshwater mussels is still in its early stages as a science. With some mussel species, even the basic question of what host fish species the mussel requires is unknown. Prior to these studies, that was the case for the Tar River Spinymussel, an endemic NC species approaching extinction. Laboratory experiments were needed to determine which host fish that species requires. Artificial propagation to support this species and others could not occur until this research and similar research with other species was conducted. Some fish host research had been done with the Dwarf Wedgemussel in North Carolina (Michaelson and Neves 1995), but this research used an incomplete list of fish species and used fish from outside North Carolina. Because fish species from various river basins had been shown to vary in their ability to serve as hosts of the same species of mussel (Eckert 2003,), we believed additional hosts may have existed beyond what was reported by Michaelson and Neves (1995).

Mussels in this subfamily often use a wide range of host fish species (Gray et al. 1999, Eads et al 2006). Conducting more complete host fish research on this species was needed to support the propagation of these species for eventual augmentation of that species in the state. Once juvenile mussels have been propagated, many obstacles to their successful grow out until they reach reproductive age challenge their survival in captivity (and the wild). Basic requirements such as diet, temperature preferences and how these vary between species need additional study. Some species have faired well in captivity while others have not, but the reasons for these differences are unknown. Our early success with some Anodontinae indicated that lower temperatures may be beneficial to some mussels, but well executed experiments were needed to determine optimal temperature for growth and survival of these species. These studies have continued the progress made at the College of Veterinary Medicine Freshwater Mussel Propagation Laboratory with the support of NCDOT, the US Fish and Wildlife Service, NC Wildlife Resources Commission, the university and the NC State Museum of Natural Sciences. Additional research is needed to apply these initial successes to other federally listed mussels and other species approaching critical levels of imperilment in the state.

RESEARCH OBJECTIVES

The objectives of this research were to:

1-Attempt to determine the required host fish species of two federally endangered mussel species in North Carolina – The Tar River Spinymussel (*E. steinstansana*), and the Dwarf Wedgemussel (*A. heterodon*),

2-Propagate and culture federally endangered freshwater mussels in North Carolina,

3- Conduct laboratory experiments to determine the effects of temperature on the growth and survival of mussel species propagated, and

SITES AND FACILITIES

Fish holding and quarantine, and fish host trials were conducted in various size aquaria (~ 1- 600 gal.) in the Mussel Barn (~ 1,450 sq. ft.), a wet laboratory on the campus of the North Carolina State University College of Veterinary Medicine 35 ^{o}47´58.54´´N, 78^{o}42´03.02´´W. Temperature, substrate, holding and equipment trials were also conducted in the Mussel Barn. Studies with shredding insects were conducted in a second, wet laboratory (~ 2000 sq. ft) on the CVM campus. Both facilities have central air conditioning and heating and relatively constant ambient temperatures. With one exception, some fluctuation was noted in recorded temperatures in the larger of the two buildings during the winter months, until a heating unit was repaired. Two hatcheries were used to support these studies, one in Table Rock, NC (36 ^{o}56´23.23´´N, 80^{o}03´25.94´´W) NC, the second in Marion NC (35 ^{o}43´40.15´´N, 82^{o}01´21.80´´W). Both are operated and maintained by NC Fish and Wildlife Commission staff.

Figure a-6: Mussel Barn, NCSU College of Veterinary Medicine

Figure a-7: Interior of the mussel facility at Table Rock Hatchery.

Figure a-8: Interior of the mussel facility at Marion Hatchery.

Chapter 1

Dwarf wedgemussel
Alasmidonta heterodon

Introduction

The dwarf wedgemussel (*Alasmidonta heterodon*) is a federally endangered species that occurs along the Atlantic slope from the Neuse River Basin in North Carolina north to the Petitcodiac River Basin in New Brunswick, Canada (Bogan 2002). In NC, the species is becoming increasingly difficult to find with two previously thriving populations currently being impacted by drought and beaver activity (personal observation). One fish host determination study had been previously published for this species (Michaelson and Neves 1995), but the number of fish species tested was limited, and one of the primary hosts (*Cottus bairdi*) does not co-occur with *A. heterodon* in North Carolina. The objective of this portion of the study was to evaluate a more complete list of sympatric fish species that could potentially serve as viable hosts for captive *A. heterodon* propagation. Any juveniles propagated were to support additional research or potential population augmentation.

Methods

Host Trials

2009 – In 2009, we tested a total of 18 fish species across 6 families as potential hosts for the dwarf wedgemussel. On 26 January 2009, 3 gravid *A. heterodon* were collected from Rocky Swamp just upstream of NC 561 in Halifax County, NC. Host fish for that trial were collected from the Tar River at US 158 as well as below Gooch's Mill Dam in Granville County, NC. On 27 January, a water-filled syringe was used to flush glochidia from the gills of the gravid females. Glochidia were placed with the host fish (Table 1-1) in 6 liters of water at an estimated concentration 2,000 glochidia/liter and heavily aerated to suspend the larvae. Glochidia concentration was estimated by taking a 50-ml sample from the 6-liters of water and counting the glochidia using a dissecting microscope. Glochidia were sampled from the suspension every 5 minutes to monitor whether they were snapping shut in suspension. The infestation was completed after 20 minutes once all glochidia in suspension had closed, and fish were moved into separate aquaria by species. Fish were maintained at 13 ± 1°C, and aquaria were monitored routinely for fish mortality, sloughed glochidia and transformed juveniles.

2010 – In 2010, we primarily focused on darter species and added the fantail darter (*Etheostoma flabellare*), which was not tested in 2009. On 23 February 2010, a single gravid *A. heterodon* was collected from Shocco Creek at Shocco Springs Road in Warren County, NC. Host fish were collected from the Tar River below Gooch's Mill Dam. On 25 February 2010, we extracted approximately 1,200 glochidia from the adult mussel by flushing the gills with a water filled syringe. The marsupia were not completely flushed for fear of causing excess damage to the gill tissue. Glochidia were placed in 1.5 liters of water with host fish (Table 1-2) and heavily aerated for 35 minutes. Fish were separated by species and held at 13 ± 1°C for the duration of the encystment. Aquaria were monitored routinely for fish mortality, sloughed glochidia, and transformed juveniles.

Juvenile Grow out

2009 – Juveniles were placed in an 8-liter container with a thin layer (approximately 10 mm deep) of fine sediment (< 300 µm grain size). An airstone provided light aeration, and mussels were fed daily with a mixture of live cultured algae (*Scenedesmus* sp.) as well as two commercially available algal products, Shellfish Diet 1600® and Nanno 3200® (Reed Mariculture, Campbell, CA) at a concentration of 50,000 - 100,000 cells/ml. Water changes were performed weekly. The container was held in a chilled water bath at 13 ± 1°C, and growth and survival were monitored at 1, 2, 3 and 10 months.
2010 – Juveniles were placed in 6 separate recirculating culture systems (Fig. 1-1). The systems consisted of a 45-liter reservoir and five 5-liter bowls for holding mussels. Each bowl had a thin layer of fine sediment (< 300 µm grain size) as a substrate for the mussels. A 200-µm-mesh basket was used on each bowl to catch any escaping mussels. Baskets were checked routinely, and any mussels found were returned to their respective bowls. Three of the recirculating systems were maintained at 13 ± 1°C and three were maintained at 16 ± 1°C. Systems were fed a mixture of Shellfish Diet 1800® and Nanno 3600® (Reed Mariculture, Campbell, CA) at a concentration of 30,000-50,000 cells/ml. Survival was assessed at 2 months.

Figure 1-1. Bowl recirculating system used to culture *Alasmidonta heterodon* in the laboratory in 2010.

Results

Host Trials

2009 - A total of 132 juveniles were produced from 9 different fish species, but no fish species proved to be substantially more efficient than the others (Table 1-1). Glochidia attached to darter species tended to take longer to transform than glochidia attached to other potential host species. However, substantial mortality of the hosts was observed during encystment and the total number of juveniles obtained from individual fish likely underestimated their true efficiency as hosts.

Table 1-1. Results from *Alasmidonta heterodon* host trials in 2009.

Species	Scientific Name	Number of fish	Total juveniles	Juveniles per fish	Days to Transformation
Aphredoderidae					
Pirate perch	*Aphredoderus sayanus*	2	13	6.5	17-27
Catostomidae					
Creek chubsucker	*Erimyzon oblongus*	1	0	0.0	-
Northern hogsucker	*Hypentelium nigricans*	1	0	0.0	-
Centrarchidae					
Redbreast sunfish	*Lepomis auritus*	1	3	3.0	17-20
Green sunfish	*Lepomis cyanellus*	1	2	2.0	20
Bluegill	*Lepomis macrochirus*	1	12	12.0	15-24
Largemouth bass	*Micropterus salmoides*	1	0	0.0	-
Cyprinidae					
Satinfin shiner	*Cyprinella analostana*	3	0	0.0	-
Bluehead chub	*Nocomis leptocephalus*	5	4	0.8	20-27
Highfin shiner	*Notropis altipinnis*	9	13	1.4	14-27
Swallowtail shiner	*Notropis procne*	10	9	0.9	37-43
White shiner	*Luxilus albeolus*	3	1	0.3	31
Pinewoods shiner	*Lythrurus matutinus*	8	4	0.5	15-24
Ictaluridae					
Margined madtom	*Noturus insignis*	1	0	0.0	-
Percidae					
Carolina darter	*Etheostoma collies*	2*	0	0.0*	-
Johnny darter	*Etheostoma nigrum*	9*	15	1.7	45-52
Chainback darter	*Percina nevisense*	7*	46	6.6	42-55
Roanoke darter	*Percina roanoka*	12*	10	0.8*	45-?*

* = Darter species experienced substantial mortality during encystment. Roanoke darters experienced complete mortality around the time that juveniles began falling off.

2010 - A total of 89 juveniles were produced from four darter species. High fish mortality was seen in darter species again in 2010. The two fantail darters (*E. flabellare*) were the best hosts tested (Table 1-2).

Table 1-2. Results of *Alasmidonta heterodon* propagation trials in 2010.

Species	Scientific Name	Number of fish	Total juveniles	Juveniles per fish
Fantail darter	*Etheostoma flabellare*	2	45	22.5
Johnny darter	*Etheostoma nigrum*	14	17	1.2
Chainback darter	*Percina nevisense*	13	8	0.6
Roanoke darter	*Percina roanoka*	31	19	0.6

Juvenile Grow out

2009 - Survival was 35% at 1 month and 17% at 2 months (Fig. 1-2). Surviving juveniles continued to grow at rates similar to other species in the laboratory (Fig. 1-3), but all individuals were dead after 1 year.

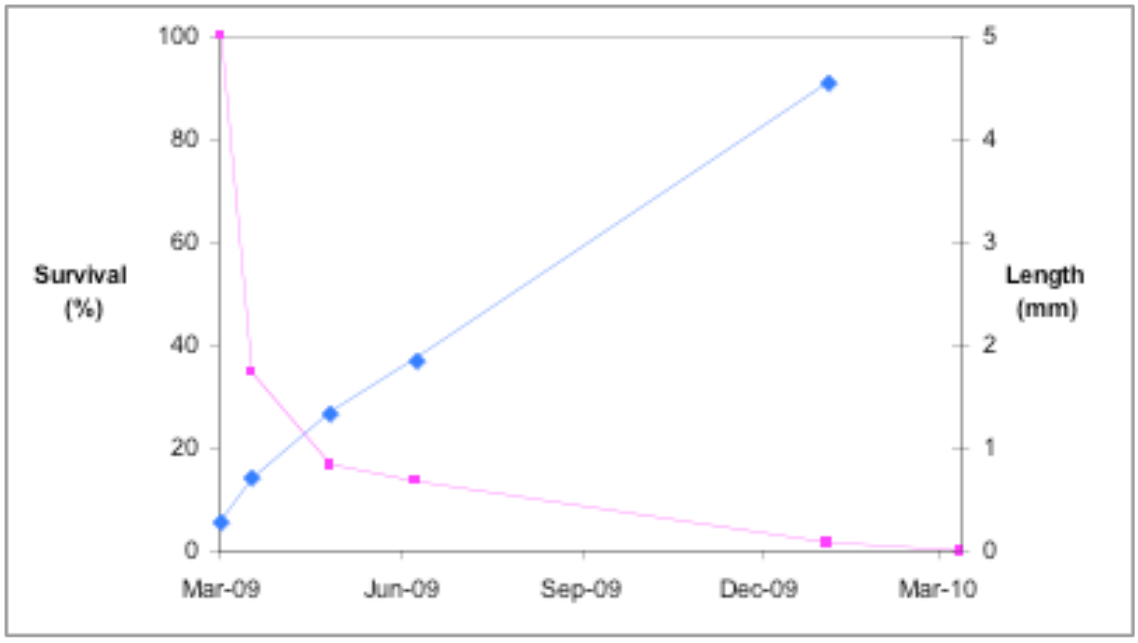

Figure 1-2. Survival and growth of cultured juvenile *Alasmidonta heterodon* in 2009.

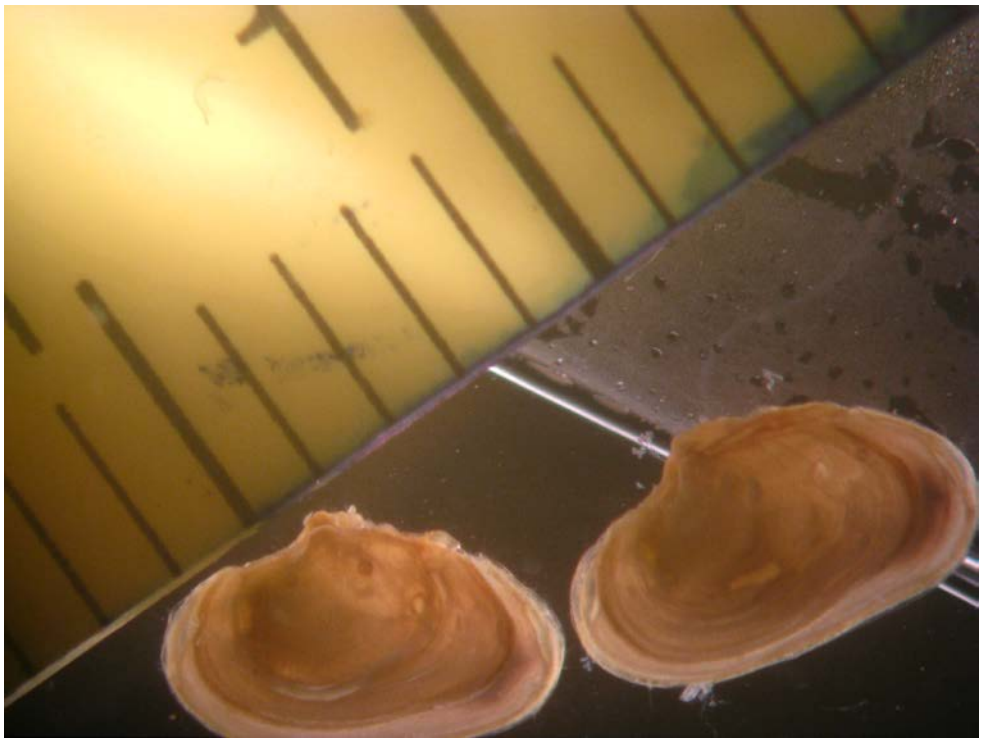

Figure 1-3. Laboratory cultured juvenile *Alasmidonta heterodon* at 10 months of age.

2010 - There were no surviving individuals after 2 months.

Discussion

Host Trials

Propagation of this species proved to be very difficult relative to most other species we have propagated in NC. The adults are hard to find and are only gravid in the winter when sampling is more difficult. Their small size yields not only a smaller brood but makes it difficult to safely extract a large number of glochidia for testing without doing damage to the mussel. The darters that served as the most efficient hosts did not fair well in captivity, and those juveniles that were produced required a long transformation period. We suggest future efforts to propagate this species utilize captive broodstock in a hatchery setting to make acquiring gravid adults easier.

Darter species appear to be the most efficient hosts for *A. heterodon*, and fantail darters were the most efficient host of all of those tested. While the one bluegill tested yielded 12 individuals, this was likely due to the relatively larger size of the individual compared to other species rather than to the efficiency of the species. Perhaps this species could be re-tested. The pirate perch yielded 6.5 juveniles per fish, perhaps in part due to the lack of scales that would prevent attachment of glochidia to the fish's skin. This species may serve as an important host in the wild since they likely share habitat where *A. heterodon* are found along undercut banks and near logs. We have observed

mottled sculpin, another scale-less species to serve as a very efficient host for a closely related species, *Alasmidonta raveneliana*. Mottled sculpin were also reported by Michaelson and Neves (1995) to be hosts for *A. heterodon*. While this fish does not co-occur with *A. heterodon* in North Carolina, it may be the best host alternative for laboratory propagation of this mussel. Additional research focused on optimum captive holding techniques for darters will be needed if the species tested are to be used for propagating *A. heterodon* in captivity.

Juvenile Grow out

Survival to 1 year and growth to 4.5 mm suggests some hope for grow out of this species in captivity. This growth rate is similar to other species propagated in the laboratory. We are unsure why the species failed to survive in 2010. Additional studies are required to determine the best diet and culture conditions for this species.

Chapter 2

Appalachian Elktoe
Alasmidonta raveneliana

Introduction

The Appalachian elktoe (*Alasmidonta raveneliana*) is a federally endangered species that occurs in tributaries to the Tennessee River in eastern Tennessee and western North Carolina. Since late 2004, the Little Tennessee River population of this species, which was once a stronghold, has experienced precipitous declines. Additionally, an acute die-off in the Cane River in North Carolina in 2008 has put further stress on this species. While these negative events have increased the need for propagation of this species, an additional opportunity exists to further establish this species. The recent re-licensing of the dam on Santeetlah Reservoir and the Cheoah River has restored minimum flows to a reach of the river that was formerly bypassed. These flows have opened up several river miles of potential habitat for re-establishing a viable *A. raveneliana* population. The purpose of this portion of the study was to evaluate the efficacy of reported hosts that can support the metamorphosis of *A. raveneliana* glochidia to juveniles and attempt the grow out of the successfully transformed juveniles.

Methods

Host Trials

2009 - On 20 April 2009, we infested 6 species of fish (Table 2-1) previously reported as hosts (pers. comm. Jim Layzer, Tennessee Tech University) for *A. raveneliana* using four gravid females collected from the Little Tennessee River in Macon County, NC. Fish were placed in 12 liters of water with an approximate concentration of 1,500 glochidia per liter. The water was aerated vigorously to suspend the glochidia. The exposure was ended after 20 minutes due to closure of the glochidia in suspension. Fish were then separated into different aquaria by species and held at 16 ± 1°C. Tanks were monitored closely for fish mortality and transformed juveniles.

2010 – Based on the fish host studies conducted during 2009, we focused efforts during the 2010 growing season on maximizing production of *A. raveneliana* with central stonerollers (*Campostoma anomalum*). On 2 April 2010, we infested 130 central stonerollers with glochidia from 6 adults collected from the Tuckasegee River in Jackson County, NC. Fish were placed in 18 liters of water with an approximate concentration of 4,000 glochidia per liter for 20 minutes. Vigorous aeration was used to suspend the mussel larvae. One small stoneroller was examined at the end of the exposure and found to have 42 glochidia attached to the fins or gills. Fish were maintained in aquaria at 16 ± 1°C and monitored for mortality as well as transformed juveniles.

Juvenile Grow out

2009 - Transformed juveniles were placed in an 8-liter container. Raleigh city water treated with sodium thiosulfate was used as the water source. The holding container was maintained in a chilled water bath at 16 ± 1°C. A thin layer (approximately 10 mm deep) of fine sediment (< 300 µm grain size) provided substrate for the juveniles. An airstone provided light aeration, and mussels were fed daily a mixture of Shellfish Diet 1800® and Nanno 3600® (Reed Mariculture, Campbell, CA) at a concentration of 50,000 - 100,000 cells/ml. Water changes were performed weekly. Growth and survival were assessed at 1 and 2 months post-metamorphosis.

2010 - During 2010 we split juvenile *A. raveneliana* into two separate bowls within the recirculating systems described in Chapter 1 that was maintained at 16 ± 1°C (Fig. 1-2). Each bowl had a thin layer of fine sand (< 300 µm grain size) as a substrate for the mussels. The system was fed a mixture of Shellfish Diet 1800® and Nanno 3600® (Reed Mariculture, Campbell, CA) at a concentration of 30,000-50,000 cells/ml. Survival was assessed at 1 and 2 months.

Results

Host Trials

2009 - A total of 1,143 juveniles were acquired from 5 of the species tested. The mottled sculpin (*Cottus bairdi*) proved to be the most efficient among those tested in 2009, but were among the more difficult to keep alive through the lengthy encystment period (Table 2-1). Juveniles transformed on sculpin between days 17 and 42 with a peak between days 30-37. Over that time period, we lost 53% of the sculpin infested. Central stonerollers survived captivity and encystment very well and served as a moderately efficient host. Metamorphosis happened much more quickly on darter species (<20 days), but the quality of the counted juveniles was not confirmed. The number of juveniles (Table 2-1) recorded for greenfin and gilt darters may have been inflated by a number of glochidia that were sloughed without going through full metamorphosis.

Table 2-1. Host trials for *Alasmidonta raveneliana* in 2009.

Species	Scientific Name	Number of Fish Infested	Number of Surviving Fish	Total Juveniles	Number of Juveniles per Fish
Mottled sculpin	*Cottus bairdi*	19	9	556	61.8
Central stoneroller	*Campostoma anomalum*	34	30	309	10.3
Gilt darter	*Percina evides*	10	0	125*	?
Greenfin darter	*Etheostoma chlorobranchium*	23	8	144*	?
River chub	*Nocomis micropogon*	14	14	9	0.5
Tuckasegee darter	*Etheostoma gutselli*	3	0	-	-

* = May not represent fully transformed juveniles

2010 - Following the infestation, a high number of glochidia could be easily seen attached to the fins of the stonerollers, but by day 3, many of these were sloughed. We collected a total of only 653 juveniles from 106 stonerollers (6.2 juveniles per fish) despite a heavy initial infestation.

Juvenile Grow out

2009 - At 30 days post dropoff there were only 8 surviving *A raveneliana*. Mean length was 510 ± 48 µm (Figure 2-1). The culture tank was heavily infested with Daphnia (*Daphnia* spp.), which would not only compete with juveniles for food but would also attach themselves to juveniles. A video of this behavior can be seen here: http://www.youtube.com/watch?v=XvzsfilGyPY. At two months, there were no survivors.

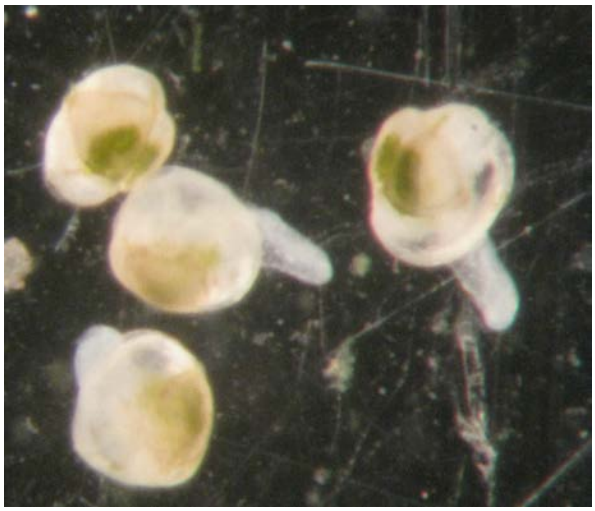

Figure 2-1. 1-month-old *Alasmidonta raveneliana*

2010 - At 1 month, there were 89 surviving juveniles (13.6%). Mussels had algae in their guts at that time and continued to actively feed, but all were still less than 400µm in length. At 2 months, there were no survivors.

Discussion

Host Trials

While mottled sculpin proved to be the most efficient host of those tested, they did not survive well in the systems in our laboratory during this study. One contributing factor is that sculpin are either in reproductive condition or are preparing to reproduce during the early spring when *A. raveneliana* were propagated. Collection, transport and holding of the sculpin in captivity during this period of reproduction when marked hormonal changes are occurring may reduce survival in captivity. In one study, Barrett and Grossman (1988) found that the handling of sculpin had a greater impact on survival in captivity than electrofishing. Because of the decreased survival in sculpin in 2009, we

chose to focus on the central stoneroller as a host in 2010. Because the 2009 infestation was relatively light, we believed that an increased load on the stonerollers would result in an increased yield of *A. raveneliana*. This, however, was not the case. Despite observing a high amount of attachment on the fins of stonerollers in 2010, many of these were sloughed and the number of juveniles per fish actually decreased from 10.3 in 2009 to 6.2 in 2010.

Future propagation efforts should focus on using mottled sculpin as hosts while taking measures to increase their survival. Additional studies in 2011 in our lab found that sculpin survived better in deeper tanks with turbulent flow compared to the shallow, wide tanks with more laminar flow used in 2009. Additionally, we found that gravid female sculpin had a greater tendency to die in captivity, so those should be released back into the stream at the time the host fish are collected.

Juvenile Grow out

In two separate years, no surviving individuals were found at 2 months. In 2009, we attribute that to the proliferation of Daphnia in the culture containers. This likely caused either a competition for food or an inability to feed due to the action of the Daphnia attaching to juveniles mussels. In 2010, we later found that the commercial diet we were adding to the bowl recirculating systems was not persisting in the system at the levels we thought we were feeding. We have found that the food available in suspension differs between different mussel grow out systems. Factors such as submersible pumps, screens, shape of the reservoir and its turbulence and flow rates all play a role in the fate of algae in a system. We recommend constant monitoring of available food in a grow out system as possible. Several techniques can be used for counting algae at the time of feeding. Procedures such as counting with a hemocytometer are extremely labor intensive and not cost effective when resources for facility personnel are limited. Alternatively several automated cell-counting devices such as Coulter counters, or new digital technology-based instruments (Cellometer, Nexilon Bioscience Inc.) should be available for propagation facility personnel for the daily monitoring of algae-based diets fed to juvenile mussels. Although expensive daily monitoring will prevent underfeeding, which affected juvenile survival in 2010, and will also facilitate daily scientific assessment of feeding loads. Because our laboratory has successfully cultured *Alasmidonta varicosa*, *Strophitus undulatus*, and *Lasmigona decorata* to adulthood, we believe *A. raveneliana* should also thrive in captivity under the right conditions. Additional trials are needed to determine optimal conditions for this species.

Chapter 3

Carolina Heelsplitter
Lasmigona decorata

Introduction

The Carolina heelsplitter (*Lasmigona decorata*) is a federally endangered species limited to only a few populations in North and South Carolina (Bogan 2002). The North Carolina populations, which all occur in Union County, have declined significantly during the last decade due to habitat degradation as well as severe drought conditions. The species has been especially difficult to find in the wild in North Carolina since 2007.

In 2007, laboratory host trials were conducted to determine which host fishes were utilized by the species (Eads et al. 2010). That work not only served as a foundation for this study but also produced animals which continued to be reared for this work. The first objective of this portion of the study was to refine grow out techniques by evaluating the effects of water temperature during attachment to the host fish as well as during the early juvenile stage on growth and survival. A second objective was to evaluate the potential for grow out of the species in a hatchery setting. We also conducted surveys to attempt to collect additional broodstock for future propagation efforts. A final objective added during the study was to refine the use of serotonin for extraction of glochidia from gravid mussels.

Methods

Temperature trial

An experiment was conducted to evaluate the effects of water temperature during host attachment and early grow out on juvenile growth and survival (Fig. 3-1). *Lasmigona decorata* broodstock were held at the Marion Conservation Aquaculture Center (CAC) where two individuals from South Carolina became gravid. Those mussels were transported in aerated coolers of water to the laboratory at NC State University on 27 January 2009. On 29 January 2009, we immersed each separately in 1 liter of water with a concentration of 130 mg/L serotonin (300 mg/l serotonin creatinine sulfate, (Acros Organics) at 13°C. One individual was removed from the exposure after 90 minutes and the other was removed after 180 minutes. Both were placed back in freshwater and maintained at 13 ± 1°C. The following morning, all glochidia released after the serotonin exposure were used to infest 120 golden shiners (*Notemigonus chrysoleucas*), which had been purchased from a commercial source. The fish were placed in a cooler with the glochidia in 28 liters of water at 16°C with an approximate glochidial concentration of 850/liter. After 90 minutes the glochidial exposure was ended, and the fish were separated into three groups of 40 fish each. One group was acclimated to 13°C, and one was acclimated to 19°C over 3 hours. The third group remained at 16°C, and all fish were transferred to separate recirculating systems at their respective temperatures. The fish tanks were then monitored closely for transformed juvenile mussels.

Juvenile mussels from fish at each of the three temperatures were then divided equally into 12 groups and randomly assigned one of three grow out temperatures (13, 16, or 19°C). This yielded a total of 9 treatment groups with 4 replicates each from each combination of the three temperatures in both the attachment and grow out stages (Fig. 3-

1). Mussels that changed temperatures from the fish stage to the grow out stage were slowly acclimated over 3 hours to their next highest or lowest temperatures. Those mussels going from 13 to 19° or from 19 to 13° were first acclimated to and held for 24 hours at 16°C to better acclimate the mussels for the drastic temperature change.

For grow out, we used 8-liter containers of water with a fine layer of sediment (< 300 µm grain size) as substrate for the mussels (Fig. 3-2). An airstone was used to provide aeration and water movement. Temperature was maintained by placing the culture vessels in a water bath chilled to the appropriate temperature. Mussels were fed a mixture of Shellfish Diet 1800® and Nanno 3600® (Reed Mariculture, Campbell, CA) at a concentration of 50,000 - 100,000 cells/ml. Growth and survival was measured at 1 and 2 months.

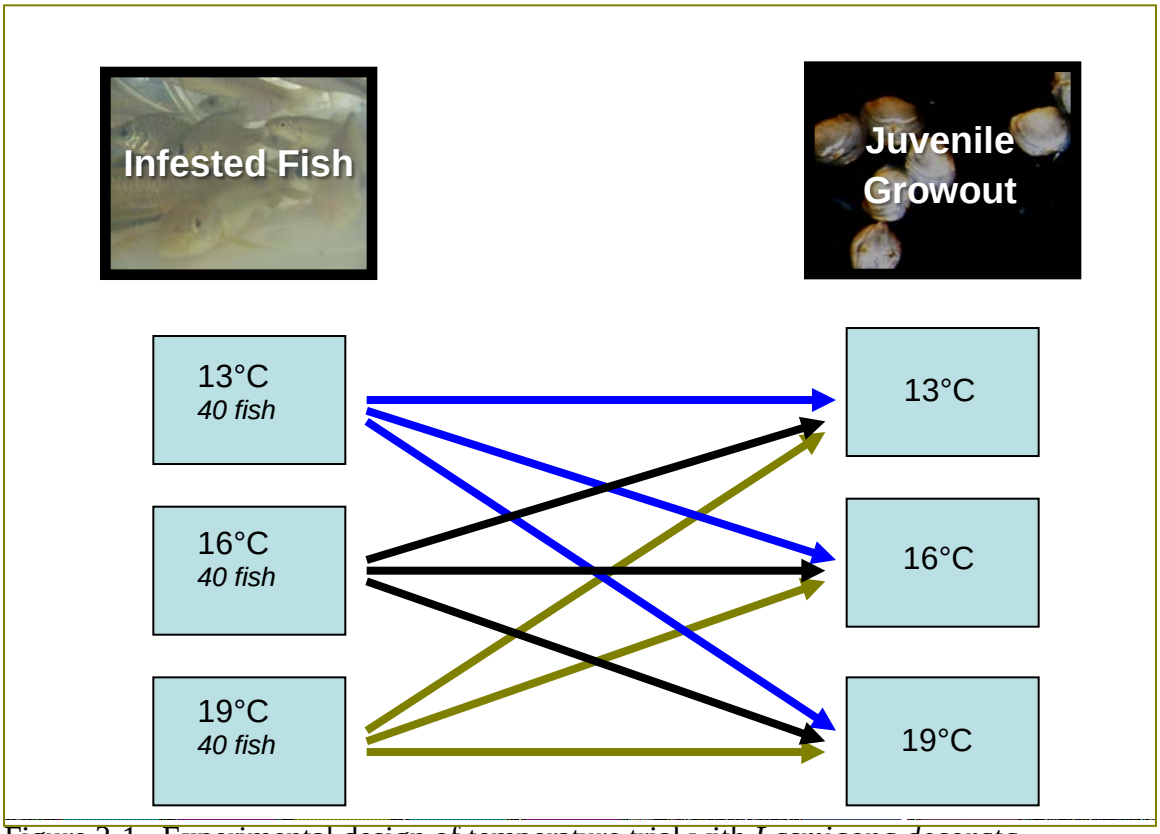

Figure 3-1. Experimental design of temperature trial with *Lasmigona decorata*

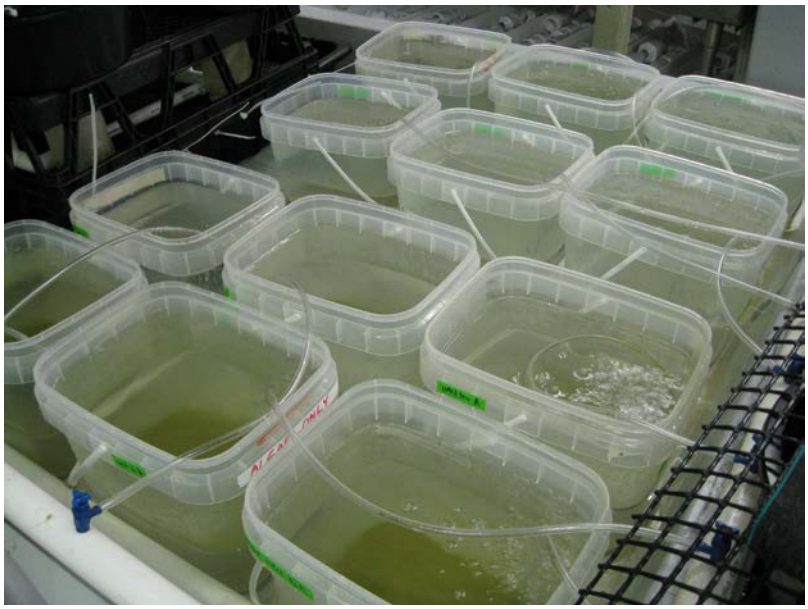
Figure 3-2. Grow out containers like these were used for juvenile *Lasmigona decorata*.

Captive holding and rearing in a hatchery setting

At the beginning of the project period (August 2008), all *L. decorata* being held at the Table Rock Fish Hatchery in Morganton, NC were transferred to the Marion CAC. This included 131 juveniles propagated from Sixmile Creek (mean length = 28.6 ± 3.6 mm) and 5 juveniles propagated from Duck Creek during 2007 (mean length = 27.2 ± 3.6 mm). Adult broodstock individuals transferred included 1 from Sixmile Creek, 2 from Duck Creek and 5 from an unnamed tributary to Bull Run Creek in South Carolina. Propagated individuals were kept separate from adult broodstock, and mussels were also separated by basin stream of origin. All mussels were held in 26-liter tanks that received a continual flow of hatchery pond water. Growth, survival and reproductive status were monitored at Marion over the project period. Adult broodstock that became gravid were used for propagation trials.

Extracting glochidia with serotonin

The manual extraction of glochidia by means of flushing the marsupial gills with a water-filled syringe can be damaging to the gills. This is especially true in the case of mussels that package their larvae into conglutinates. The monoamine neurotransmitter Serotonin (5-hydroxytryptamine) has previously been used effectively to facilitate the release of glochidia from gravid female mussels (Eads et al. 2010). In an attempt to assess the potential use of serotonin for the extraction of *L. decorata* glochidia, we immersed 3 adult *L. decorata* being held at the hatchery in varying concentrations of serotonin in 2009 and 2010. On 29 January 2009, two adults from South Carolina were immersed in 130 mg/l serotonin for the temperature trial as described above. The individual with tag number M212 was removed after 1.5 hours, and the individual tagged F066 was removed after 3 hours. Those same two adults were returned to the hatchery, became gravid later that year and were immersed again in 10 mg/l serotonin the

following year (2 March 2010) for 6 hours. On 3 February 2010, we immersed a gravid adult *L. decorata* from Duck Creek in 2 liters of water at 13°C with a concentration of 20 mg/L serotonin. After 3 hours, the concentration was reduced to 13 mg/L. At 5 hours, the mussel was placed back in freshwater, and we counted the number of glochidia that had been released. At 24 hours, we counted the glochidia again.

We conducted an experiment in April 2009 with two additional conglutinate-producing species to better inform our use of serotonin. We exposed two species of mussel, the fluted kidneyshell (*Ptychobranchus subtentum*) and the creeper (*Strophitus undulatus*), to varying concentrations of serotonin and the serotonin reuptake inhibitor fluoxetine to compare and contrast those chemicals as tools for chemically inducing glochidial release. We collected gravid *P. subtentum* from the North Fork Holston River in Smyth County, VA on 3 April 2009. The *S. undulat*us were collected from two streams in Virginia by the Virginia Department of Game and Inland Fisheries and transported to NCSU in aerated coolers of stream water.

On 8 April 2009, 42 gravid *P. subtentum* and 20 gravid *S. undulatus* were placed individually into glass jars (Fig. 3-3) with 800 ml of laboratory water and varying concentrations of serotonin or fluoxetine (Table 3-1). Serotonin concentrations were achieved by adding the appropriate amount of serotonin creatinine sulfate (Acros Organics). Fluoxetine concentrations were achieved by using fluoxetine hydrochloride (Sigma-Aldrich). An airstone provided aeration to each jar. After 8 hours, we ended the adult serotonin exposure with a 100% water change on all jars using only fresh laboratory water. Expelled conglutinates were removed from each jar and counted at 1, 3, 5, 8, 12, and 24 hours. At the end of 24 hours, we noted the physical condition of all mussels and checked to see whether they were still gravid.

All conglutinates removed from the jars during the exposure were held in a 50 ml subsample of the treatment water they came from for the entire 24-hour period. We tested viability of the glochidia from each replicate at 1, 8, and 24 hours by exposing them to a saturated salt solution. If no conglutinates were released by an individual at that time period, glochidia were taken from those previously released and held in the 50 ml treatment subsample. Glochidia were considered viable if they were open prior to salt exposure and then closed upon introduction of the salt.

After the exposure, all *S. undulatus* were returned to their streams of origin, but *P. subtentum* were maintained at the Marion CAC for 7 months. At the end of that period, growth and survival were assessed.

Figure 3-3. Experimental setup used for fluoxetine and serotonin exposures with *Ptychobranchus subtentum* and *Strophitus undulatus*.

Table 3-1. Experimental design of serotonin and fluoxetine exposures for *Ptychobranchus subtentum* and *Strophitus undulatus*.

Species	Chemical	Concentration (mg/L)	Number of Replicates
Ptychobranchus subtentum	serotonin	20	6
		40	6
		100	6
	fluoxetine	0.5	6
		2	6
		5	6
	none (control)	-	6
Strophitus undulatus	serotonin	20	5
		40	5
		100	5
	none (control)	-	5

Field surveys

On 9 March 2009, in cooperation with the NC Wildllife Resources Commission, March 2009, we surveyed two sites on Duck Creek where *L. decorata* had been

previously found (9 person-hours). We also surveyed Goose Creek below the confluence of Duck Creek (9 person-hours). On 1 October 2009, we surveyed Goose Creek from the Brief Road crossing up to US 601 (22 person hours). We surveyed Crooked Creek (Union County, NC) for the potential to hold the species on 21 and 22 July 2010 (24.67 person-hours).

Results

Temperature Trial

The length of time to metaphorphosis to the juvenile stage was inversely related with temperature (Table 3-2). All mussels attached to fish at 19°C had transformed to the juvenile stage by day 15. Encystment on host fish lasted up to 26 days at 16°C and up to 48 days at 13°C. While the number of juveniles collected decreased with increasing temperature, this could not be evaluated for statistical significance since the fish holding systems did not allow for replication to evaluate that metric as an endpoint. At both one and two months, survival was best at lower temperatures and tended to be higher in treatments that did not involve acclimating the juveniles to a different temperature (Figs. 3-4, 3-5). Growth increased slightly with increasing temperature (Figs. 3-6, 3-7). A proliferation of Chironomidae larvae throughout the culture vessels during the second month of the experiment negatively affected growth and survival; however, survival was best when mussels were held on the fish at 13°C. No mussels held at 19°C survived to 2 months. Of those surviving 2 months, growth was greater at 16°C.

Table 3-2. Time to metamorphosis and the number of juveniles collected from *Lasmigona decorata* attached to fish at three different temperatures.

Temperature (°C)	Start Day	Peak Days	Finish Day	Total # of Juveniles
13	9	26-31	48	997
16	7	15-19	26	901
19	7	8-9	15	789

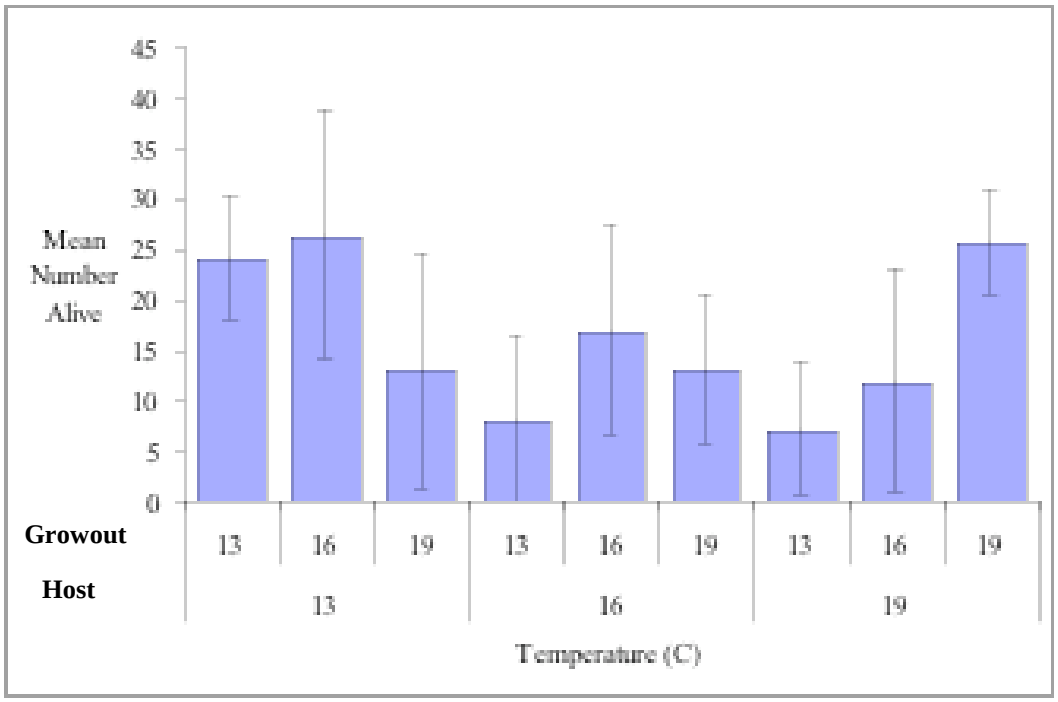

Figure 3-4. Survival at 1 month of juvenile *Lasmigona decorata* held at three different temperatures during host attachment and early grow out. Error bars represent 95% confidence intervals.

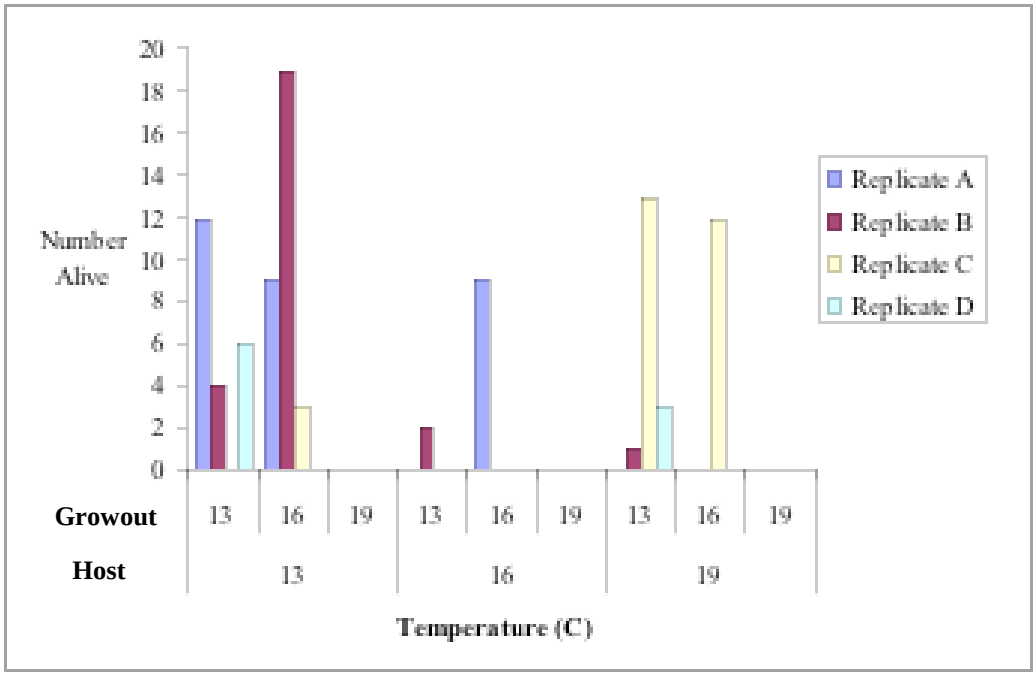

Figure 3-5. Survival of individual replicates at two months of juvenile *Lasmigona decorata* held at three different temperatures during host attachment and early growout. Because several replicates had no survival, these data were non-normal and could not be presented as means.

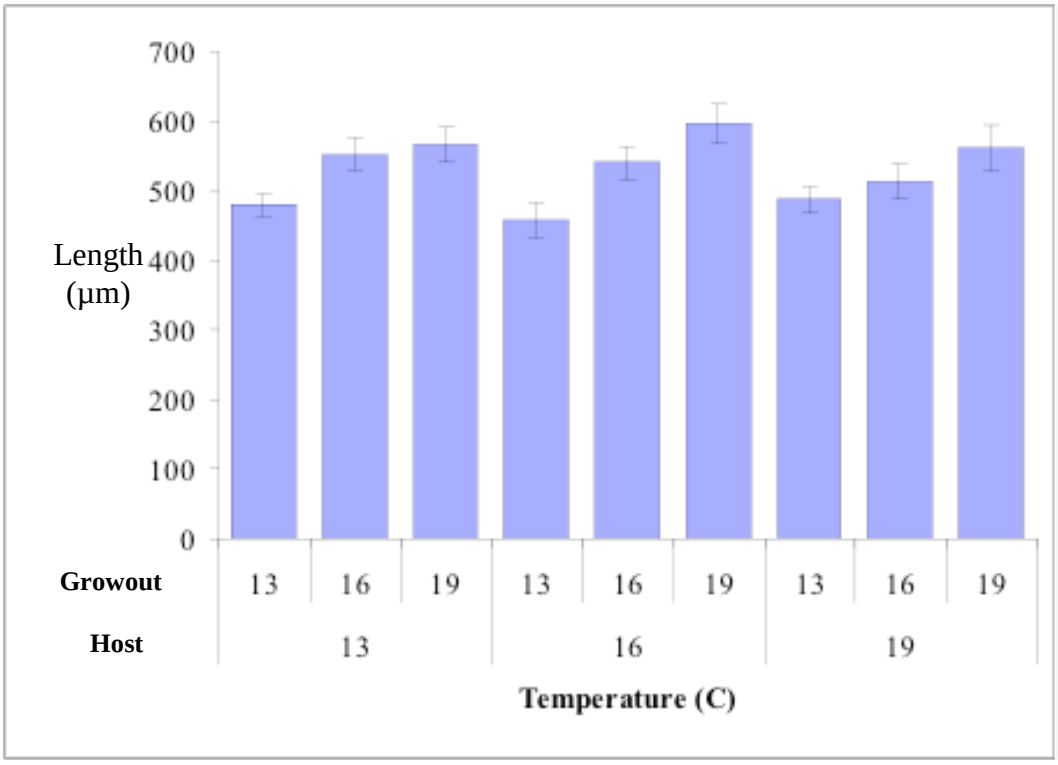

Figure 3-6. Length of 1-month old *Lasmigona decorata* held at three different temperatures during host attachment and during early grow out. Error bars represent 95% confidence intervals.

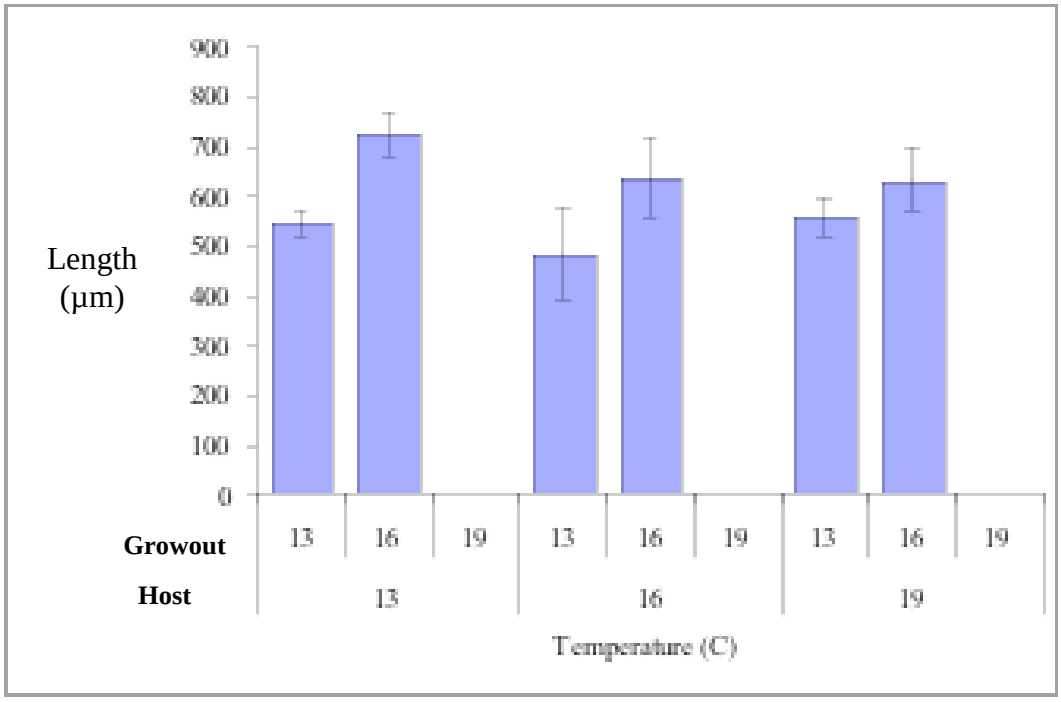

Figure 3-7. Length of 2-month old *Lasmigona decorata* held at three different temperatures during host attachment and during early grow out. Means represent 95% confidence intervals.

Hatchery broodstock and grow out of juveniles

In November 2010, 122 of the 131 Sixmile Creek *L. decorata* (93.1%) taken to the Marion CAC in August 2008 were alive. All 5 of the propagated individuals from Duck Creek were alive in November 2010. Growth was slower than expected with propagated *L. decorata* growing from a mean length of 25 mm to a mean length of 44 mm in those 2 years (Fig. 3-8). In November 2009, 45 of the 125 Sixmile Creek individuals were found to be gravid for the first time. Only 1 of the 5 Duck Creek individuals were gravid at that time.

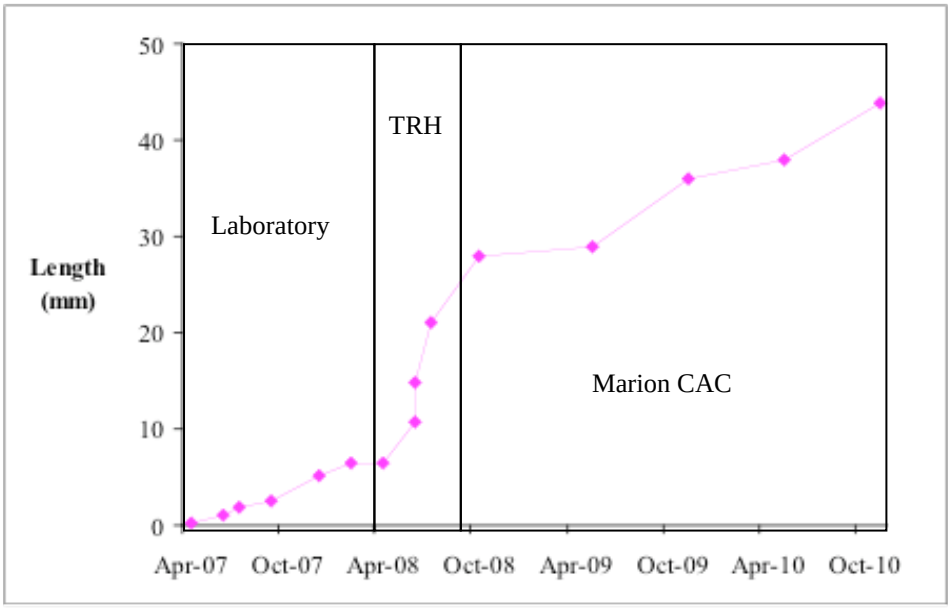

Figure 3-8. Length of *Lasmigona decorata* propagated in 2007. Mussels were held initially in the laboratory, then at the Table Rock Hatchery (TRH), then at the Marion CAC.

The two known females from South Carolina both died in 2010. Both valves of the one wild-caught female from Duck Creek were cracked when it was checked for gravidity in the fall of 2009. That mussel died early in 2010, so there are currently no known wild-caught females in captivity.

Field surveys

We found 1 adult *L. decorata* in Duck Creek on 9 March 2009. That individual was transferred to the Marion CAC and placed in a tank with the other adults from Duck Creek. To date, that individual has not become gravid. No individuals were found in October 2009, and no individuals were found in Crooked Creek.

Extracting glochidia with serotonin

We saw significant variation between how individual *L. decorata* responded to serotonin. The individual tagged M212 was highly affected by serotonin and had released virtually its entire brood 24 hours after the initial immersion in both years. The other individual (F066) was affected very little by serotonin both times it was immersed. Foot swelling, a clinical finding that has been seen previously when using serotonin in mussels was evident in M212 but minimal in F066. And most importantly, F066 released very few glochidia either year. Despite the large differences in concentration used on these two individuals in 2009 and 2010, the resulting glochidial release was virtually the same. The 2009 exposure seemed to cause no ill effects as both individuals spawned and became gravid again the next fall. Unfortunately, both of those adults died within a 2 weeks of being returned to the Marion CAC after serotonin exposure in 2010.

The individual immersed in 20 mg/l serotonin for 5 hours had released only a portion of its brood after 24 hours. We estimated that it had released 2,000-2,500 glochidia during the immersion and another approximately 20,000 glochidia over the next 20 hours in freshwater. Conglutinate release seemed to involve a different mechanism in *L. decorata* than in the other two species tested. To release conglutinates, *L. decorata* had to forcefully close their valves to eject their larvae. Conglutinates streamed freely from the excurrent aperture of *P. subtentum* and *S. undulatus* without any valve movement required.

Serotonin at 20 and 40 mg/l proved to be very effective in inducing release of conglutinates for both *P. subtentum* and *S. undulatus* (Figs. 3-10, 3-11). Once mussels were immersed in serotonin at those concentrations, swelling of the foot and release of mucuous near the foot (Fig. 3-9) began to occur after approximately 15-30 minutes. Release of conglutinates began around 30 minutes as well. There was a negative response to increased concentrations. The highest concentration (100 mg/l) produced the same physical reaction of edema and mucous release, but *P. subtentum* released significantly fewer conglutinates and had more of the individuals still gravid after 24 hours (Table 3-3). While *S. undulatus* still released their entire brood at the high concentration, the release was slower (Fig. 3-11).

At the 24 hour point, the swelling in those mussels immersed in 20 or 40 mg/l serotonin had been fully relieved while mussels immersed at 100 mg/l still exhibited edema. Serotonin did not affect viability of the glochidia even up to 24 hours as tested with the salt solution. The highest fluoxetine concentration (5 mg/l) yielded glochidial release in *P. subtentum* similar to that of serotonin, but the reaction was much slower. The highest release in the fluoxetine treatments occurred at the 24 hr evaluation, while the serotonin treatment produced the greatest release between 1 and 5 hours. And while 5 mg/l fluoxetine triggered release, the glochidia exposed to it had significantly decreased viability at 24 hours (Fig. 3-12). When those glochidia were exposed to the salt solution, they did not close their valves. After the adult *P. subtentum* were held in the hatchery for 7 months, overall survival was 83.3%, and no significant differences were found between treatments (Table 3-3)

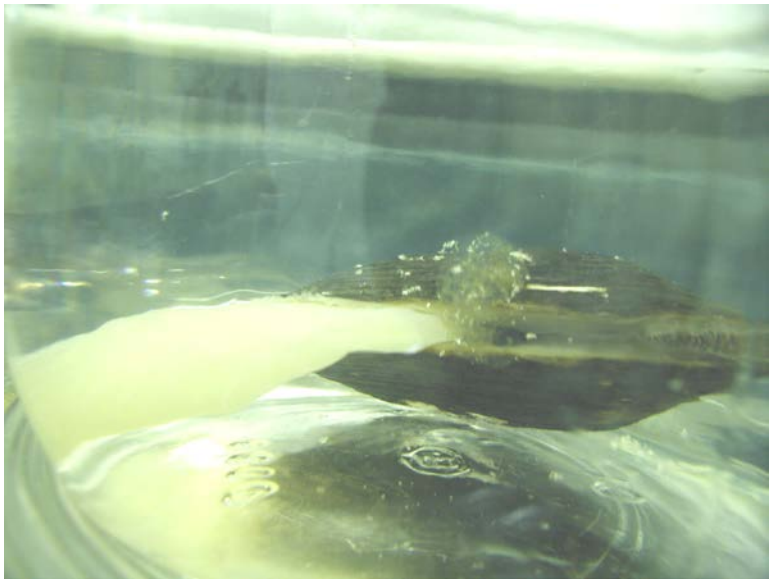

Figure 3-9. Swollen foot and mucous release of *Lasmigona decorata* exposed to serotonin.

Table 3-3. Proportion of *Ptychobranchus subtentum* and *Strophitus undulatus* still gravid after 24 hours following exposure to varying concentrations of serotonin and fluoxetine.

Species	Chemical	Concentration (mg/l)	Proportion of individuals still gravid after 24 hours	Proportion of individuals alive after 7 months
P. subtentum	serotonin	20	1/6	5/6
		40	2/6	6/6
		100	4/6	5/6
	fluoxetine	0.5	6/6	4/6
		2	5/6	5/6
		5	1/6	4/6
	none (control)	-	6/6	6/6
S. undulatus	serotonin	20	0/5	-
		40	0/5	-
		100	0/5	-
	none (control)	-	5/5	-

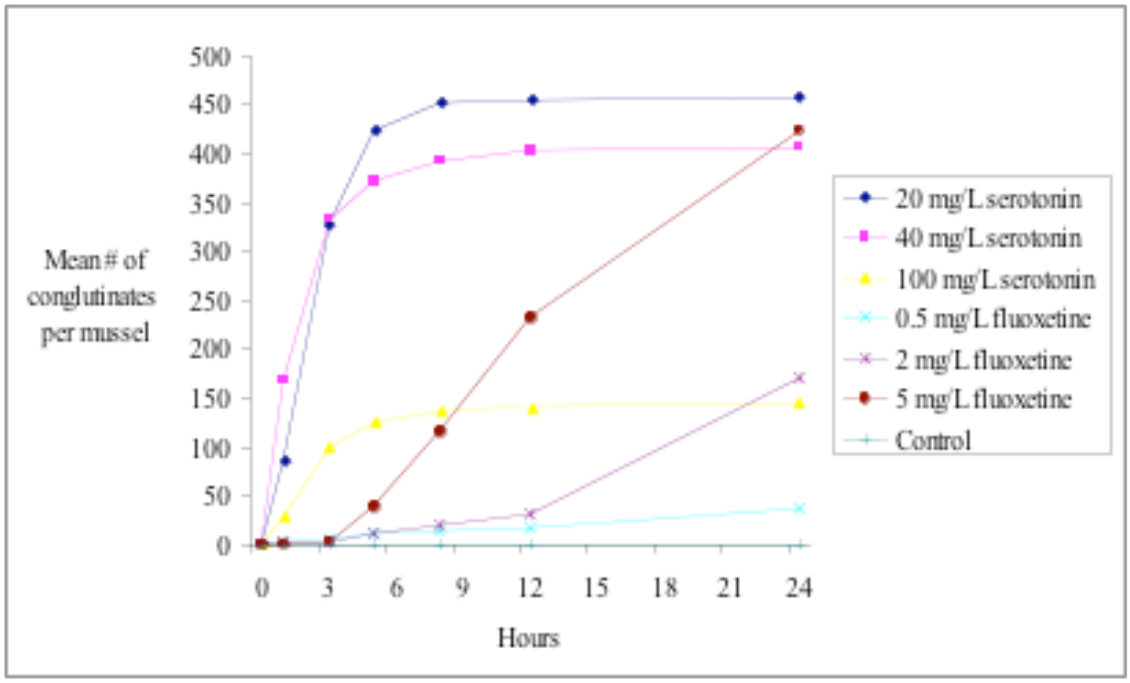

Figure 3-10. Mean number of conglutinates released by *Ptychobranchus subtentum* over 24 hours when exposed to varying concentrations of serotonin and fluoxetine.

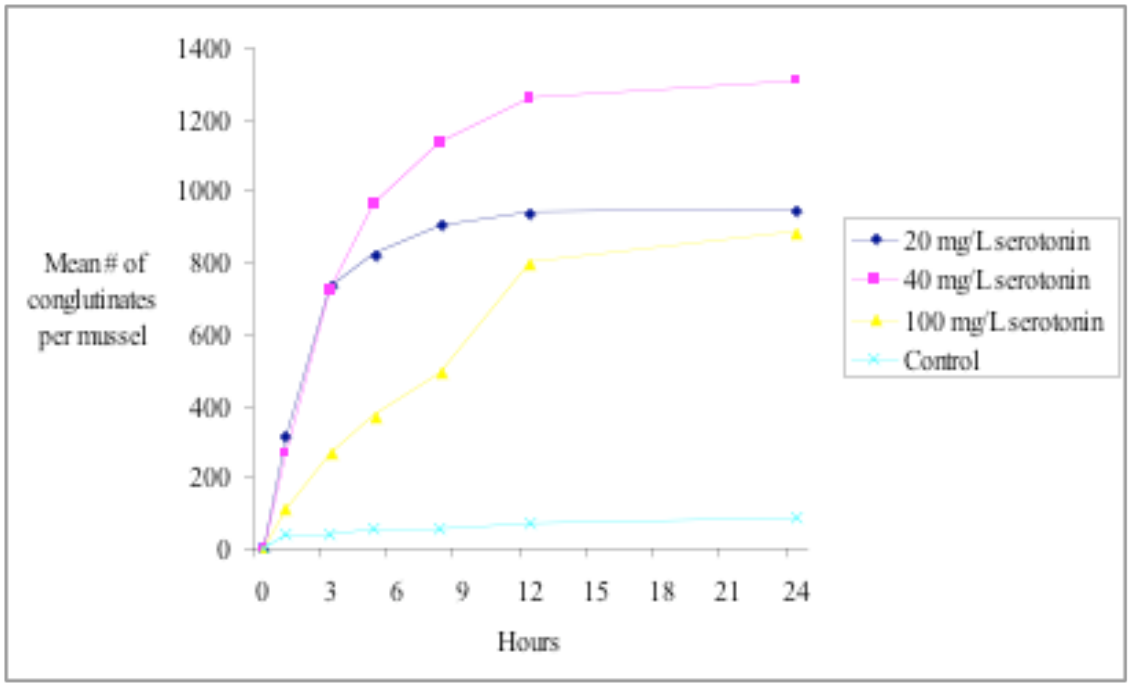

Figure 3-11. Mean number of conglutinates released by *Strophitus undulatus* over 24 hours when exposed to varying concentrations of serotonin. None of the individuals exposed to serotonin were still gravid after 24 hours.

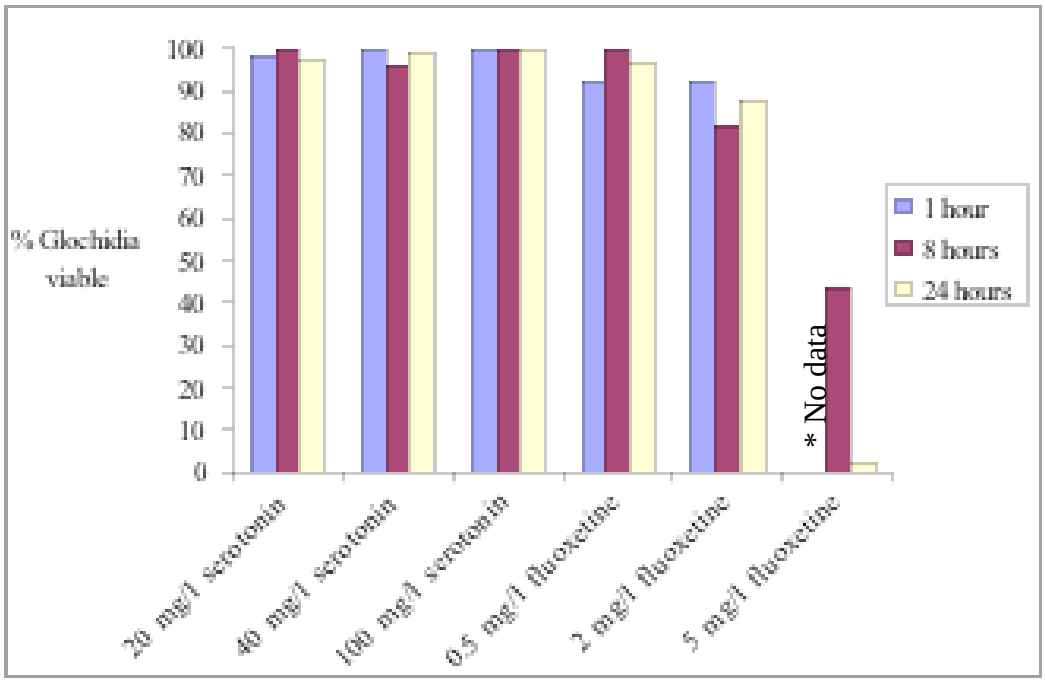

Figure 3-12. Viability of *Ptychobranchus subtentum* glochidia evaluated at 1, 8, and 24 hours following exposure to various concentrations of serotonin or fluoxetine. No glochidia were released from the 5 mg/l fluoxetine concentration at 1 hour.

Discussion

Temperature Trial

Survival of *L. decorata* increased at lower temperatures, but growth was slowed. The results of this trial suggest that this species is best propagated by holding the fish at 13°C during glochidial attachment and starting the juveniles off at that temperature. The water could then slowly be warmed over several days up to 16°C and maintained there while they are held in the laboratory.

Chironomidae larvae were shown to have a substantial negative effect on the culture environment when they occurred in large quantities. Their mode of interference may have been either through physical disturbance of the mussels or alteration of food resources or water quality within the sediment. Within treatments, those replicates that had fewer Chironomids tended to be the ones that had surviving juvenile mussels. After this experiment, we started using fine mesh screen to cover all juvenile grow out systems to prevent adult Chironomids from laying eggs in the grow out chambers. That measure has significantly decreased the amount of insect larvae in the culture vessels.

Captive holding and rearing in a hatchery setting

While survival has been very good at Marion to this point, growth of *L. decorata* has been somewhat slow compared with other species held there. Larger, thin-shelled species like *L. decorata* typically show rapid growth rates in the wild. Apparently something is missing in their diet in the hatchery or environmental conditions within the

hatchery don't mirror what is needed for optimum growth. Fortunately, nutrition at Marion has been adequate for maintenance, reproduction and some growth. Wild-caught females spawned each year during captivity. Additional research focused on the dietary and nutritional requirements of both adult and juvenile *L. decorata* is needed to maximize their growth their growth and survival and the overall benefit of rearing them in captivity.

Field surveys

Currently, there are no known wild-caught females in captivity. Habitat in Goose and Duck Creek continue to degrade as base flow has declined and erosion and sedimentation have altered stream sediments. Recent trips to locations where *L. decorata* have routinely been collected during prior surveys have identified one sole individual. Despite the extreme drought of 2007, and the drying of much of that creek, the species continued to be extant in 2009; however, intensive, multi-day surveys of both Goose and Duck Creek would likely be necessary to locate any other individuals for propagation. While Crooked Creek had stable mussel habitat in places and could potentially support *L. decorata*, the lack of diversity of other species suggests this creek is less than ideal as a potential location to find additional *L. decorata*, or consider augmenting the remaining population with animals propagated in captivity.

Extracting glochidia with serotonin

This research was initiated due to previous difficulty encountered when attempting to extract glochidia from *L. decorata*. With long-term brooders such as *L. decorata*, we typically flush glochidia from the gills using a water-filled syringe. Our original propagation efforts in 2007 (Eads et al. 2010) found that this species packages their larvae with unfertilized eggs into amorphous conglutinates. Those large masses could not be easily extracted with syringe. The first adult we tried that method with suffered significant tearing of the gill tissue due to the repeated attempts at extraction. The other alternative to extraction is letting the mussels release larvae on their own. That process is can take several weeks to complete. Additionally, only small amounts of larvae are released at a given time, which makes significant propagation efforts and related research difficult to carry out. We needed another tool to safely extract an entire brood in a short period of time.

With *Ptychobranchus subtentum* and *Strophitus undulatus*, we found serotonin to be a very safe and effective tool for that purpose. With concentrations of 20-40 mg/l, we were able to collect virtually their entire brood within 8 hours and a large percentage of it within 5 hours. No real differences were seen in *P. subtentum* survival between treatments after 7 months. We found that fluoxetine was somewhat effective at our highest concentration (5 mg/l) but acted slowly and was harmful to the glochidia.

Unfortunately, the same concentrations were less predictable, triggered a slower glochidial release, and likely caused some mortality when used with *Lasmigona decorata*. In some cases, entire broods were collected within 24 hours. In other cases, we collected very few glochidia. Two individual *L. decorata* were affected very

differently by serotonin, and this was consistent across two separate trials in two separate years. Our initial trial in 2007 with serotonin (500 mg/l immersion for 3 hours) was successful, and the adult was held long-term in the hatchery with no apparent ill effects (Eads et al. 2010). Our 2009 exposures (300 mg/l for 1.5 and 3 hours) seemed to also cause no ill effects since the mussels not only survived but became gravid again the next fall. In 2010, we used a much lower concentration (10 and 20 mg/l) but slightly longer immersion times (5 and 6 hours), and all three of those individuals died within a few weeks. We cannot determine the reason that mussels died in 2010. *Lasmigona decorata* has thin shells and one of those mussels had a severely cracked shell from being opened for examination. The others could have been injured during transfer to freshwater. Perhaps the combination of concentration and immersion time created a toxic effect.

Physical injury should certainly be guarded against with future use of serotonin. With the substantial swelling that takes place, the mussel must be handled with great care. Any attempts to move the mussel during this time could result in physical injury. If the mussel were lifted without fully supporting the swollen foot and its extra water weight, it is possible that the mussel could be pulled from its shell or be injured internally. More research is needed on the safety of this chemical with this species.

Chapter 4

Tar River Spinymussel
Elliptio steinstansana

Introduction

The Tar River spinymussel (*Elliptio steinstansana*) was described as a species endemic to the Tar River in North Carolina in 1983 by (Johnson and Clarke 1983). Clarke (1983) found that the species was already in decline. Since 1983, surveys indicate the species continues to decline within its range and has now reached a state of critical imperilment. Captive propagation may now be a key component to restoration of this species in North Carolina.

Alderman (1989) suggested *E. steinstansana* was closely related to the James spinymussel (*Pleurobema collina*) and likely used similar host fish. Hove and Neves (1989) determined the host fishes of *P. collina* to be a group of minnow species (Cyprinidae) from multiple genera. Indeed, in 2008, our laboratory determined the hosts of *E. steinstansana* to be a group of minnows very closely related to the hosts of *P. collina* (Eads and Levine 2008). In 2008, we propagated a total of 38 newly transformed juveniles from the initial host fish trial.. This effort was initiated to bring wild *E. steinstansana* from the Tar River Basin into captivity as broodstock, continue to enhance propagation and culture techniques and raise individuals to augment declining populations. Funds available for this effort were complemented by Prevention Extinction funds provided by the North Carolina Wildlife Resources Commission. Working jointly with commission staff we conducted surveys to acquire the needed adult broad stock and then worked to refined captive propagation techniques for this species that was nearing extinction.

Methods

Field Surveys and Brood Stock Collection

From July 2007 - October 2010, we conducted or participated in 38 surveys covering 364.5 person-hours across 16 sites and 5 streams (Table 4-1) finding a total of 22 *E. steinstansana* and collecting 18 of those. We collected a total of 15 *E. steinstansana* from Little Fishing Creek, 2 from Fishing Creek and 1 from the Little River in the Neuse Basin. From Little Fishing Creek, 7 of those were collected immediately below the Glenview Road Bridge (SR 1338). Another 7 individuals were collected from approximately 1.0 km downstream of that road crossing (N 36.17752, W 77.87872). One individual was located approximately 0.6 km upstream of the same road crossing (N 36.19078, W 77.87283). In Fishing Creek, we collected one individual from immediately below Avent Rd (SR 1338 on the Halifax-Nash County Line) (N 36.16927, W 77.92306) and one approximately 1.4 km downstream of Melton Bridge Road (SR 1342 on the Halifax-Nash County Line) (N 36.14479, W 77.82648). The *E. steinstansana* from the Little River was collected 1.0 km upstream of Micro Rd (SR 2130) in Johnston County (N 35.61176, W 78.21117).

Table 4-1. Surveys conducted for the Tar River spinymussel (*Elliptio steinstansana*) from 2007-2010.

Date	Stream	Location	Total Survey Hours	# of TRSM Found	# of TRSM Collected
7/9/2007	Swift Creek	Red Oak Rd	12	0	0
7/9/2007	Swift Creek	East Hilliardston Rd	6	0	0
7/11/2007	Swift Creek	Red Oak Rd	12.5	0	0
7/11/2007	Little Fishing Creek	Ward Rd	7.5	0	0
7/12/2007	Little Fishing Creek	Glenview Rd	25	1	1
7/18/2007	Little Fishing Creek	Glenview Rd - downstream	25	3	3
7/19/2007	Little Fishing Creek	Glenview Rd - downstream	6	1	1
7/29/2007	Little Fishing Creek	White Oak/Ward Rd	5	0	0
7/30/2007	Fishing Creek	Lonesome Pine Rd	18	0	0
8/4/2007	Tar River	NC 33	10	0	0
8/29/2007	Fishing Creek	Etheridge Farm Rd	5.33	0	0
4/11/2008	Little Fishing Creek	Glenview Rd	12	1	1
5/5/2008	Little Fishing Creek	Glenview Rd	5	1	1
5/6/2008	Little Fishing Creek	Glenview Rd - downstream	10	1	0
5/14/2008	Little Fishing Creek	Glenview Rd	6	0	0
5/28/2008	Fishing Creek	Bellamy Mill Rd	2	0	0
5/28/2008	Little Fishing Creek	Glenview Rd - upstream	3	0	0
5/28/2008	Fishing Creek	Etheridge Farm Rd	2	0	0
5/29/2008	Little Fishing Creek	Glenview Rd - downstream	10	3	3
6/13/2008	Fishing Creek	Melton Bridge Rd - NC 48	7	2	0
10/7/2008	Little River	Shoeheel Rd - Micro Rd	10	1	0
4/21/2009	Fishing Creek	Melton Bridge Rd - NC 48	7	1	1
4/22/2009	Little Fishing Creek	Glenview Rd - downstream	4.67	0	0
4/23/2009	Fishing Creek	NC 43-Avent Rd	15	0	0
4/28/2009	Little River	Micro Rd	7.67	1	1
4/29/2009	Fishing Creek	NC 561 - NC 43	10	0	0
5/12/2009	Little Fishing Creek	Glenview Rd	7.5	4	4
5/12/2009	Little Fishing Creek	Glenview Rd - upstream	2.25	1	1
5/13/2009	Fishing Creek	Avent Rd	1.5	1	1
5/13/2009	Fishing Creek	Avent Rd - downstream	0.67	(1)*	0
5/14/2009	Tar River	NC 33	3	0	0
5/14/2009	Swift Creek	West Logsboro Rd	3	0	0
5/14/2009	Fishing Creek	Bellamy Mill Rd	4.5	0	0
5/12/2010	Little River	Micro Rd	29	0	0
6/17/2010	Little River	Micro Rd	23.75	0	0
6/23/2010	Swift Creek	Red Oak Rd	15	0	0
10/11/2010	Little Fishing Creek	Glenview Rd	12.85	0	0
10/12/2010	Fishing Creek	Melton Bridge Rd-NC 48	7.75	0	0
		TOTAL	**364.5**	**22**	**18**

* = shell only

Broodstock Holding

From July 2007 - August 2008, brood stock were held in a trough at the Table Rock Fish Hatchery. The Table Rock Fish Hatchery (TRH) is located near Morganton, NC on Irish Creek in the Catawba River basin. The main water supply is a small (1.4 hectare) reservoir on the creek. This water was mixed with water from a warmer, more nutrient-rich pond (0.4 hectare) and was piped continuously through the mussel culture trough. Mussels were placed in a substrate of coarse sand and fine gravel in the bottom of the trough.

Due to mortality of *E. steinstansana* at TRH, the remaining broodstock was moved to the new Marion Conservation Aquaculture Center (CAC) in August 2008. All individuals from Little Fishing and Fishing Creeks were held indoors in a round 26-liter tub (Fig. 4-1) and were fed through continuous flow of pond water through the tub. A layer of very coarse sand and fine gravel was placed in the tub for substrate for the mussels. The individual from the Little River was held separately in another identical tub. A total of 5 of those from Little Fishing Creek died at Table Rock (1 in October 2007, 1 found 8 August 2008, and 3 found 25 August 2008). All *E. steinstansana* were moved to the new Marion CAC on 26 August 2008 where 1 additional adult died only a few days later – likely already stressed from the cause of mortality at Table Rock. All survived from that time up until the end of January 2011 when one of the females (Tag # C277/278) was found freshly dead in the broodstock tank. At the time of writing of this report, there were 10 brooding individuals remaining from Fishing and Little Fishing Creeks and a single individual from the Little River.

Figure 4-1. Tanks (26-liters) used for holding brooding Tar River spinymussels (*Elliptio steinstansana*) at the Marion Conservation Aquaculture Center.

Handling of host fish

In 2009, host species were captured by a combination of electrofishing and seining from small streams surrounding Raleigh, NC in the Neuse and Tar River Basins. Fish were transported back to the laboratory in aerated coolers of water. Approximately 2 g/l of salt (Instant Ocean®) was added to each cooler to support osmoregulation of fishes. Because we were originally unaware that the *E. steinstansana* would spawn and release broods repeatedly, we often struggled to keep enough host fish on hand for propagation efforts in 2009. This resulted at times in having to use fish for propagation on the same day or soon after they were collected. Fish were often stressed by capture and transport and disease and mortality were observed in some fish after glochidial attachment.. To reduce fish stress in 2010, electrofishing was eliminated from our collection protocol, and only seining techniques were used. We also began adding Stress Coat® and Finer Shiner™ (Aquatic Ecosystems, Apopka, FL) to transport coolers to support fish health. We began collecting and quarantining large numbers of host fish in advance of their use. All host fish used in 2010 were held for at least 2 weeks prior to infestation and treated with formalin (Paracide F, Argent Labs, Redmond, WA) to prevent the entry of flatworms into the juvenile culture systems (Zimmerman et al. 2003).

Spawning and brood collection

From late March through late July in 2009 and 2010, the broodstock tank at the Marion CAC was checked daily for released conglutinates by NCWRC staff (Fig. 4-2). Known females were also checked bi-weekly for gravidity, and gravid females were transported to the Aquatic Epidemiology and Conservation Laboratory (AECL) at NC State University. There, they were held in individual 8-liter tanks without substrate and monitored daily for release of conglutinates. Because of difficulty distinguishing between gravid and non-gravid *E. steinstansana*, gravid mussels were occasionally left at Marion by mistake, and several broods were released there in 2009. Most of those broods were used to infest fish at Marion, which were subsequently transported to the AECL in aerated coolers within 2 weeks of glochidial attachment. The host fish at Marion were collected either from the creek running through the hatchery or from the Neuse or Tar River Basins and transported to Marion for holding. Some broods were transported from Marion to the AECL within 24 hours in cooperation with NCWRC staff for infection of fish we attempted to transport all broods released at Marion to the AECL within 24 hours of release. In 2010, we began rotating known females between the CAC and AECL on a rotation so that they were in Marion for 2 weeks and at the AECL for 2 weeks. The females could spawn and become gravid again in two weeks at Marion and then release their brood in the two weeks they spent at the AECL. That rotation was effective for April and May, but spawning was not as frequent in June and July. Any broods released at Marion in 2010 were transported to the AECL as possible. No fish were infested with *E. steinstansana* at the CAC in 2010.

For most broods, we estimated the number and percentage of viable glochidia and non-viable eggs by counting the total number of conglutinates in a brood as well as the number of viable and non-viable glochidia on 6-10 of those conglutinates (Fig. 4-3). Initially, viable glochidia were separated from conglutinates by aspirating them in and out of a syringe or small pipette. In 2010, we found it most efficient to place the

conglutinates on a 500-µm sieve and wash them through with a squirt bottle. That action was sufficient to separate viable glochidia from the conglutinates and non-viable eggs.

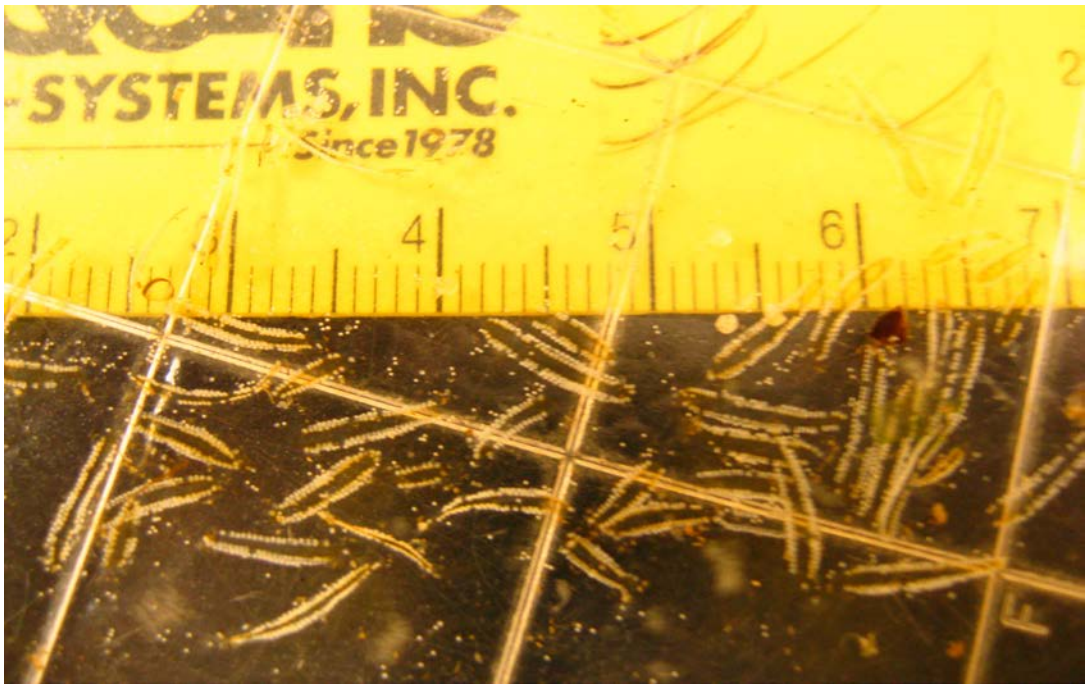

Figure 4-2. Conglutinates released by the Tar River spinymussel (*Elliptio steinstansana*)

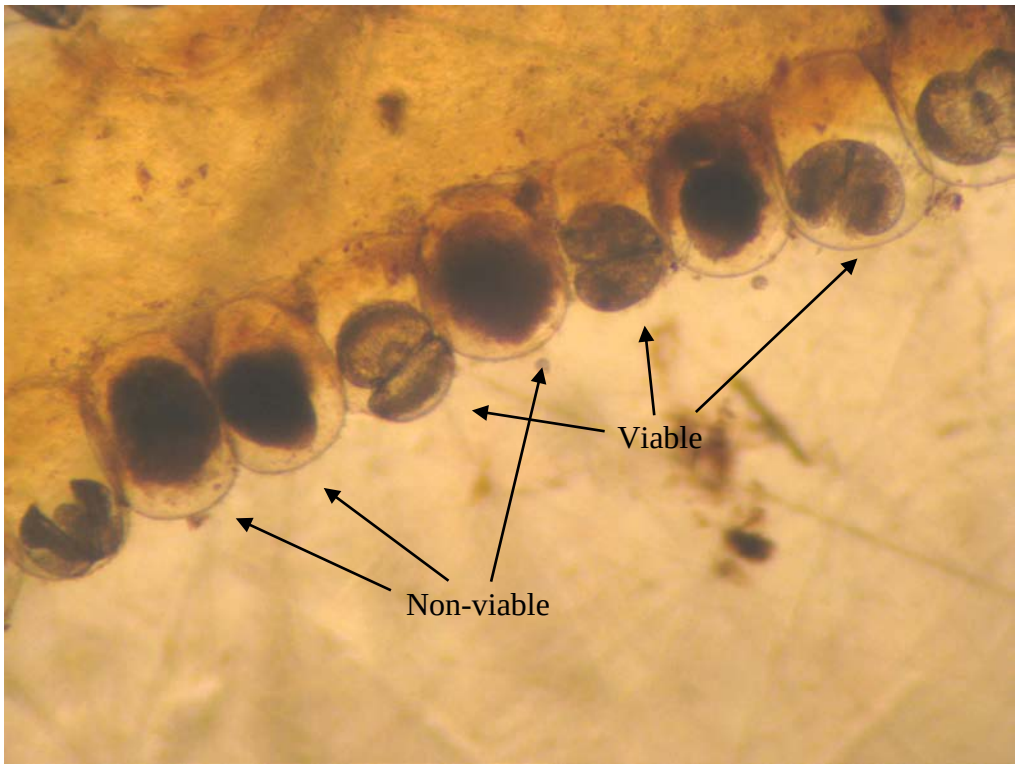

Figure 4-3. Viable glochidia and non-viable eggs in a conglutinate released by

the Tar River spinymussel (*Elliptio steinstansana*).

Because of both the rarity and the small size of *E. steinstansana* broods, we originally thought that pipetting the glochidia directly onto the gills of the host fish would result in the highest percentage of glochidia attached. The first 5 infestation events of 2009 were done by pipetting glochidia onto the gills of anesthetized host fish. Over time, we discovered that attachment of glochidia pipetted onto gills was poor, and the greatest attachment was achieved by suspending glochidia with the host fish by means of aeration. All subsequent infestations in 2009 (Table 4-2) and 2010 (Table 4-3) were done by placing the host fish in a small amount of water with the glochidia and aerating vigorously. In 2009, a total of 27 broods were released at either the CAC or AECL, and fish were infested with glochidia 23 of those times. We exposed a total of 248 bluehead chubs (*Nocomis leptocephalus*), 189 white shiners (*Luxilus albeolus*), 61 satinfin shiners (*Cyprinella analostana*), 23 pinewoods shiners (*Lythrurus matutinus*), 5 swallowtail shiners (*Notropis procne*), 3 comely shiners (*Notropis amoenus*), and 1 creek chub (*Semotilus atromaculatus*) to the glochidia. In 2010, a total of 18 broods were released, and 15 of those broods were used to infest a total of 249 white shiners. Fish were separated by species into round holding tanks of various sizes and monitored for transformation of juvenile mussels. Each holding tank was designed to remove transformed juveniles from the bottom of the tank by means of circular flow and double standpipe designed to remove water from the bottom of the tank. Outflow from each tank flowed through a 100-µm mesh basket to catch transformed juveniles. Baskets were rinsed into a petri dish daily during excystment of juvenile mussels from the fish, and juveniles from each tank were enumerated using a dissecting microscope.

Feeding conglutinates to host fish

We set up four 38-liter glass aquaria, each with 3 individuals of one of four host species: bluehead chub, satinfin shiner, white shiner, and pinewoods shiner. For two weeks, we attempted to train these fish to eat frozen bloodworms upon their introduction to the tank. On 3 July 2009, conglutinates from a brood released at the AECL that day were introduced into the aquaria of the bluehead chub and satinfin shiner. A total of 15 conglutinates were introduced, one at a time, to the bluehead chubs, mixed with a small amount of frozen bloodworms to encourage feeding. We introduced 8 conglutinates to the satinfin shiners in the same manner. The white shiners remained easily spooked and did not adapt to the glass aquaria. They would not eat bloodworms upon introduction to the tank, so they were not included in this trial. All of the pinewoods shiners died before the trial occurred, so this species was also not tested.

Table 4-2. Summary of Tar River spinymussel (*Elliptio steinstansana*) propagation efforts in 2009. Infestation done by hand represents those done by pipetting glochidia onto gills of anesthetized fish. Batch infestation represents suspension of glochidia by aeration with host fish. Fish scientific names are abbreviated: *Nocomis leptocephalus* (*Nl*), *Luxilus albeolus* (*La*), *Lythrurus matutinus* (*Lm*), *Cyprinella analostana* (*Ca*), *Notropis procne* (*Np*), *Notropis amoenus* (*Na*), and *Semotilus atromaculatus* (*Sa*).

Infestation date	Number of conglutinates	Estimated % of glochidia viable	Estimated total number of glochidia	Fish Infested	Infestation Method
5 April	No est.	No est.	No est.	42 *Nl*, 20 *La*	Both
8 May	57	0 %	0	None	none
11 May	No est.	No est.	300	8 *Nl*, 8 *La*, 11 *Lm*, 2 *Ca*	By Hand
15 May	No est.	No est.	750	15 *Nl*, 5 *La*, 2 *Lm*	By Hand
18 May	100	No est.	500	26 *Nl*	By Hand
22 May	36	80 %	No est.	15 *Nl*, 7 *La*, 4 *Lm*, 4 *Ca*, 2 *Np*, 3 *Na*	By Hand
29 May	73	86 %	3650	14 *Nl*, 8 *La*, 3 *Lm* 12 *Ca*	Batch
1 June	116	43 %	2900	13 *Nl*, 2 *La*, 1 *Lm*, 1 *Ca*, 1 *Np*, 1 *Sa*	Batch
10 June	40	100 %	2000	12 *Nl*, 13 *La*, 1 *Lm*	Batch
12 June	77	No est.	2156	6 *Nl*, 20 *La*	Batch
15 June	100	95%	5000	10 *Nl*, 29 *La*	Batch
16, 17 June	119	No est.	No est.	12 *Nl*	Batch
18 June	216	No est.	No est.	26 *Nl*, 20 *La*	Batch
22 June	130	No est.	4940	9 *Nl*, 12 *La*, 1 *Np*	Batch
23 June	75	70 %	2625	18 *Nl*	Batch
24 June	97	64 %	4074	20 Ca 10 *La*	Batch
25 June	100	No est.	2800	12 *Ca*	Batch
29 June	No est.	No est.	No est.	None	none
3 July	115	87 %	4255	3 *Nl*, 10 *La*, 3 *Ca*	Batch, Feed
8 July	54	No est.	No est.	3 *Nl*, 7 *La*	Batch
10 July	No est.	95 %	No est.	3 *Nl*, 7 *La*	Batch
14 July	No est.	No est.	No est.	1 *Nl*, 4 *La*, 1 *Ca*, 1 *Np*	Batch
20 July	71	No est.	2911	3 *Nl*, 8 *La*, 6 *Ca*, 1 *Lm*	Batch
22 July	No est.	52 %	No est.	12 *Nl*, 9 *La*	Batch
27 July	No est.	No est.	No est.	None	none
TOTAL	**1576**		**38861**	**248 *N. leptocephalus*** **189 *L. albeolus*** **61 *C. analostana*** **23 *L. matutinus*** **5 *N. procne*** **3 *N. amoenus*** **1 *S. atromaculatus***	
MEAN	**92.7**	**70.2 %**	**2590.7**		

Table 4-3. Summary of Tar River spinymussel (*Elliptio steinstansana*) propagation efforts in 2010. Only white shiners were used as hosts in 2010. The two broods released on 5 May were combined to infest 26 fish.

Infestation date	Number of conglutinates	Estimated % of glochidia viable	Estimated total number of glochidia	Number of fish infested	Female mussel
7 April	No est.	No est.	No est.	12	Unknown
9 April	No est.	No est.	No est.	22	Unknown
14 April	66	41%	726	25	B302
19 April	107	44%	2915	25	F453
19 April	120	72%	5160	25	C275
20 April	113	56%	3390	25	B301
5 May	102	34%	960	26	B298
5 May	127	45%	3963		C278
7 May	100	73%	3183	15	Unknown
14 May	107	32%	3192	15	B302
16 May	108	10%	216	1	F453
21 June	60	87%	1160	18	Unknown
23 June	100	73%	2200	12	B302
9 July	60	90%	1500	14	Unknown
14 July	72	37%	1296	14	Unknown
TOTAL	**1242**		**28565**	**249**	
Mean	**95.5**	**53%**	**2380**		

Growout of juvenile mussels

Of the 38 juvenile *E. steinstansana* that were propagated in 2008, 3 of those survived in the laboratory to the spring of 2009. On 9 April 2009, those 3 juveniles were taken to the Marion CAC for growout and placed in one of the 26-liter tanks with a medium-coarse sand substrate where they received constant flow of pond water. Those 3 individuals remained in that tank for the remainder of this project.

All juveniles propagated in 2009 were maintained in the laboratory. The water source in the laboratory was City of Raleigh, NC municipal water filtered through activated charcoal and dechlorinated using sodium thiosulfate ($Na_2S_2O_3$). All water used for juvenile mussels was also treated with an ammonia-locking reagent (Ammo-Lock®, Aquarium Pharmaceuticals, Inc.). Of those propagated in 2009, newly transformed juveniles from the first 11 successfully propagated broods were placed in 8-liter tanks (Fig. 4-3) with a thin layer of fine sediment (<300 µm grain size) and moderate aeration. Mussels were fed daily a mixture of a cultured algae (primarily *Scenedesmus*) and commercially available algal products (*Nannochloropsis*, *Isochrysis*, *Pavlova*, *Tetraselmis*, and *Thalassiosira weissflogii* from Reed Mariculture Inc., Campbell, CA) at a concentration of approximately 100,000 cells/ml. A 75% water change was performed weekly. Newly transformed juveniles from the next six broods were placed in a 19-liter downweller system modified from what was described by Barnhart (2006) (Fig. 4-4). Each downweller unit contained 5 culture cells constructed from 5-cm PVC pipe and 105-µm nytex screen. The feeding and water change regimes were the same as described

for the 8-liter tanks. Mesh screens were rinsed three times per week. Juveniles in the downweller systems were evaluated every 1-2weeks while they persisted in the system. Due to poor survival in the downweller system, all remaining juveniles were moved to 8-liter tanks with sediment after 6 weeks from the initial placement into the system.

Figure 4-3 Holding tanks (8-liters) for newly transformed Tar River spinymussels (*Elliptio steinstansana*) in 2009.

Figure 4-5. Downweller culture system used holding newly transformed juvenile Tar River spinymussels (*Elliptio steinstansana*).

In 2010, all newly transformed juveniles, except for 2 cohorts, were initially placed in a downweller system at approximately 22-23°C in cells with 105-µm mesh. We fed daily with a mixture of cultured algae (*Neochloris oleoabundans*, *Bracteacoccus grandis*, and *Oocystis polymorpha*) and the same commercial algal products described above at a concentration of approximately 50,000 cells/ml. We cleaned the screens daily and did a 100% water change weekly. Each brood was held separately in a single cell, and we graduated the screen-size for each brood whenever the smallest individual was contained by the next screen-size available. A cohort generally moved up to 150-µm mesh at one week, 200-µm mesh at 2-3 weeks, and 300-µm mesh at 6 weeks of age. Screen cleaning

became less frequent and was done only as needed once the mussels moved up to 300-µm mesh.

We moved a total of four juvenile cohorts propagated in 2010 to the Marion CAC. On 15 June 2010, we transferred three broods to the CAC at ages 3, 5, and 7 weeks. The 3-week-old mussels were placed in their I-unit in a stainless steel 125-µm-mesh cage (18 x 18 cm) with a thin layer of medium sand (500-800 µm grain size). The I-unit was fed by pond water that was filtered to 5 µm and then supplemented with the commercial algal products described above. The 5- and 7-week-old juveniles were placed in 250-µm-mesh cages (10 x 15 cm) with a thin layer of medium sand (500-800 µm grain size) and placed in one of the 26-liter growout tubs. Unfiltered pond water flowed directly into the top of those cages, and the top of the cages extended above the water line. Survival of each of those cohorts was assessed in August 2010. On 11 August 2010, we transferred an additional 17 *E. steinstansana* (10-weeks old) to the CAC and placed them loose in the medium-coarse sand in a 26-liter tub. Growth and survival of those juveniles was assessed on 3 November 2010.

We conducted two trials in 2010 to attempt rearing juvenile *E. steinstansana* in sediment in the laboratory setting. The first test, which began 19 July 2010, was also designed to evaluate 4 different diets as well. We divided 400 juveniles (<10 days old) from 2 separate cohorts into 20 groups of 20 individuals each. Each group was randomly assigned to one of each of the combinations of four diet treatments (commercial algae - low conc., commercial algae – high conc., cultured algae-low conc., cultured algae-high conc.) and five sediment treatments (100% sand, 75/25 sand-silt, 50/50 sand-silt, 25/75 sand-silt, and 100% silt) (Table 4-4). The commercial diet consisted of the Nanno 3600® and Shellfish Diet 1800® from Reed Mariculture, Inc. described above. The cultured diet consisted of a mixture of the 3 species cultured at the AECL (*Bracteacoccus grandis*, *Neochloris oleoabundans*, and *Oocystis polymorpha*). The low concentration diets were fed at approximately 30,000 cells/ml/day, and the high concentration was twice that. Each of the four diet treatments were fed to one of four separate recirculating systems (65 liters total volume). Each contained a 40-liter reservoir with five 5-liter round bowls designed to hold the juvenile mussels. Water was introduced to each bowl to create a circular flow, and a center standpipe directed water back to the reservoir below (Fig. 4-5). A 100-µm mesh basket was used to catch mussel that went down the standpipe. The sand was a medium sand (500-800 µm grain size), and the silt was collected from New Hope Creek (Orange County, NC), sieved to less than 300 µm and autoclaved. Mussels were fed daily and water changes were performed weekly. Growth and survival were assessed at 3 months.

A second trial was performed using the same sediment treatments in another identical 65-liter recirculating system using a single diet consisting of a mix of the commercially available and cultured species described above at approximately 60,000 cells/ml. We placed thirteen 2-month-old individuals (mean length = 1.26 ± 0.26 mm) into each sediment treatment. Feeding was done once daily and water changes were done weekly. After 50 days, growth and survival of each of the treatments were assessed.

Table 4-4. Design of sediment diet and sediment trial for newly transformed Tar River spinymussels (*Elliptio steinstansana*).

Diet treatment	Commercial diet	Commercial diet	Cultured diet	Cultured diet

	(30,000 cells/ml)	(60,000 cells/ml)	(30,000 cells/ml)	(60,000 cells/ml)
Bowl 1	100% sand	100% sand	100% sand	100% sand
Bowl 2	75% sand 25% silt	75% sand 25% silt	75% sand 25% silt	75% sand 25% silt
Bowl 3	50% sand 50% silt	50% sand 50% silt	50% sand 50% silt	50% sand 50% silt
Bowl 4	25% sand 75% silt	25% sand 75% silt	25% sand 75% silt	25% sand 75% silt
Bowl 5	100% silt	100% silt	100% silt	100% silt

Figure 4-5. Recirculating system used in *Elliptio steinstansana* sediment and feeding trial.

Results

Field Surveys and Broodstock Collection

The Tar River spinymussel appears to be continuing on a trend of population decline in the wild. A substantial amount of effort was required to find individuals even at sites where they were known to exist. Only 2 of the 23 individuals found had any remants of spines left, and all of them appeared old based on shell erosion. They were found most consistently in Little Fishing Creek near Glenview Road (SR 1338). While habitat in Little Fishing Creek appeared favorable for persistence of the species there, the density of the invasive *Corbicula fluminea* was extremely high.

Our survey results suggest continued population declines in both Swift Creek and the Tar River, but we did find the species at three new sites on Fishing Creek. Three thorough searches of Swift Creek at Red Oak Road (SR 1003) yielded no *E. steinstansana*. The sand-gravel bar where the species was found on the Tar River at NC 33 in 2001 was dry during the drought of 2007. Additionally, a survey there in 2009 found poor habitat, no *E. steinstansana* and only 6 *Elliptio complanata* in 3 person-hours of surveying. Fishing Creek from Avent Rd (SR 1506) downstream through the fall line between Melton Bridge Rd and NC 4/48 had patches of very nice habitat with several areas of stable, clean coarse sand and fine gravel. We found *E. steinstansana* at 3 sites in this reach. Additional surveys in that area would likely yield more individuals. The reach between Avent and Ward Roads contained especially nice habitat and should be surveyed more thoroughly. The habitat in the reaches we surveyed upstream of Avent Road was marginal to poor. Large reaches were very flat and stagnant or were unstable with substantial sediment movement. Even the few stable areas that had flow held very few *Elliptio* relative to what we expected from simply observing the stability of the habitat. A covering of fine silt and periphytic algae in those reaches may have been indicative of non-point nutrient and sediment inputs from agriculture *Broodstock spawning*

After monitoring the gravidity of our broodstock every two weeks over two brooding seasons, we believe we had 6 females and 5 males on hand. Those that were never observed to be gravid were assumed to be males. In 2009, there were a total of 27 brood releases from early April through late July. Averaged over 6 females, that suggests each one released around 4-5 broods over the brooding season. That number could have been higher, but six of the adults were not in the tank until early May 2009. April and May were the most active months of 2010, so these mussels may be capable of releasing up to 6 broods in a season when healthy. There was relatively little spawning in June 2010 (2 brood releases) compared to the same month in 2009 (13 brood releases). Consequently, the overall number of broods produced was lower in 2010 compared to 2009. Additionally, the percentage of each brood that was viable and the total number of viable glochidia per brood decreased in 2010. The adults grew significantly in length in 2010 and did not grow in 2009 (Table 4-5).

Table 4-5. Length data for Tar River spinymussel (*Elliptio steinstansana*) broodstock by date held at the Marion Conservation Aquaculture Center.

Tag Number	Gender	5/27/2009	11/3/2009	5/4/2010	11/9/2010
C275-276	female	no data	37	37	41
C277-278	female	no data	37	no data	40
B299	male	36	36	36	41
B295-296	male	38	38	38	38
B297	male	29	30	30	35
B300	male	32	32	32	35
F453-454	female	35	35	35	38
B298	female	40	40	no data	40
B301	female	32	32	31	36
B302	female	no data	37	37	41
Untagged mussel - etched	male	no data	38	37	42
B293-294 – Little R	unknown	36	37	38	42

Host comparisons in 2009

We propagated a total of 2,827 juveniles in 2009 off of 6 host species. In addition to the four host species originally found to serve as hosts (*N. leptocephalus*, *L. albeolus*, *C analostana*, and *L. matutinus*) (Eads and Levine 2008), we also found that creek chub (*S. atromaculatus*) and the swallowtail shiner (*N. procne*) also facilitated transformation of glochidia to the juvenile lifestage. Those species, however, appeared to be less than ideal as hosts. White shiners were the most effective over time, yielding an average of 15.8 juveniles per fish (SE = 3.3) across all tests (Fig. 4-5). Pinewoods shiners were especially difficult to maintain in captivity, and only one lived all the way to yield juvenile mussels. That one yielded 12 juvenile *E. steinstansana*.

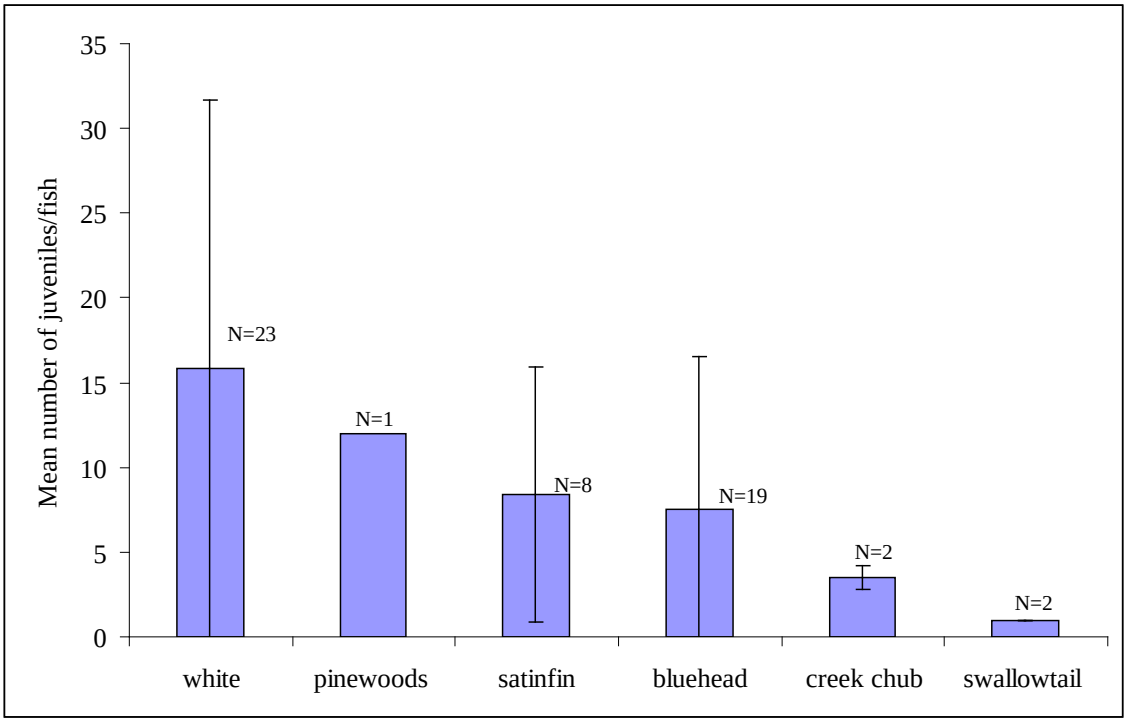

Figure 4-5. Mean number of juveniles (± SD) that excysted from an individual fish of the six host species that facilitated transformation.

Handling of host fish

Host fish survival greatly increased in 2010. Of the approximately 500 white shiners collected and held for propagation of this species as well as *Elliptio lanceolata* from March-July 2010, we estimate that only 5-10 of those individuals died in captivity. By eliminating electrofishing techniques from sampling and quarantining and acclimating the fish before use, we apparently improved the health and survival of the shiners used as hosts for the glochidia.. There were no fungal, bacterial, or protozoan outbreaks of these fish in 2010. Additionally, the quarantine and formalin treatment virtually eliminated predacious flatworms from the culture facility.

Feeding conglutinates to host fish

Feeding conglutinates to bluehead chubs and satinfin shiners yielded very few juvenile *E. steinstansana*. The 3 bluehead chubs yielded 4 juveniles after being fed 15 conglutinates, and 3 satinfin shiners yielded only 2 juveniles after being fed 8 conglutinates. Bluehead chubs readily took the conglutinates upon their introduction to the tank and would even spit the conglutinate out and attempt to eat it a second and third time. Satinfin shiners did take the conglutinates but were not as aggressive and began to distinguish between the conglutinates and the bloodworms that were introduced with them. This was not an effective method for infecting these two species. Each of the conglutinates had an average of 37 (SD = 11.5) viable glochidia.

Propagation efforts in 2010

Even though we had fewer broods to work with in 2010, we were able to be more efficient at production. We propagated a total of 3,201 juveniles from 15 viable broods that were available for use (Table 4-6).

Table 4-6. Total number of juvenile Tar River spinymussels (*Elliptio steinstansana*) propagated in 2009 and 2010.

2009			2010		
Infestation Date	**Female Tag Number**	**Number of juveniles propagated**	**Infestation Date**	**Female Tag Number**	**Number of juveniles propagated**
5 April	Unknown	0	7 April	Unknown	441
8 May	Unknown	0	9 April	Unknown	58
11 May	Unknown	0	14 April	B302	82
15 May	Unknown	1	19 April	F453	42
18 May	Unknown	0	19 April	C275	202
22 May	Unknown	8	20 April	B301	
29 May	B301	9	5 May	B298	479
1 June	B302	50	5 May	C278	
10 June	Unknown	342	7 May	Unknown	616
12 June	Unknown	23	14 May	B302	563
15 June	Unknown	332	16 May	F453	6
16, 17 June	Unknown	244	21 June	Unknown	118
18 June	Unknown	531	23 June	B302	532
22 June	C275/276	195	9 July	Unknown	62
23 June	Unknown	36	14 July	Unknown	47
24 June	C277/278	153			
25 June	F453	0			
29 June	Unknown	0			
3 July	B302	197			
8 July	Unknown	41			
10 July	Unknown	316			
14 July	Unknown	21			
20 July	Unknown	147			
22 July	Unknown	181			
27 July	Unknown	0			
TOTAL		**2827**			**3201**
MEAN		**113**			**185***

= represents the total number of juveniles divided by the 15 broods used to produce those juveniles.

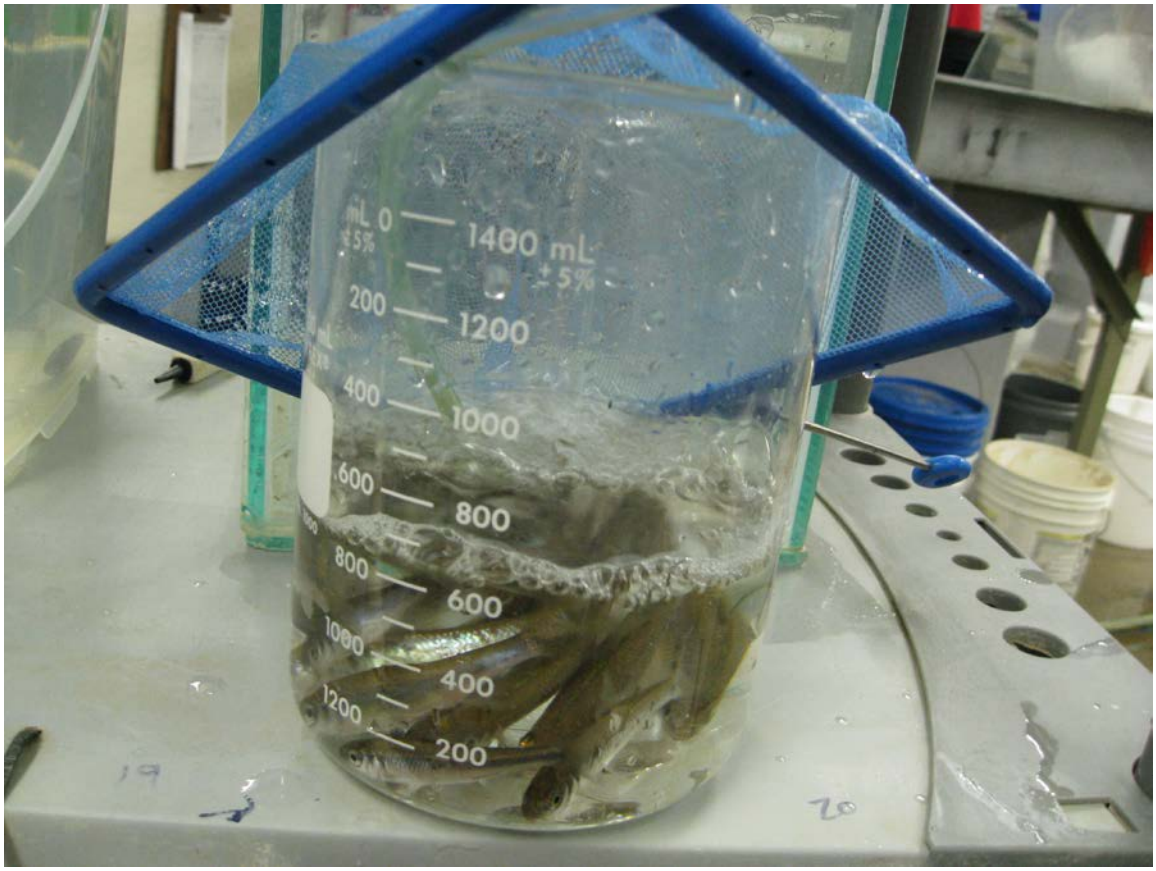

Figure 4-6. Infestation of 14 white shiners (*Luxilus albeolus*) with glochidia of the Tar River spinymussel (*Elliptio steinstansana*).

We raised the mean number of juveniles propagated per brood from 113 in 2009 to 185 in 2010. By May 2010, we learned that the best way to increase production from a single brood was not to increase the number of fish used but to decrease the amount of water used to suspend the glochidia. On 7 May 2010, we placed 15 medium to large white shiners in 1.25 liters of water with a brood of approximately 3,200 glochidia (Fig. 4-6, above). That effort yielded 616 juveniles, which was the highest number of any of the propagation efforts. One week later, 15 white shiners suspended with an equal number of glochidia in 1 liter of water yielded 563 juveniles. Twelve white shiners in 1 liter of water on 23 June 2010 yielded 532 juveniles. We reduced the water volume on the last two efforts in July 2010 to only 700 ml but produced only 62 and 47 juveniles respectively (Fig. 4-7). Those broods only had 1,500 or fewer glochidia and potentially weren't as healthy as some of the others.

Growout of juvenile mussels

The 3 individuals propagated in 2008 that were moved the CAC in May 2009 all survived the length of the project and exhibited rapid growth (Fig. 4-7). The first spines formed in mid-June 2009 when the mussels were approximately 6-8 mm in length (Figs. 4-8, 4-9). At the end of 2009, they had reached 15, 17, and 19 mm in length respectively (Fig. 4-10). In 2010, they did not add additional spines but continued rapid growth and measured 32, 33, and 34 mm in length at the end of the year (Fig. 4-11).

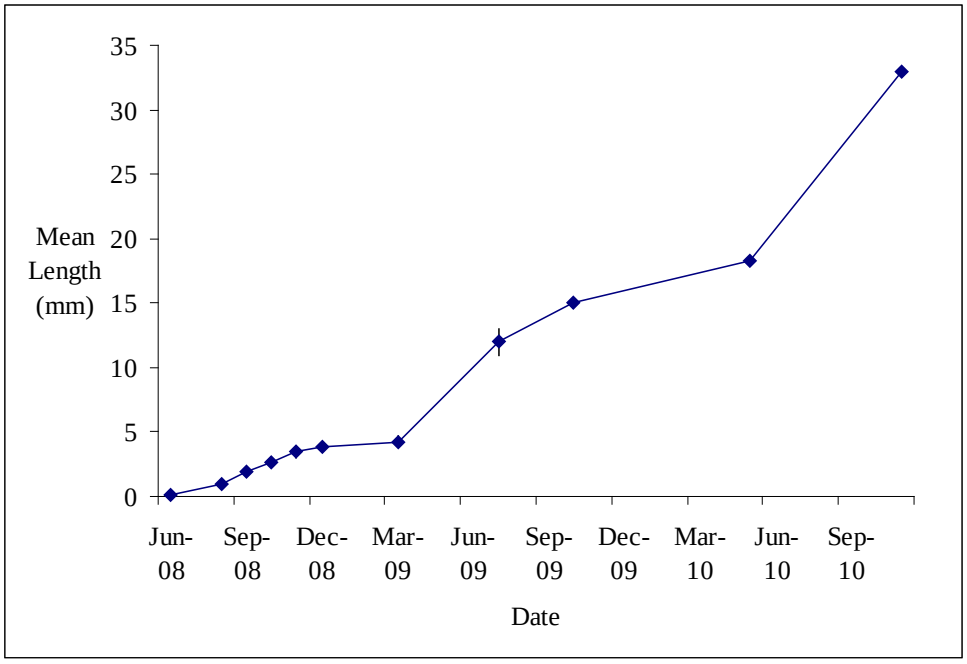

Figure 4-7. Length of Tar River spinymussels (*Elliptio steinstansana*) propagated in 2008 and monitored through the end of the growing season in 2010.

Figure 4-8. Three *Elliptio steinstansana* (1-year-old) with their first spines. Picture taken 18 June 2009.

Figure 4-9. *Elliptio steinstansana* propagated in 2008. Picture taken 18 June 2009.

Figure 4-10. *Elliptio steinstansana* propagated in 2008. Picture taken 12 November 2009

Figure 4-11. *Elliptio steinstansana* propagated in 2008. Picture taken 11 August 2010

We had 100% mortality of all mussels propagated in 2009. Those cultured in the downweller system were monitored regularly and were declining rapidly in number even though they exhibited growth comparable to other species cultured successfully in the laboratory. The 6 broods cultured in the downwellers remained in that system for 3-6 weeks, and survival ranged from 0 to 2.2%. It was determined that daily cleaning was needed for the 105-µm screens to allow constant flow. Additionally, the cultured algae we were feeding (*Scenedesmus*) tended to colonize and likely contributed to the clogging of screens. On 31 August 2009, the 8 remaining juveniles from the downweller system were moved to an 8-liter tank with a sand substrate. On 17 September 2009, we sorted through all of the 8-liter containers and found no live individuals. We did, however, find a high density of predacious flatworms in all of the tanks. We attributed the 100% mortality to predation.

In 2010, juveniles cultured in the downwellers had approximately 10% survival after 2 months (Fig. 4-12). The largest percentage of the mortality occurred during the first 2 weeks of life. There was no survival 2 months later among the 3 broods placed at Marion on 15 June at 3, 5, and 7 weeks of age. The I-unit, which held the youngest cohort of juveniles had experienced an unknown problem, and NCWRC staff noticed significant mortality of other species in that system as well during a short time frame (T.R. Russ, NCWRC, pers. comm.). The 10 x 15-cm cages holding the 5 and 7-week-old juveniles collected a large amount of organic debris, aquatic insects, leeches and worms. The sediment in those cages became clogged and likely was not conducive to growth of small mussels. In contrast, 13 of the 17 individuals taken to the Marion CAC on 11 August were alive on 9 November, having exhibited significant growth. On 11 August, this brood was 10 weeks old with a mean length of 2.6 ± 0.5 mm, but in November mean length was 5.4 ± 0.9 mm. One individual (6 mm long) had started developing spines.

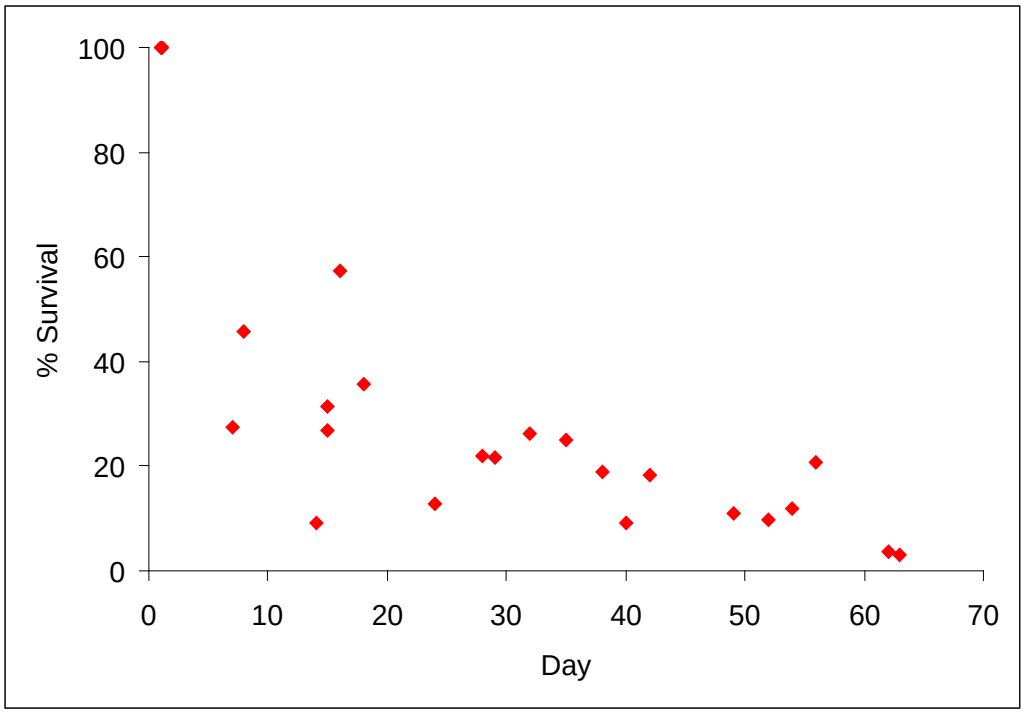

Figure 4-12. Survival of Tar River spinymussels (*Elliptio steinstansana*) cultured in a downweller system.

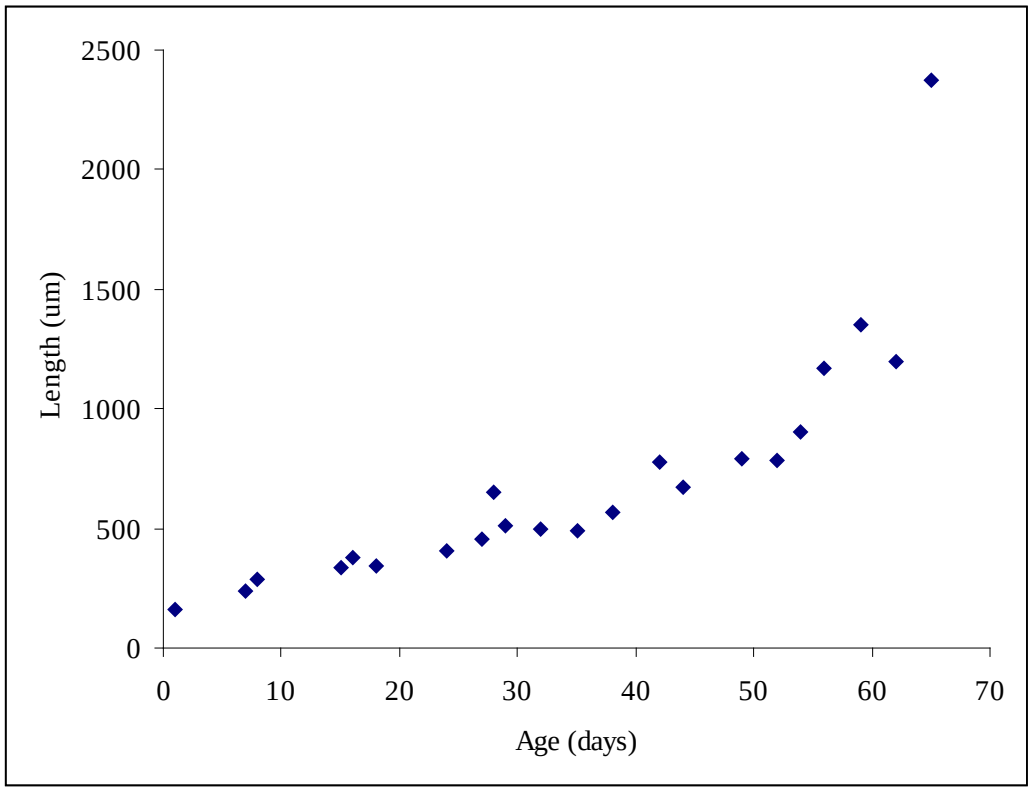

Figure 4-13. Growth of *Elliptio steinstansana* cultured in a downweller system.

Mussels placed in sediment in 2010 did better if they were placed there at 2 months. At 3 months, there were no surviving juveniles from the sediment and feeding trial that began with newly transformed juvenile mussels. In the sediment trial that began with 2-month old juveniles, overall survival was 81.5%, and mussels grew to a mean length of 2.94 ± 0.42 mm after 50 days (Table 4-7). There were no significant differences in length between treatment. The experiment was not replicated to further assess survival due to the shortage of individuals available.

Table 4-7. Survival and growth after 50 days of juvenile *Elliptio steinstansana* placed in 5 different sediment treatments at 2 months of age.

Treatment	Proportion alive	Mean Length ± SD (mm)
100% sand	9/13	3.19 ± 0.44
75% sand 25% silt	13/13	2.81 ± 0.49
50% sand 50% silt	12/13	2.97 ± 0.53
25% sand 75% silt	11/13	2.92 ± 0.27
100% silt	8/13	2.84 ± 0.19

Discussion

Field Surveys and Broodstock Collection

The Tar River spinymussel is getting very difficult to find in the wild. The two remaining streams in North Carolina where *E. steinstansana* could still be found somewhat regularly were in the lower portion of Little Fishing Creek near Glenview Road and Fishing Creek from Avent Road down to the fall line downstream of Melton Bridge Road. While habitat conditions in these locations appeared suitable to maintain the species, the invasive *Corbicula fluminea* are extremely dense and may pose a threat to the remaining viability of the species there. In a laboratory setting, *Corbicula* were shown to ingest as well as displace newly transformed juvenile Unionids (Yeager et al. 1999). Alternatively, *Corbicula* could be competing with native Unionids for food resources (Atkinson 2011), but this is unknown and may vary from stream to stream as well as across Unionid species. *Fusconaia masoni*, a species that often co-occurs with *E. steinstansana* did show some signs of recent recruitment in Little Fishing Creek but are in relatively low numbers in Fishing Creek. Because of a lack of survey effort in Fishing Creek historically, we cannot comment on population trends in this area. Still, no evidence of recent recruitment was seen. Also, the lack of healthy *F. masoni* populations may indicate that the declines may be cross-species in nature and associated with detrimental changes in habitat.

Broodstock spawning

Brooding patterns in freshwater mussels are not fully understood. The documentation of the release of 4-5 broods in a single season by individual females is a notable discovery. Price and Eads (2011) observed that other short-term brooders from the genus *Elliptio* also spawn more than once in a brooding season. In 2010, the increased transport of females between Marion and Raleigh may have been the cause for decreased spawning activity. We were concerned that this may have indicated a loss of condition over time at the hatchery, but the adults grew significantly from July-September in 2010.

The comparatively higher growth observed during 2010 may simply represent a shift in their resources from reproduction to growth, or it may have been a result of more favorable conditions in 2010. A cold-water line was added to the mussel growout facility late in 2009 to avoid extreme summer temperatures and potential dips in dissolved oxygen in the lower pond. In the hottest part of the summer of 2009, some mortality of other mussel species occurred at the hatchery. Perhaps a more favorable temperature regime resulted in more growth in 2010. Hatchery temperature data were not available at the writing of this report.

Handling of host fish

The health of host fish is critical to the success of propagation efforts. If these sensitive fish become diseased while mussels are attached, juvenile mussels are lost.. The utmost care should be used in all stages of fish collection and holding. These hosts should only be seined. During collection, we recommend holding the fish instream in a mesh cage to minimize the time they spend in a collection bucket. Collection efforts should be scheduled to avoid periods of hot air temperatures, and minimize transport times. We typically transport white shiners at a density of no greater than 1 individual per liter. After arrival in the laboratory the fish should be carefully acclimated to laboratory conditions, quarantined for preferably a minimum of three weeks prior to use, and treated with formalin according to Zimmerman and other (2003). The formalin treatment will also reduce the load of trematodes and other gill parasites, and minimize their effect on gas exchange through the gills. We suggest that laboratories develop specific standard operating practices for handling, transporting, quarantining and housing fish prior to host infestation with glochidia.

Host comparisons in 2009

Additional research is needed into the importance of pinewoods shiners (*Lythrurus matutinus*) as a host. Since we were able to greatly improve host fish survival in the laboratory in 2010 with white shiners, we suggest future attempts to hold pinewoods shiners may be more successful. Satinfin shiners were inconsistent as hosts, as some performed very well yielding 15-20 juveniles from a single fish and others performed poorly yielding only 1-3 juveniles. While bluehead chubs are abundant, easily collected and can reach larger sizes, they were not as consistently good hosts.

Feeding conglutinates to host fish

Feeding conglutinates to bluehead chubs and satinfin shiners was a very inefficient way of getting glochidia on the gills of those species. If we had fed the entire brood of 115 conglutinates (estimated at 4,255 glochidia) to these two species, this would have yielded only an estimated 25-40 individuals. That would be less than 1% transformation of the original brood size. Perhaps the more efficient hosts at transformation also yield the greatest attachment through this more natural infection method.

While white shiners were shown to be the best host in the laboratory, it is unknown which is the most important host to the survival of the species in the wild. Determining which species facilitates the greatest transformation of juveniles after eating the conglutinates may be an important factor in determining the role of individual as hosts species in streams. We were unable to train white shiners in a glass aquaria to feed on bloodworms immediately upon their introduction to the tank. This fish species remained was easily startled, and exhibited erratic "flight" behavior when lab personnel attempted to feed the fish. Because of this, they were not included in the trial when *E. steinstansana* conglutinates were fed to the different host species. Pinewoods shiners did not survive the experiment and were also not included in this trial. When white shiners were held in large (190 and 380 liter), opaque gray tanks in 2010 they became more adjusted to laboratory feeding routines and regularly approached the water surface anticipating feed. Future trials feeding conglutinates should first acclimate both white and pinewoods shiners in large, dark-colored tanks and conduct the feeding trials in the holding tanks. Once the fish have fed, they could be moved to other tanks more suitable for recovery of transformed juveniles.

Propagation efforts in 2010

In 2010, we discovered that the most effective way to increase attachment was to decrease the water volume used to infect the fish to increase glochidial concentration. We currently aim to achieve a concentration of 4,000 glochidia per liter. In 1 liter of water, we suggest using 10-15 medium-sized white shiners for propagation of this species.

While the host fish were being exposed to the glochidia, the glochidia would snap shut in suspension over time in response to either the physical agitation of aeration or mucous sloughed by the fish. We routinely monitored the glochidia in suspension every 5-15 minutes during exposure and would end the exposure once all the glochidia were closed. Most infestation efforts in 2010 lasted approximately 45-50 minutes. We witnessed some foaming of the water, which was possibly indicative of sloughing of mucous and a stress response by the fish. When the water foams, glochidia will snap shut in suspension before they are able to attach to the fish. Infestations may best be done in a completely dark chamber to decrease fish stress.

Growout of juvenile mussels

In 2009, we had no survival of any of the juveniles propagated. Those held in the downwellers grew well but exhibited high mortality, so they were moved to sediment

based growout tanks. Unfortunately, all of those tanks became infested with predacious flatworms. In 2010, we took several measures to improve survival of juveniles that proved to be somewhat effective. We quarantined all host fish and treated them with formalin to prevent the entry of flatworms into the culture systems (Zimmerman et al. 2003). We bleached the central reservoir and distribution lines, which were observed to have flatworms in 2009. We also added 10-µm filtration to the line coming from that reservoir. As a result, we saw virtually no flatworms in the juvenile culture systems. We converted almost exclusively to the downweller systems to allow more frequent observation and began cleaning the screens in them daily. We stopped feeding with *Scendesmus* algae and also reduced the overall daily feed to 50,000 cells/ml from 100,000 cells/ml. This kept the screens much cleaner and facilitated better flow. Still, while survival improved in 2010, it still was less than what has been seen in other mussel species cultured in the laboratory. The reasons for that are unknown. Perhaps only 10% of those broods were fit enough to survive or could adapt to the culture environment. Or perhaps, there is a nutritional short-fall that causes a majority of the brood to be undernourished. Much research is still needed to understand why some species and cohorts do well in culture and why others do not.

Those individuals propagated in 2008 and moved to the Marion CAC in 2009 grew rapidly and have already approached the size of the wild-caught adults from Little Fishing and Fishing Creeks. This indicates that the hatchery environment offers a more nutritious or abundant food source than is found in those streams. The oldest juveniles transferred to the hatchery in 2010 faired the best. We suspect that the high survival of this brood was in part due to the fact that it was 3-5 weeks older than those moved to the CAC in June. We also suspect that placing these juveniles loose in the 26-liter tanks provided them with a cleaner substrate in which to thrive. Marion CAC staff has witnessed good survival and growth of juveniles of other species in their 26-liter tubs when placed there at 500-1000 µm (T.R. Russ, pers. comm.), and we suspect the same would be true of *E. steinstansana*. We would recommend future cohorts be transferred to this type of setting at 1 mm in length (55-60 days old). At that stage, they were entering a period of rapid growth and should not be as vulnerable to predators in the hatchery setting.

There was also a drastic difference in survival in sediment between 2-month old and newly transformed juveniles cultured in sediment in the laboratory. Overall survival of mussels after 2 months of age (81.5%) in sediment was encouraging. Mussels performed similarly between sediment treatments, but more research could be done in this area. More research is needed to assess the health and survival of *E. steinstansana* in various sediment types. We are unsure of why there was no survival in sediment of juveniles placed there at less than 10 days old. There were no predacious flatworms in the system and no significant proliferation of Chironomids or other fauna such as nematodes. That lifestage is the most sensitive and could have responded negatively to any number of conditions from food shortage to poor water quality or flow in the interstitial spaces

Chapter 5
Master's Student Graduate Work
Potential contribution of shredding insects to unionid diets

Introduction

Shredding aquatic insects play an important functional role in river ecosystems (Vannote et al 1980). They break down leaf litter and other forms of large organic matter when feeding, which creates fine particulate organic matter (FPOM). The FPOM suspended in the water column has a large surface area, making it an important carrier of nutrients in streams (Yoshimura et al 2008). Through fragmentation and feces, the shredder process provides nutrients for consumption by other organisms downstream. Leafpack is the largest component of course particulate organic matter in temperate streams (SOURCE). It leaches the most within the first 24 hours of being submerged in the stream, losing 5-27% of its dry weight in this time (Anderson and Sedell 1979). Leaf leachate consists of polysaccharides (carbohydrates), amino acids (protein) and fatty acids (lipids) (Volk et al 1997, from Yoshimura 2010). Leaf leachates can make up 42% of the total dissolved organic matter (DOM) in a stretch of stream (McDowell and Fisher 1976, from Yoshimura 2010). After the leaf leaches, microbes degrade the leaf surface, a process which is mostly complete within two weeks (Cummins 1974). This microbial transformation of the leaf material is a key determinant of stream DOM composition (Yoshimura 2010). Pathways for removal of this DOM in streams include flocculation, microbial utilization, and photodegradation (Ch 6 Osburn and Morris of book edited by Helbling and Zagarese 2003).

Although leaves break down on their own through leaching and microbial colonization, decomposition of this course particulate organic matter is faster with the help of shredding insects. In one study leaves lost 50% of their weight in 70-90 days when held without shredders, but lost that same weight in 40 days when shredders were present (Anderson and Sedell 1979). Cummins and coworkers found that 20% of initial leaf weight loss went to shredder processing and approximately 10% was lost due to leaching (1973). Percent dry weight loss of leaves after 40-60 days was higher with shredders present than in controls with no animals (40-64% versus 26%, respectively). A large amount of FPOM is generated by the process of feeding and defecation of shredders on course particulate organic matter (CPOM) (Wallace and Webster 1996). Shredding insects play a large part in leaf decomposition and FPOM production.

Shredding insects also accelerate microbial growth (Covich et al 1999). Microbial colonization is similar for all species of leaves that fall into the stream (Cummins et al 1973). Aquatic shredding insects generally select leaves for feeding based on the degree of degradation (SOURCE). Anderson and Sedell found that bacteria are relatively sparse on detritus until it is passed through the invertebrate gastrointestinal tract. They showed that maximum microbial activity on FPOM is reached after 2-3 days of passing through the animal (1979). Crowl and coworkers (2001) completed a study with detrital shrimp and noted that microbial production on FPOM occurs more slowly than production in the presence of shredding shrimp. The acceleration of microbial growth by shredders creates increased nutrient availability to other aquatic organisms. Different insects contribute by different degrees to the organic matter in streams. Covich and coworkers (1999) suggest that the presence or absence of one species can dramatically affect decomposition rates. The three dominate families of shredding insects in North America are *Trichoptera*, *Tipulidae*, and *Plecoptera* (Anderson and Sedell 1979). Cummins and coworkers (1973) found that *Tipula* decreased leafpack dry weight more than all insect detritivores tested (including *Pycnopsyche* or *Pteronarcys).*

They more than doubled the dry leaf weight loss from leaching (64% to 26%), and note that *Tipula* and *Pycnopsyche* alone could process at least half of the detritus into particulates for the stream that was studied (Cummins et al 1973).

Although the role of aquatic shredding insects in the mechanical and microbial processing of leaves (and organic matter production) is well documented, their affect on chemical composition of DOM over time is poorly described. Our goal for this study was to compare both the quantitative and qualitative production of FPOM and DOM by three sympatric species of shredding insects: *Tipula*, *Pteronarcys* and *Pycnopsyche*. Flow cytometry and absorbance and fluorescence spectroscopy have been used widely to study quantitative and qualitative water chemistry. We used flow cytometry to estimate particle production by these species. Absorbance and fluorescence spectroscopy was used to examine changes in the concentration, molecule complexity, and chemical composition of DOM over time.

Methods

The study was conducted in August and September of 2009. All insects and leafpacks used in the study were collected from various NC streams within a week before experiments began. Leafpacks were collected in stream locations where shredding insects were present. We hand-selected leaves that had the same degree of degradation as packs where the shredding insects were found.

Set-up:

Twelve 1-liter glass jars were used to hold four treatments, each with three replicates. A treatment designated as the control included leafpack but no insects. The remaining three treatments were each of the three insect genera used, *Pteronarcys*, *Tipula*, and *Pycnopsyche*. Each treatment had three individuals of the same genus per jar. The jars were held indoors, in a temperature and light controlled facility. The full spectrum bulbs were set on a 12-hour on, 12-hour off cycle. The air temperature ranged from 69 to 73 degrees Fahrenheit.

Study jars were thoroughly cleaned and rinsed with an acid wash and water and allowed to dry prior to their use in the study. Each treatment jar contained 500mL of well water and an airstone. Leafpack was homogenized by pulsing with a kitchen blender and then filtered through a 200um sieve. Only particles larger than 200um were used for the experiment. After blending and filtration, 15g wet weight of leafpack was added to each jar. Aluminum foil was placed over the jar top to reduce evaporation. The airstone air flow was adjusted every day to keep the airflow as consistent as possible across all jars. Insects were acclimated in well water for at least 24 hours before adding to the experiment jars. The insects were not disturbed for the duration of the experiment.

Water samples:

The same day insects were added and 2 days after tanks were initially set up (day zero), 35mL water was removed from the control treatment and tested using flow cytometry, absorbance and fluorescence (using the methods described below). This water was replaced with the same volume of fresh well water. The sample was removed

by sucking the water off slowly near the surface to avoid disturbing the settled material (including the leafpack) on the bottom of the jar. The samples taken at day 5, 13, and 21 were retrieved the same way, but across all 12 jars. A 250mL water change was completed on day 8, with collection of the samples on that day. Part of each water sample was filtered through a 200um sieve before passing through the flow cytometer to get an estimate of particle count. The remaining water was passed through a 0.7um Whatmann glass microfiber filter to examine the dissolved component through absorbance and fluorescence of the sample.

3 Methods of testing:

Flow cytometry.

Flow cytometry was conducted using a Becton Dickinson FACS Calibur, running CellQuestPro Software. Total particle count was received per flow run time, which was held constant throughout the experiment at two minutes. At the high flow setting that was used, the instrument drew up the sample at 60uL per minute.

Absorbance of DOM.

Absorbance of DOM was determined using a Cary 300 UV Spectrophotometer. Each 800-200nm scan was run at 600nm/min. Data was collected at 1nm intervals. A 10cm cylindrical cuvette was used to hold the sample. MilliQ water was used as a blank and substracted from the sample data to get the raw absorbance value.

Fluorescence of DOM.

A Cary Eclipse Spectrofluorometer was used to obtain fluorescence contouring data to create Excitation-Emission Matrices (EEMs). Lamp intensity was 900 volts, and emission spectra from 300-600nm was measured at excitation wavelengths incrementally from 240-450nm at 5nm intervals. Emission wavelengths were sampled every 2nm. A 1cm pathlength cuvette was used to hold the sample. MilliQ water was run before every set of samples to normalize the data for the Raman water scatter peak. Quinine sulfate was used as a standard. All absorbance and fluorescence data were exported as CSV files and post processed with Microsoft Excel and/or Matlab.

Results

Flow cytometry

Particle count was highest at every timepoint for the *Tipula* treatment. The presence of craneflies increased production of <200 μm FPOM more than other shredders or leaves decomposing alone (Fig. 5-1). There were no significant differences between the control treatment and *Pycnopsyche* or *Pteronarcys*. All particle counts declined after the day 8 timepoint because of the water changes in the tanks on that day.

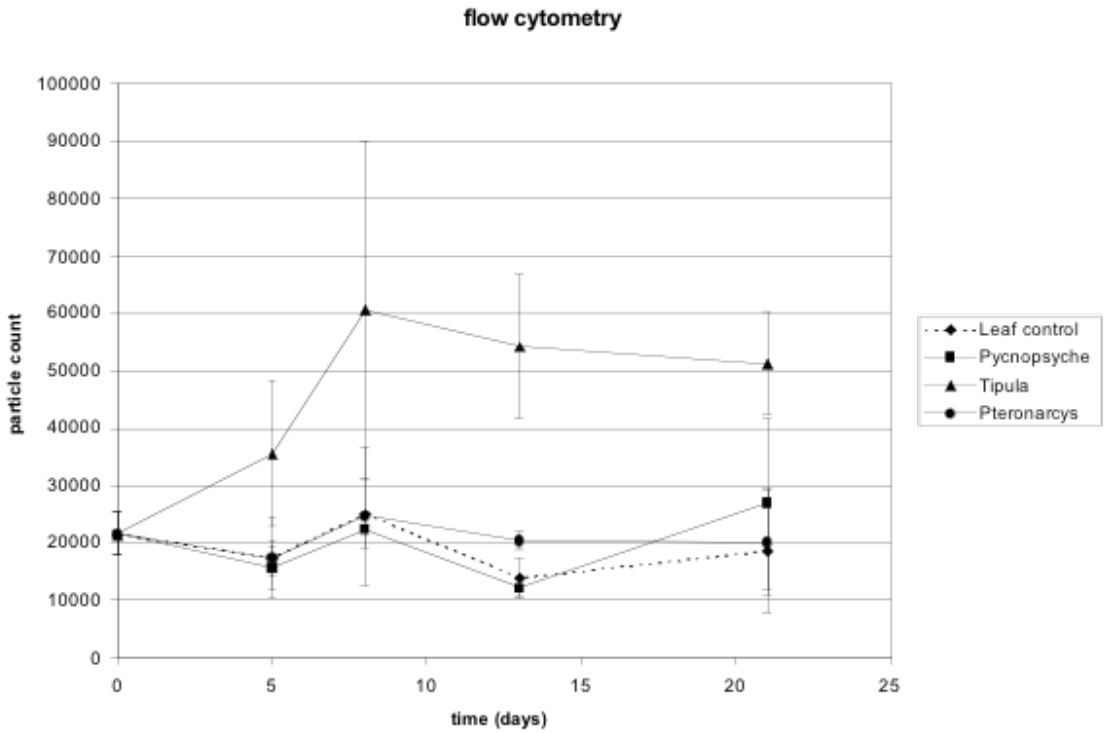

Figure 5-1: Fine particulate organic matter particle counts determined by flow cytometry.

Absorbance of DOM

Tipula demonstrated considerable increased absorption across the range of wavelengths. (Figure 5-2).

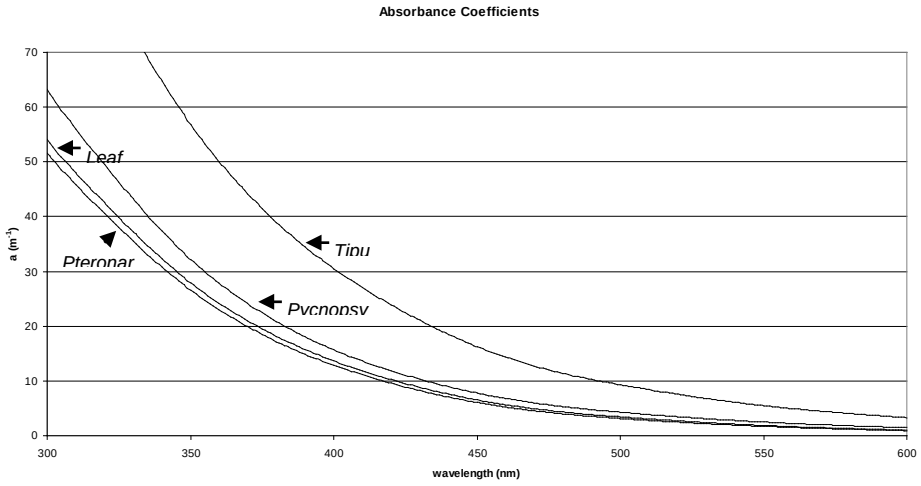

Figure 5-2: Absorption of DOM by Tipula. A single representative replicate from the day 21 samples.

Absorption coefficients at 350nm and 440nm were examined as reference points for all absorption data (Figures 5-3, 5-4). The *Tipula* treatment was the only treatment that had absorption coefficients significantly above the control values. The higher the

absorbance the greater concentration of the absorptive material in the water samples (Beers Law). The craneflies had increased levels of light-absorbing colored DOM (CDOM) over all the treatments tested in our lab. The cranefly water samples were visibly darker in color than any of the other samples by day 13 and 21

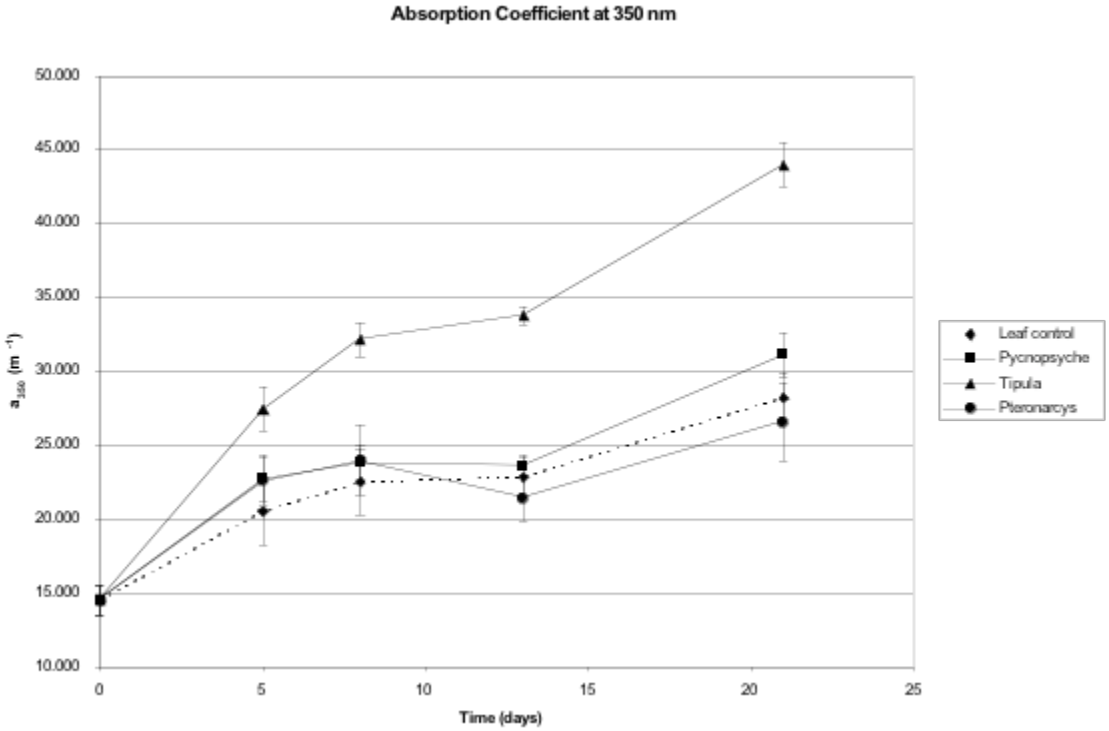

Figure 5-3: Estimated dissolved organic matter based on absorbance at a_{350} (350nm).

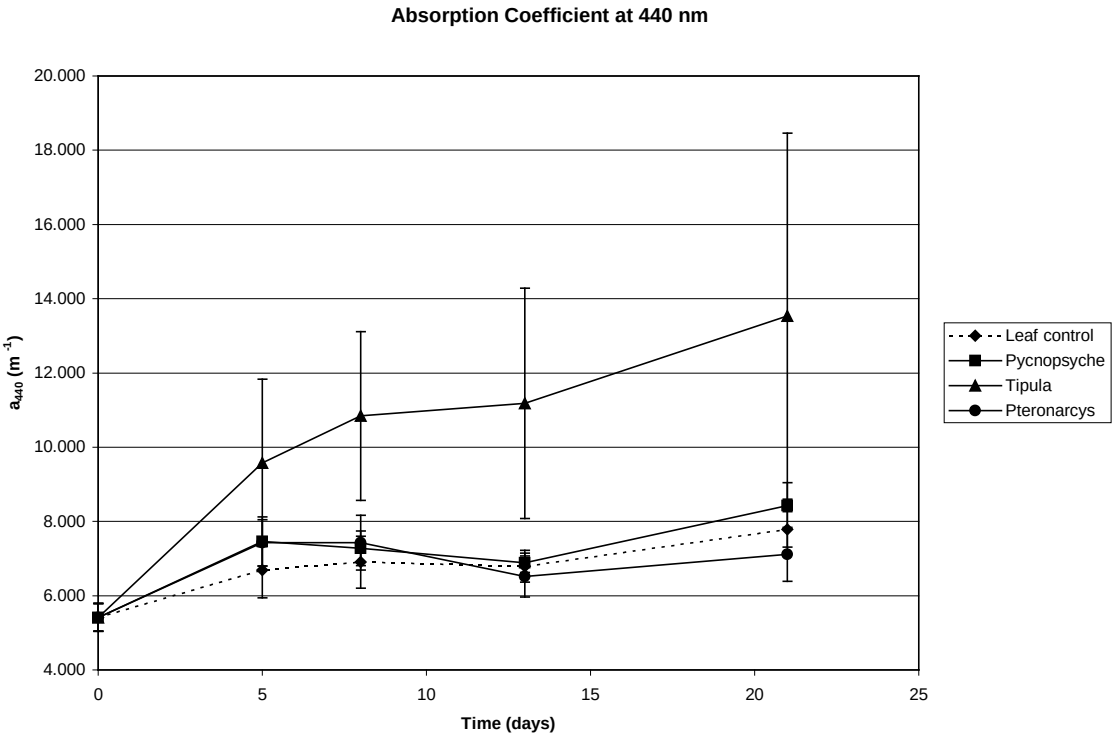

Figure 5-4: Estimated dissolved organic matter based on absorbance at a_{440} (440nm).

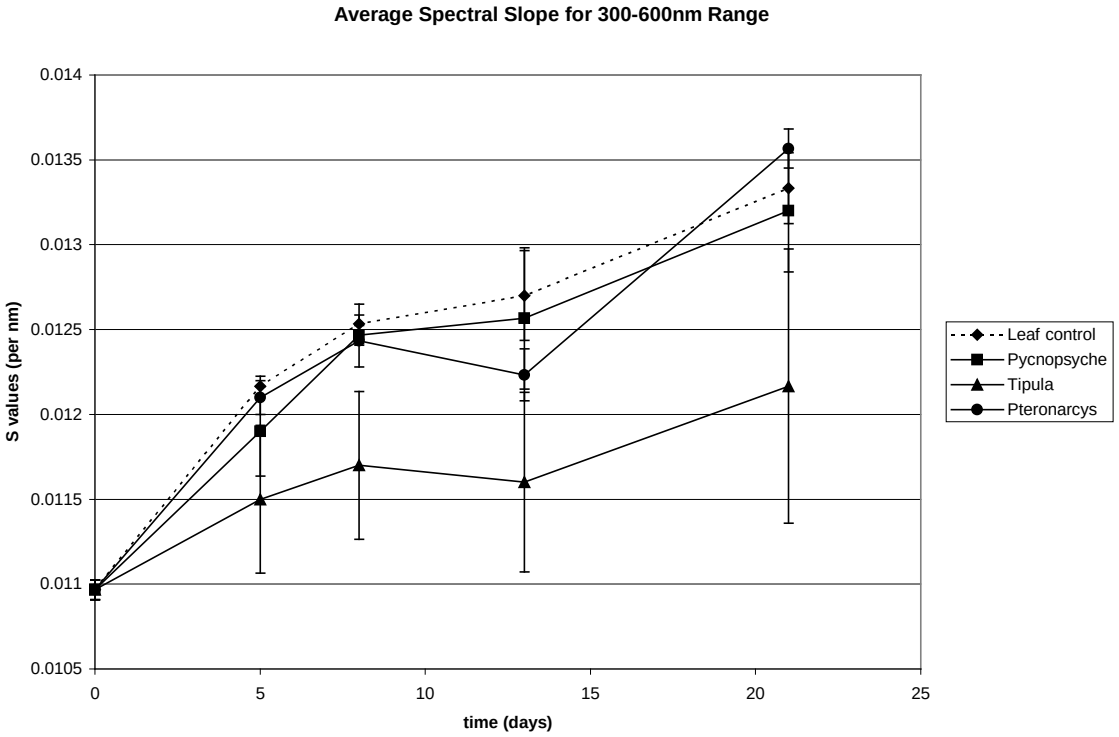

Figure 5-5: Comparison of spectral slope data associated with the shredding insects (Spectral slopes$S_{300\text{-}600}$).

Spectral slope value is often used as a characterization tool for CDOM and has been correlated to molecular weight (MW) of colored dissolved organic matter The spectral slope (S value) is a measure of CDOM, assessed by evaluating the steepness of the absorbance over a range of wavelengths ((Twardowski et al 2004); Helms et al 2008). Spectral slope is lower for samples with molecules that are more complex due to the increased absorption of these molecules at longer wavelengths. In this study, S was calculated as the slope from log transformed absorption coefficients between 300-600nm. The slope values were determined by fitting a regression line to the log-linerarized absorption spectra in Microsoft Excel. In natural fresh waters this value ranges from 0.012 nm^{-1} to 0.020 nm^{-1}(Kirk 1994). In this study, the values were on the low end of this range. The general increasing trend of S values over time possibly was due to intense microbial degradation of DOM in all of the treatments (Fig. 5-5, above). Microbial degradation breaks DOM molecules down into smaller pieces, and the graph demonstrates this as the amount of smaller molecules increased over time for all treatments. However, the spectral slope values for the cranefly treatment were lower (less steep) than those from the other insects and the control at every timepoint. This was a result of increased longwave absorption and the presence of more complex molecules in the *Tipula* treatment. The other shredders tested did not demonstrate S values very different from the control treatment.

We calculated S values for the 300-600nm (Fig. 5-5), 275-295nm range (Fig. 5-6) and the 350-400nm range (Fig. 5-7).

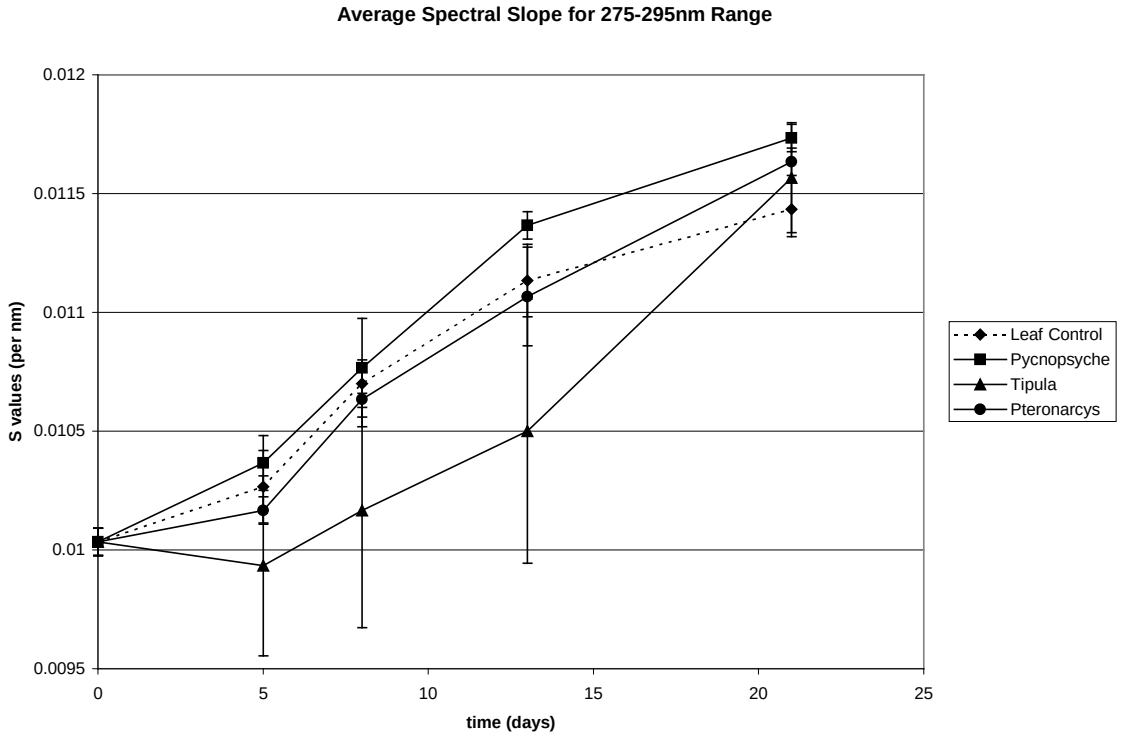

Figure 5-6: Comparison of spectral slope data associated with the shredding insects (Spectral slopes $S_{275\text{-}295}$).

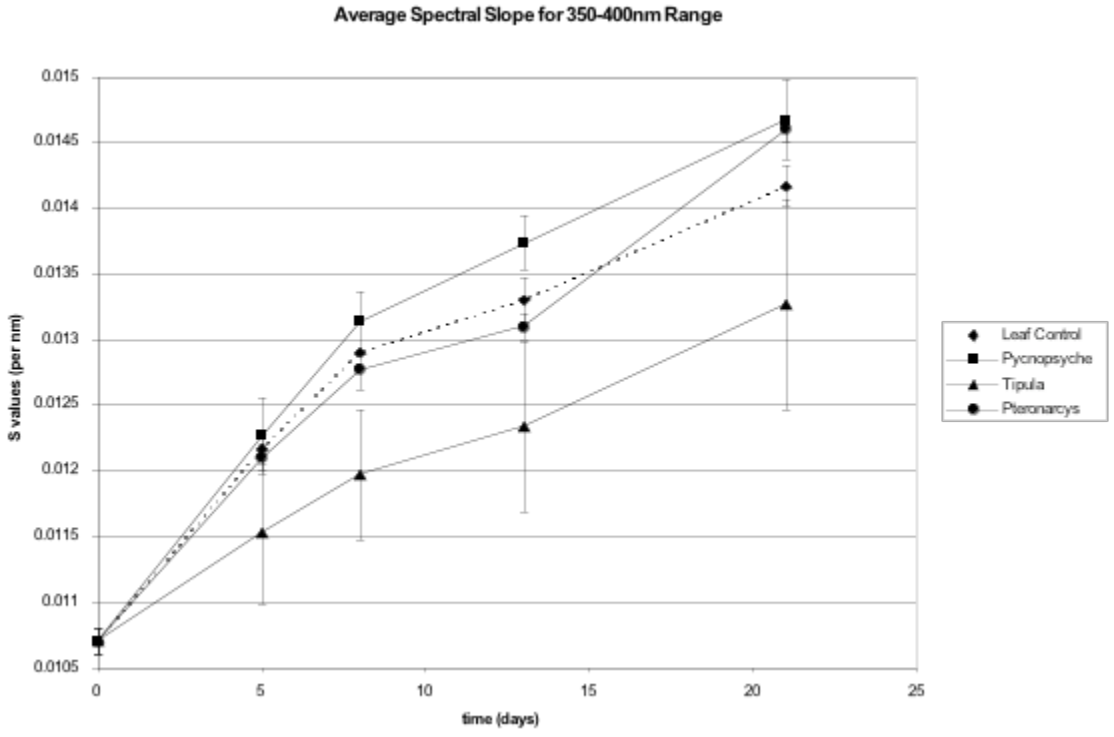

Figure 5-7: Comparison of spectral slope data associated with the shredding insects (Spectral slopes$S_{350\text{-}400nm}$).

All treatment were undergoing microbial degradation and producing smaller MW molecules as time went on (Figures 5-6, 5-7). The *Tipula* treatment had significantly lower S values than the other treatments, indicating the presence of larger MW DOM. *Pycnopsyche* had significantly higher S values than the control, indicating increased levels of degradation of DOM in this treatment. A ratio of the slopes from the two smaller ranges was calculated and graphed (figure 5-8). This dimensionless ratio, S_R, is used to examine changes in DOM quality by inherent sensitivity to changes in long wave absorption (Helms et al 2000). The general decreasing trend of all treatments except *Tipula* follows Helms and coworkers (2008) for DOM undergoing increasing microbial degradation (Fig. 5-8). The *Tipula* treatment had higher S_R values at every timepoint.

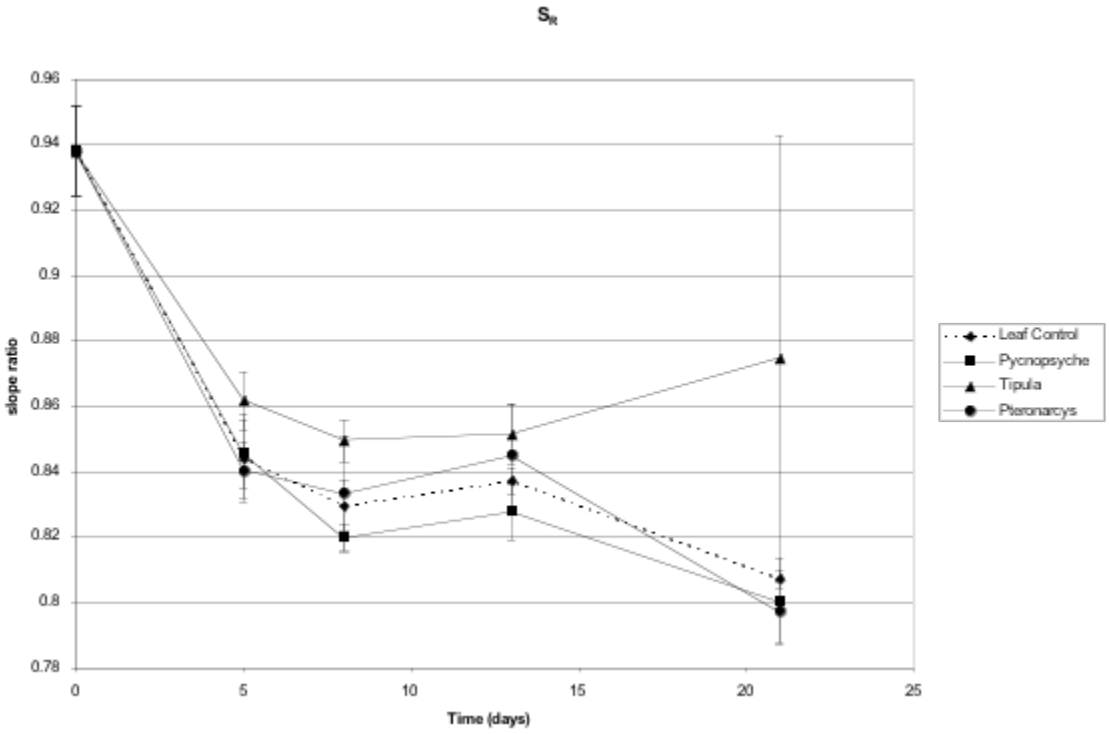

Figure 5-8: Comparison of DOM quality associated with the shredding insects.

Fluorescence of DOM

Fluorescence contouring data can be used to identify different types of fluorescent compounds in a water sample (Fellman et al 2010). Scanning emission over a range of excitation wavelengths provides data for production of an excitation-emission matrix (EEM). The EEMs produced show peaks for different compounds (fluorophores) and their position identifies generally what type of compounds they are. We focused on two basic types of DOM fluorescent signals in this study; humic-like (peaks C and A) and protein-like (peaks T and B). Humics exhibit emissions at longer wavelengths and have broad emission maxima due to their complex molecular nature (Fellman et al 2010). Peaks T and B (tryptophan-like and tyrosine-like, respectively) are a mixture of amino acids with the same fluorescence characteristics (Fellman et al 2010). There is some ambiguity in determining these peaks (Coble 1996). For the humic-like peaks, fluorophore C is at 350nm excitation and 420-480nm emission, and fluorophore A is at 260nm excitation and 380-460nm emission (Coble 1996). For the protein-like peaks,

fluorophore T is at 275nm excitation and 340nm emission, and fluorophore B is at 275 nm excitation and 310 nm emission (Coble 1996). We used Matlab to create the 3-D EEMs for our samples and obtained the peak fluorescence values for T, B, C and A at those regions of the graph (Table 5-1).

Table 5-1. Comparison of fluorescence contouring data reflecting different types of DOM associated with the three species of shredding insects. Peak fluorescence values (RU) for T, B, C, and A. Values are the average of the three replicates.

	Time (day)	**T**	T stdev	**B**	B stdev	**C**	C stdev	**A**	A stdev
	0	0.23	0.02	0.13	0.02	0.54	0.05	0.45	0.03
	5	0.32	0.07	0.17	0.04	0.93	0.14	0.90	0.15
Leaf control	8	0.40	0.08	0.24	0.03	1.18	0.15	1.23	0.19
	13	0.35	0.09	0.20	0.03	1.13	0.08	1.22	0.12
	21	**0.50**	0.10	**0.24**	0.05	**1.71**	0.16	**2.09**	0.24
	0	0.23	0.02	0.13	0.02	0.54	0.05	0.45	0.03
	5	0.34	0.01	0.19	0.02	1.02	0.02	0.99	0.01
Pycnopsyche	8	0.38	0.01	0.22	0.01	1.13	0.06	1.25	0.02
	13	0.40	0.04	0.22	0.03	1.14	0.04	1.40	0.04
	21	**0.73**	0.07	**0.33**	0.02	**1.87**	0.05	**2.62**	0.09
	0	0.23	0.02	0.13	0.02	0.54	0.05	0.45	0.03
	5	0.43	0.06	0.22	0.01	1.15	0.12	1.15	0.15
Tipula	8	0.54	0.05	0.31	0.04	1.34	0.14	1.53	0.21
	13	0.58	0.13	0.31	0.08	1.38	0.23	1.71	0.36
	21	**0.99**	0.47	**0.43**	0.13	**2.26**	0.54	**3.92**	1.77
	0	0.23	0.02	0.13	0.02	0.54	0.05	0.45	0.03
	5	0.33	0.01	0.19	0.01	1.04	0.06	0.98	0.05
Pteronarcys	8	0.40	0.07	0.22	0.02	1.14	0.09	1.24	0.11
	13	0.38	0.05	0.20	0.03	1.06	0.05	1.19	0.12
	21	**0.53**	0.05	**0.29**	0.03	**1.66**	0.14	**2.13**	0.25

Changes in the biochemical characteristics of DOM over time can be examined with fluorescence optics (Fellman et al 2010). The fluorescence values for the specific regions of the EEM (T, B, C and A) increased in all treatments over time (5-1). However, the values increased in the *Tipula* treatment more rapidly (by day 8) and were larger than any of the other insect treatments or control values, especially by the end of the study (Table 5-1, bold and underlined data). By looking at the fluorescence data at designated wavelengths over time, we noted changes in chemical composition of DOM and when they occurred. These composition changes are demonstrated by shifts in the position of the excitation or emission maxima over time (Del Castillo et al 1999).

Table 5-2. Comparison of the changes in excitation maxima (nm) demonstrated by each species.

	day 0	day 5	day 8	day 13	day 21
	335	330	325	315	325
Leaf control	325	335	330	315	315
	335	330	320	325	315
	335	330	325	325	**240**
Pycnopsyche	325	320	325	315	310
	335	330	325	310	320
	335	325	325	325	320
Tipula	325	330	325	**240**	**240**
	335	335	315	330	**240**
	335	325	325	325	330
Pteronarcys	325	330	325	315	320
	335	325	310	325	310

A shift in Excitation maxima for some of the cranefly and caddisfly treatments was observed from 325nm to 240nm by the end of the experiment (Table 5-2, bold and underlined data). This suggests a change in the chemical composition of those samples from one type of humic substance to a different type. This shift from C-type to A-type represents a shift from labile to more aromatic humic molecules (Fellman et al 2010). This change happened faster for the cranefly, with day 13 showing the first account of the shift. Not all replicates showed the shift. The emission maxima however, showed no difference over time, staying around 420-430nm.

Discussion

Tipula treatments produced the highest concentration of <200 μm FPOM and colored DOM of the three insects tested and the leaf control. It seems the craneflies were fast and efficient processors of leafpack under our lab conditions. When the experiment was disassembled a month after the last data were obtained, the *Tipula* had shredded all of their leafpack into a fine dust, where no large pieces remained. The leafpacks in the other tanks were still mostly intact with large pieces still apparent. The spectral slope data show that all treatments were undergoing intense microbial degradation (Figs. 6-5 through 6-8). Additionally, in the presence of craneflies, the DOM contained molecules that were more structurally complex. Each of these different processes was reflected over time. Spectral slope values increased over time for all treatments, reflecting more and more microbial degradation. The S value stayed consistently lower for the cranefly treatment over time, indicating that more complex molecules continued to be created or persist.

The molecular complexity indicated by the CDOM absorption and fluorescence results from the cranefly treatments is probably a result of the unique gut structure of *Tipula*. *Tipula* have a distinct bacteria-loaded intestinal anatomy that is different from the other shredders (Sinsabaugh et al 1985). The symbiotic bacteria in their gut probably play a role in the increased molecule complexity of the DOM. More research is needed

to determine what role the different intestinal bacteria have in the creation of more complex DOM molecules.

The fluorescence data showed that the *Tipula* treatment had higher levels of both humics and protein than the other shredders or the leafpack control over time. In fact, the *Tipula* treatment had almost a two-fold increase in the amount of both humic and protein material by the end of three weeks time over the other treatments. The protein-like fluorescence peaks have been used to label DOM as derived from microbial sources or linked to bacterial production or respiration (Fellman et al 2010). Humic-like fluorescence is usually controlled by hydrology, whereas protein-like components of DOM are controlled by biological processes (Fellman et al 2010). Humics are also the end product of microbially mediated degradation of plant material (Aitkenhead-Peterson et al 2003, from Yoshimura 2010). From this information we can deduce that there was more microbial decomposition and more complex molecules produced in the cranefly treatments. The increase in protein-like material in the *Tipula* treatment is likely excretory, since the T region fluorophores are indicative of recent bacterial degradation activity (Moran et al 2000) and indicate compounds of recent biological origin (Parlanti et al 2000). Protein in adequate quantities and of appropriate form and digestibility is essential for bacterial growth, and the form in which it is available plays a role in determining the species composition of microbial communities. Simon and Azam (1989) state that protein concentrations can be used to estimate the amount of bacteria present. An increase in protein also can indicate an increase in bacteria. Tanoue et al (1995, from Parlanti et al 2000) reported that bacterial membranes may be a major source of dissolved protein in sea water. Regardless of what the protein compounds are, the significant addition of protein to natural systems is ecologically important. *Tipula's* increased contribution to protein production should be quantified to determine the importance of *Tipula,* specifically, in protein cycles in streams. Additional studies are needed to assess how *Tipula* could be used to enhance the availability of good quality protein for juvenile mussels in propagation facilities.

The other shredding insects that were tested did not demonstrate changes in the molecular complexity of DOM different than normal leaf decomposition. Nor did they produce the increased levels of decomposition or protein production like *Tipula*. It is possible that these species were not as actively feeding in our lab due to sub-optimal environmental conditions. *Pycnopsyche* were the smallest of the shredders used in this study. Their lower biomass may have lessened their effect compared with other shredders. Also, some of the *Pycnopsyche* did not fare well during the experiment and up to 30% of animals may not have been shredding by day 21. Smaller biomass and lower survival may be reasons that *Pycnopsyche* did not have as strong of an effect as *Tipula* on production of FPOM and DOM in this study. Future research should equilibrate *Tipula* and *Pycnopsyche* biomass and determine the effects on DOM production and quality. *Pteronarcys,* which are generally found in faster and cooler water temperatures, probably did not function well in the higher temperatures that were used in this experiment. It is also possible that this animal does something different to the DOM that we could not determine with the detection methods used in this study. Cummins et al (1973) found that *Tipula* had maximized their leaf conversion at elevated temperatures, like our lab conditions. *Pteronarcys* may need cooler temperatures and/or higher dissolved oxygen to maximize their processing of detrital material, and may actually be using some nutrients in the water of this experiment for general body maintenance. Perhaps they would be

more useful for the propagation of species of mussels that prefer cooler temperature mountains streams.

The studies were conducted under controlled laboratory conditions. The natural environment in which these animals live is much more variable and inconsistent. Different times of the year and different seasons create changes in food availability and quality for shredding insects that alter the structure of shredding insect populations and their local abundance. Temperature, and physical environmental factors as well changes in the presence and structure of microbial communities and other invertebrates may dramatically affect the shredder's ability to process leafpack, as well as how they process it. The subsequent contribution to the ecosystem of these shredding insects will also vary with the variable environmental conditions. The implications for *Tipula* in stream ecosystems produced from this data need to be thoroughly tested under field conditions. *Tipula's* unique contributions to freshwater DOM and FPOM likely have positive impacts on juvenile freshwater mussel nutrition. The increased growth and survival of Lampsiline mussels in the presence of *Tipula* over controls in our lab is a good indication of this relationship and argues for further examination of the role *Tipula* could play in the grow out of freshwater mussels propagated in captivity.

Additional research is needed to determine exactly how *Tipula* benefit juvenile mussel diets. Additional work is necessary to identify what components of the *Tipula* shredding process benefit mussels, and in what ways. Analysis of the feeding process of juvenile mussels would help clarify the specific food items that those mussels target. Since mussel bodies are 50% protein (Nichols and Garling 2000), the protein provided by the craneflies is likely an important nutrient for mussels. Although we don't know exactly how and why *Tipula* benefit mussels, we have seen a positive connection. Propagation facilities working with rare mussel species will want to consider the use of *Tipula* in their rearing systems for juvenile mussels. Although only the *Lampsiline* species showed positive results in our study, it is possible that other mussel species would benefit as well. Since *Tipula* are relatively easy to find and collect in the field and no detrimental effects of *Tipula* on mussels were found in our study, the addition of *Tipula* to lab rearing mussel systems seems worthwhile. To avoid predation problems from adding leafpack to early stage juvenile mussel tanks, filtered water taken from tanks with shredding *Tipula* could be added to the juvenile mussel tanks as a food supplement.

Population augmentation, streamside infestations and translocation efforts may want to examine the proposed sites for shredding insects. Increased survival and growth of some species of juvenile mussels in the presence of *Tipula* in our lab indicate the possibility of the same effects in the field. Until this is tested, it seems justified to choose sites with *Tipula* in close proximity. Relocation efforts of freshwater mussel populations have been largely unsuccessful (Nichols and Garling 2000). This demonstrates that we are uncertain of all the necessary requirements for their habitat. If *Tipula* are indeed contributors to freshwater mussel diet, surveys should show the presence of *Tipula* at potential sites for relocation of mussels if the relocation is to be successful. Dunn and Sietman (1997) state that the poorly understood ecology of mussels limits success of translocation efforts. The identification of natural food resources is critical for successful picking of new sites for translocated or augmented populations. Suitable habitat selection for translocation, relocation, or augmentation begins with suitable food sources (Vaughn et al. 2008).

Chapter 6

Master's Student Graduate Work

To be submitted to Limnology and Oceanography

Potential contribution of Tipula to freshwater mussel growth and survival

Introduction

Aquatic ecosystems are a complex assemblage of fauna influenced by the dynamic biotic, physical and chemical processes defining habitats (Bucci *et al* submitted). The majority of aquatic species include a variety of food items in their diet. Aquatic invertebrates have flexible diets and can adapt to changes in resources (Rosi-Marshall and Wallace 2002). When one item becomes rare, they find other things to eat. Algae are an important component of freshwater mussel diets. Algae are routinely ingested and found in the stomachs of most freshwater mussel species when stomach contents are examined. Captive propagation of freshwater mussels has been adapted as a conservation tool for helping mitigate the systemic decline that has been observed in freshwater mussel species. Captive reared juvenile mussels are routinely fed commercially available or cultured algae. The survival and growth of many species in captivity is poor, and our limited understanding of Unionid diets (Nichols and Garling 2000) may be contributing to the poor success of some species in propagation facilities.

Mussels ingest whatever is in the water column with a passive flow of stream water. Cilia on the mussel gills sort potential food particles by size as the inhalant material is flushed over the gills, and send the right size particles to the stomach. The examination of stomach contents is routinely used to identify what aquatic species are consuming. However, the presence of a food item is in the stomach of a freshwater bivalve does not confirm that the food item is digested, assimilated and playing a role in the animals nutritional health. Even if algae are selected for ingestion, Nichols and Garling (2000) found that algal carbons were not assimilated in body stores. Many particles pass through the gut untouched (Miura and Yamashiro 1990), and it is difficult to determine beneficial food items of mussels from dissection alone.

Recent research using stable isotope analysis has found that some mussels choose bacteria over algae in the FPOM for their main dietary item in nature (Nichols and Garling 2000, Christian *et al* 2004). Algae may be important for immediate energy needs or provide a good substrate for the beneficial bacteria that are contributing to the nutritional health of mussels. But bacteria may be providing the nutritious and energy-storing component of freshwater mussel diets. Only bacteria derive vitamin B12 and some specific fatty acids which are key dietary components for mussels (Nichols and Garling 2000, Vaughn *et al* 2008). Food items in stream FPOM are highly varied and come from many sources.

Shredding aquatic insects are significant contributors to the particulates in the water column, and can be found in the same habitats or upstream of freshwater mussels. Shredding insects only use 40% of the CPOM they process (Cummins 1974); the rest is egested as feces. Shredding insect feeding is also known to create small pieces of leaf detritus that they do not ingest. These processes contribute greatly to the amount of FPOM in the stream (2-7mg in feces alone per large shredder per day, McDiffett 1970). Fragmented, well-conditioned CPOM and invertebrate feces probably represent the highest quality components of native stream detritus (Cummins and Klug 1979). Since shredding insects can make up 20% of the macroinvertebrate biomass in streams (Cummins *et al* 1989), their contribution to FPOM is quite significant.

Aquatic insect shredders accelerate the detritus breakdown process by increasing surface area for microbial colonization, which further decomposes the material and releases nutrients

(McDiffett 1970). Shredder insects have been shown to accelerate microbial growth (Covich *et al* 1999), which includes bacteria, a potential food source for mussels. It has been suggested that bacteria are relatively sparse on detritus until it is passed through invertebrate guts (Anderson and Sedell 1979). After 2-3 days of passing through the gastrointestinal tract, maximum microbial activity on FPOM is reached (Anderson and Sedell 1979). These microbes enhance detrital food quality (Tenore *et al* 1982). Bacterial populations in digested food of *Tipula* and *Pycnopsyche* are 2-5 orders of magnitude more dense than bacterial populations in ingested food (Cummins and Klug 1979). These bacteria, feces, and other microbes brought about by the shredder feeding process may provide a source of nutrition for freshwater mussels.

There is an intimate relationship between shredding insects and collecting insects in that shredding insects increase growth and survival of collecting insects through their particle production (Heard and Richardson 1995). A substantial body of work has previously focused on the role of shredding insects in the life history and diet of filter feeding aquatic insects (Heard and Richardson 1995). Short and Maslin (1977) found that the presence of shredding insects increased food availability to collecting insects. They claim that shredding insects are of great importance in leaf processing with respect to nutrient availability to collecting insects. Cummins (1973) found that filter-feeding mayflies (*Stenonema*) grew in the presence of shredding insect feces due to coprophagy. Wallace and coworkers (1977) state that they found detritivore feces in the gut of a filter feeder. Cummins (1973) claim that the FPOM created by shredding insects "undoubtedly" constitute a high quality food source for collecting insects. The role of shredding insects in the life history and diets of freshwater mussels, however, is essentially undescribed.

Shredding insects, in their largest numbers, are generally found in headwater streams, and the FPOM they create is carried downstream. Mussels are found in higher order streams. Covich and coworkers (1999) say that filter feeders are typically located downstream of the shredding insects and that their loss from the system would alter food availability for suspension feeders. As water flows down river, local terrestrial input is less important to the health of the ecosystem than upstream input (Vannote *et al* 1980). Streams generally shift from heterotrophy to autotrophy at about 3rd or 4th order, then revert back to mostly upstream inputs at higher stream orders. Mussels are mostly found in higher order streams and rivers and likely depend on upstream inputs for their food supply.

The FPOM and possibly DOM generated by shredding insects could play an important role in the diet, growth and survival of freshwater mussels. In preliminary studies, we compared and contrasted the contribution of three species of shredding insects to the growth and survival of three species of freshwater mussels. Concurrent studies examining the FPOM and DOM production of *Pteronarcys*, *Tipula*, and *Pychnopsyche* documented that FPOM and DOM production of *Tipula* exceeded that of the other tested species (Greiner *et al*. in manuscript). In addition, *Tipula* generated more complex DOM molecules and greater levels of microbially derived protein than the other insect species. Consequently, subsequent studies focused on the contribution of the products of *Tipula* leaf shedding to the growth and survival of juvenile freshwater mussels. Tipula were held in containers that concurrently housed freshwater mussels and survival and growth of the mussels was measured.

Methods

Preliminary study:

A preliminary study was conducted to measure the potential benefit of three species of shredding insects, *Pteronarcys*, *Tipula*, and *Pycnopsyche*, to the growth and survival of freshwater sub-adult mussels. This study was completed at the Table Rock Fish Hatchery in Morganton, NC. Insects and leafpack for the study were collected from various local sites. Leafpack was hand-selected based on the degree of degradation, using leafpack where shredding insects were found as an example.

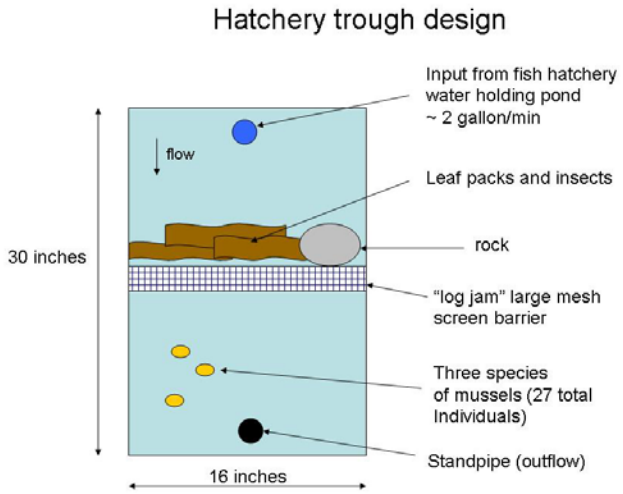

Figure 6-1: Tank design for preliminary shredding insect studies.

Figure 6-2: Preliminary shredding insect study control tank (left) and test tank (right).

Four plastic troughs were set up from June-Sept 2009 (Figs. 6-1 and 6-2). Three different species of mussels approximately 2 years of age were used in this study: *Lampsilis fasciola* (from the Pigeon River, NC), *Ligumia nasuta* and *Lampsilis radiata* (both from the Broad River, SC). Troughs labeled 1 and 3 were set up with the three species of mussels and leaf packs and insects, troughs 2 and 4 were the controls with no leaf packs or insects. The insects used were common NC shredding insect in the genus *Pteronarcys*, *Tipula*, and *Pychnopsyche*. The troughs had one

inch of sand in the bottom and were full of water to about one inch below the rim of the tank (approximately 4 inches of water). All tanks had large (¼ inch) size mesh barriers in the middle to hold the leafpack and insects upstream of the mussels in test tanks (screens were present but not jammed by leafpack in control tanks). Troughs were set up so that water came in at one end and out at the other to create a somewhat linear flow. The tanks hold approximately 8 gallons of water each. The water input was set to approximately 2 gallons per minute, from the fish hatchery (unfertilized) water holding pond. The temperature of this water varies with the natural environment, and stayed between 62 and 74 degrees Fahrenheit for the duration of the experiment. Troughs had a screen top to let in natural light, but to prevent animals from getting in or out. After one month, the flow in each tank was reduced to about 1 gallon per minute, and a shade screen was added to cover all tanks after filamentous algae became a problem. The shade screen prevented filamentous algae problems for the remainder of the experiment.

The tanks were monitored biweekly. Each time, all leafpack and insects from the experimental tanks were removed and counted. Insect densities were maintained at approximately 50 *Pycnopsyche*, 30 *Tipula*, and 15 *Pteronarcys* per test tank, adding fresh insects and leafpack as necessary. Tanks were cleaned thoroughly once a month by stirring the sand and cleaning the wire mesh barriers. On the other trips, tanks were lightly cleaned by waiving away the organic material buildup over the mussel section of the tank, but the mussels were otherwise not disturbed.

The mussels were all measured at the beginning of the experiment. Measurements were taken once a month for all months during the study to monitor growth (this was done during the same trips where the tanks were thoroughly cleaned). At the end of the experiment, tanks were disassembled and final length data was taken on all mussels.

Laboratory Studies

To examine growth and survival advantages of shredding *Tipula* for the captive rearing of juvenile mussels, a lab study was designed to determine if freshwater mussels grow faster in the presence of craneflies.

Freshwater mussels are particularly sensitive to ammonia, and ammonia toxicity is lethal to juvenile mussels. To ensure that ammonia produced by the craneflies would not affect mussel health a preliminary study of cranefly ammonia production was conducted. Three craneflies in a 2.5 gallon tub of water produce an untraceable amount of ammonia (mg/L) over a period of 7 days. The pH of these tubs also did not change, even though the water looked increasingly brown as time went on.

Lampsilis fasciola juveniles were obtained from the Aquatic Wildlife Conservation Center mussel propagation facility in Marion, VA. Juveniles were propagated from adults from the Clinch River in Russel County, VA. The mussels were approximately 8 months to a year old when removed from the facility and used in this experiment. They varied in size from 3.3 mm to 7.6 mm (measured 50 of the 200 animals used). *Villosa delumbis* were propagated in our lab at NC State University from gravid individuals previously propagated by our lab. The grandparents of the *V. delumbis* used in this study were from the Deep River, NC. Grow-out of the transformed juveniles followed standard procedure for our lab. At four months old the mussels were

implemented in this study. They ranged in size from 1.0mm to 1.4mm (measured 50 of the 200 animals used).

Forty 2.5 gallon plastic tanks were set up in the lab, where light was controlled by a 12 hour on, 12 hour off cycle. The air temperature of the lab varied daily in a cyclic manner, with cooler temps at night. The facility has central heating and cooling, so a relatively constant temperature was sustained throughout the studies. Tanks were filled with city water treated with sodium thiosulfate and AmmoLock to remove chlorine and chloramines. A small fish net with 1-mm mesh was suspended over the side of the bucket so that the net was submerged, but the rim was out of the water, keeping anything larger than 1mm in size from escaping into the rest of the tank (Fig. 7-4). 150g of autoclaved play sand >300um in size was covering the bottom of each tank. Two clean airstones were dropped in each tank; one in the suspended net and one on the sediment at the bottom of the tank. After the tanks were set up, 10 mussels per tank were added, with attempts to vary the different sizes evenly across all treatments and tanks. Three craneflies were added to the submerged nets per tank. A 35g leafpack was added to each net (see method below for preparing leafpack).

Figure 6-3: Study tanks holding shredding insects and mussels.

Figure 6-4: Closeup of test tank used to house shredding insects during studies conducted to assess the growth of juveniles when insect produced FPOM is available.

Four treatments were set up in 5 replicates for each of the two species of mussels.

1) Craneflies and leaves and commercial food
2) Craneflies and leaves with no additional food
3) Leafpack without craneflies and no additional food
4) Commercial food only, no craneflies or leafpack

The commercial food that was fed once a day (during weekdays only) was 1 drop of 1:3 NannoChloropsis to water and 1 drop Shellfish diet for each tank. A 75-80% water change on all tanks was performed once a week, slightly agitating the water to stir up the settled organic matter but not to disturb the sediment or mussels.

Fresh, partially decayed leafpack collected from the field was periodically added to replenish the food supply for the craneflies. This leafpack was collected from field sites where mussels were present. All tanks with leafpack were treated the same. Before adding leafpack to the tanks, it was thoroughly searched and all craneflies were removed. The leaves were added to a kitchen blender and pulsed a couple times to break up the leaves into smaller pieces and distribute the different species/components of the leafpack. The resulting leafpack was then rinsed through one of the unused small fishnets (1-mm mesh) to remove all particles smaller than 1mm. Only leafpack pieces larger than 1mm were added to the experiment tanks.

Cranefly assessments were completed once a week to maintain the same number of craneflies in each tank. At all times, 2-4 craneflies were in the test tanks, making sure that all treatments and replicates had the same number of *Tipula* at any one time. Cranefly survival was fairly high over the course of the experiments, on average only adding one new fly to a particular tank once every few weeks. Different cranefly sizes were distributed evenly across all tanks and treatments. Craneflies were collected at field sites where mussels were present.

During the last week of May, craneflies were found in the treatment that was set up to have leafpack and no craneflies. The flies were immediately removed from this treatment. After this event, all fresh leafpack brought into the lab was quarantined for two weeks before going through and picking out craneflies for addition to tanks to prevent future contamination of controls.

Mussel length and survival was assessed in 2 of the 5 replicates for *V. delumbis* (8 tanks total) and 1 of the 5 replicates for *L. fasciola* (4 tanks) the first week of June (the 30 day timepoint). These tanks were harvested and the animals were removed from the experiment. 28 tanks remained after this assessment. The second week in July, the replicates were all pooled so that only 8 tanks remain, one for each treatment for each species. All remaining mussels were kept in the experiment at this time, just all getting combined into one tank for each treatment. Mussels were assessed for length and survival at this time (70 day timepoint). At this timepoint, the animals measured were not harvested, just measured and counted and placed back into the tanks.

Beginning July 15th, leafpacks were stirred during the daily feeding to release particles created by the *Tipula* that were less than 1mm from the nets and into the tank. Because control tanks in experiment one were contaminated with larval cranefilies that were to small to see when the tanks were set up (noted above). A second experiment was conducted. As noted above, leafpacks used in the second experiment were held for two-weeks prior to use in the experiment to ensure the larval were of sufficient size and remove from the leafpacks.

Experiment 2: July 2010.

Treatments for this experiment were:

1) Commercial food only
2) Leafpack and commercial food, no craneflies
3) Craneflies and leafpack and commercial food

Thirty tanks were set up in the same manner as for experiment 1. Animals for experiment 2 came from our NC State University propagation facility only. *Elliptio lanceolata* came from propagated adults collected from Swift Creek (Tar basin), NC. Juvenile mussels were 1 month old before placing in the experiment. Lengths ranged from 0.2mm to 0.7mm (50 of 150 animals used were measured) (Table 6.1 below). 10 mussels were placed in each tank. *Lampsilis fasciola* were from propagated adults from the Pigeon River, NC. Juveniles were 2 months old at the start of the study. Individuals ranged from 0.6mm to 1.1mm in length (50 of 450 animals were measured). 30 *L. fasciola* were placed in each tank.

Both experiment 1 and experiment 2 were harvested blindly on Dec 7. All mussels were counted and measured. An analysis of variance was conducted to test the potential difference between the length of treatment and control animals. A Pearson Chi-Square test was used to compare the survival of treatment and control animals.

DOC samples:

At approximately one month (29 days), we collected 30mL water samples from one random replicate for each treatment from experiment 1. A control of treated lab water was also collected. Samples were placed in the fridge for two days before filtering and acidifying (with H3PO4) the samples for DOC analysis (DOC concentration and 13C ratio).

At approximately 6 months (130 days) we collected 8 samples from the experiment 1 tanks, 2 lab water samples, 2 samples of lab water treated with AmmoLock, and one from each of two replicates for each of the experiment 2 treatments (12 samples from experiment 2). Samples were refrigerated until tested. 10 days later, samples were filtered and acidified for DOC analysis.

Results

Preliminary study:

No significant differences were observed in mussel growth between tanks or treatments. Mussel survival was 100%.

The preliminary studies helped guide the design and implementation of the laboratory studies. Frequently during the two-week maintenance checks of the hatchery study, *Tipula* were found buried in the sand or otherwise missing (and therefore not shredding). Since this specific genus was found to make a growth difference in mussels in the laboratory, their effect may have been weakened during the hatchery study. Consequently *Tipula* were held in nets suspended in the tanks during subsequent experiments.

Table 6-1. Initial lengths (mm) of hatchery mussels used in preliminary studies with shredding insects.

Lampsilis fasciola		***Ligumia nasuta***		***Lampsilis radiata***	
average	stdev	average	stdev	average	stdev
26.93	1.40	37.97	1.58	21.78	1.83

Table 6-2. Lengths (mm) of hatchery mussels at the termination of preliminary studies assessing the potential value of FPOM produced by shredding insects to mussel growth.

	Lampsilis fasciola		***Lampsilis nasuta***		***Lampsilis rardiata***	
	average	stdev	average	stdev	average	stdev
tank1test	30.3	1.1	42.5	1.5	26.7	2.4
tank2control	30.5	1.7	44	1.2	26.7	1.8
tank3test	29.6	1.3	43.1	1.6	26.4	1.7
tank4control	30.1	1.4	43	1.3	27.8	1.9
ANOVA p-value	0.5526		0.1449		0.5489	

Water temperature in the Table Rock hatchery varied considerably during the studies. Water temperature was held at relatively constant levels during the laboratory studies. Frequent handling has been shown to temporarily arrest juvenile mussel growth and the delay in growth can last for several weeks (Arthur Bogan, personal communication). We delayed measurements during the third laboratory experiment (noted below) to minimize the effect of handling on mussel growth.

Laboratory studies:

Experiment 1

Mussels used in the study were measured at the beginning of the study and 30, 70, and 210 days. *Villosa delumbis* held in the presence of craneflies and fed algae grew faster than *V. delumbis* receiving just algae or craneflies, and leaves without algae. *Villosa delumbis* demonstrated increased growth in the presence of craneflies over control treatments (Fig. 6-1 -6-3). The treatments that were not fed commercial algae did not differ from the algae only control (Table 6-3). The addition of craneflies and leafpack to tanks that were fed commercial algae increased growth of the *Villosa delumbis* mussels more than just feeding them commercial algae alone. *Lampsilis fasciola* showed no difference between treatments (Table 6-3).

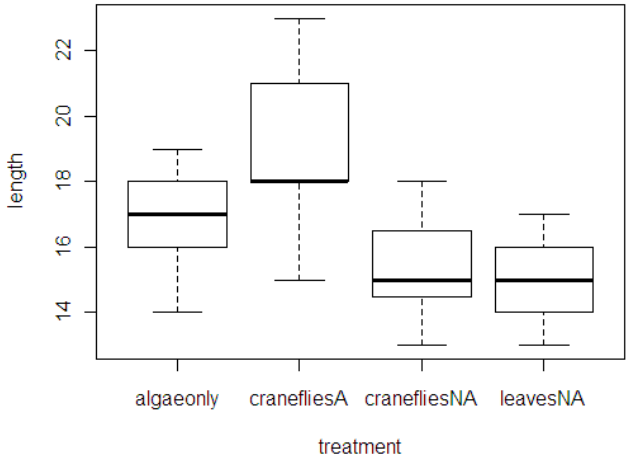

Figure 6-5: *Villosa delumbis* length (in mm x 10^1) at 30 days.

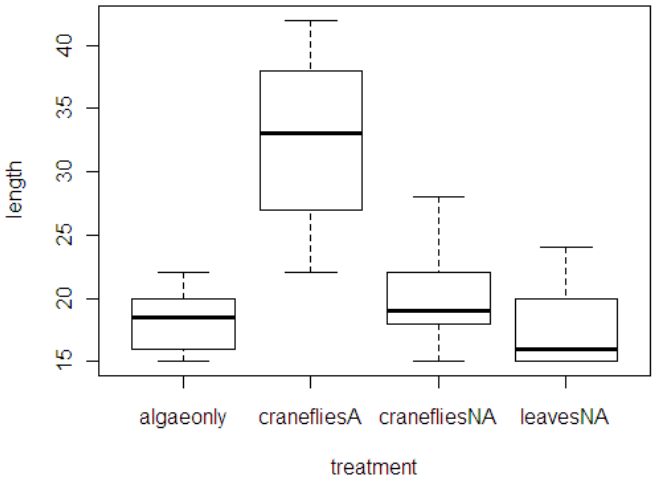

Figure 6-6. *Villosa delumbis* length (in mm x 10^1) at 70 days.

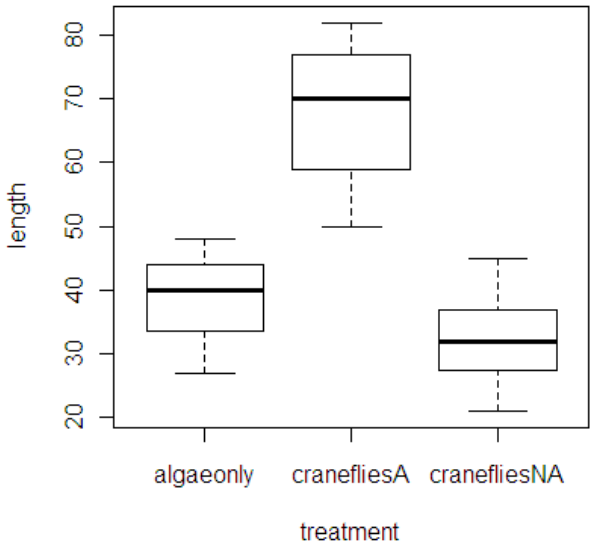

Figure 6-7. *Villosa delumbis* length (in mm x 10^1) at 210 days.

Table 6-3**.** Length of *Villosa delumbis* held in tanks with or without craneflies (in mm x 10^1).

	Villosa delumbis			*Lampsilis fasciola*		
30 day harvest						
	average	stdev	TukeyHSD	average	stdev	TukeyHSD
algaeonly	17	1.6	A	54	13.5	A
cranefliesA	19	2.2	B	44.8	12.3	A
cranefliesNA	15.5	1.6	AC	56.5	12.4	A
leavesNA	15	1.3	C	55.1	13.3	A
ANOVA p-value	1.12E-06			0.46		
70 day						
	average	stdev	TukeyHSD	average	stdev	TukeyHSD
algaeonly	18.3	2.1	A	60.7	12.1	A
cranefliesA	32.4	5.6	B	61	12.6	A
cranefliesNA	20.1	3.3	A	54.8	11	A
leavesNA	17.6	3.2	A	52.9	10.9	A
ANOVA p-value	2.20E-16			0.07		
210 day						
	average	stdev	TukeyHSD	average	stdev	TukeyHSD
algaeonly	39	6.9	A	66.4	12	A
cranefliesA	68	11	B	70.1	14.3	A
cranefliesNA	32.3	6.9	A	66.7	9.5	A
leavesNA	-	-	-	54.5	14.8	A
ANOVA p-value	1.28E-11			0.45		

Survival was lowest for the leavesNA treatment for both species of mussels (see table 6-4). For *Villosa delumbis*, survival in the cranefliesNA treatment was not significantly different than treatments fed commercial algae (see table 6-5). *Lampsilis fasciola* had decreased survival in both treatments that were not fed commercial algae.

Table 6-4. Percent survival of *Villosa delumbis* and *Lampsilis fasciola* mussels at the end of the study.

Villosa delumbis		***Lampsilis fasciola***	
(n=30/trt)		(n=40/trt)	
algaeonly	40%	algaeonly	38%
cranefliesA	43%	cranefliesA	38%
cranefliesNA	37%	cranefliesNA	10%
leavesNA	0%	leavesNA	5%

	Villosa delumbis		***Lampsilis fasciola***	
	X-Squared	*p-value*	*X-Squared*	*p-value*
3 degrees of freedom	17.46	5.68E-04	20.93	1.09E-04
2 degrees of freedom (removed leavesNA treatment)	0.2778	0.8703	9.9316	6.97E-03

Table 6-5. Pearson's Chi-Square test results comparing the survival of *Villosa delumbis* and *Lampsilis fasciola* held with or without craneflies.

Experiment 2

Handling of juveniles can temporarily arrest their growth. Consequently in the second experiment we only measured animals at 130 days after the initiation of the study. Because Villosa delumbis of a suitable size were not available for the second study, *Elliptio lanceolata* and *Lampsilis fasciola* were used.

Elliptio lanceolata showed no difference between the craneflies treatment and the algae only control, however the algae only control showed better growth than the leaves control (Figure 6-8).

Lampsilis fasciola demonstrated higher growth rates in the presence of craneflies than mussels fed only the commercial diet (Figure 6-9). The control treatments were not different from one another; nor was the leaves control different from the craneflies treatment (Table 6-6). Therefore, we cannot say that the mussels in this experiment were benefiting from the craneflies directly, since the craneflies and leaves only treatments were not statistically different from each other. However, as shown with experiment 1, the addition of craneflies and leafpack to tanks that are already fed commercial algae increases growth of the mussels more than just feeding them commercial algae alone.

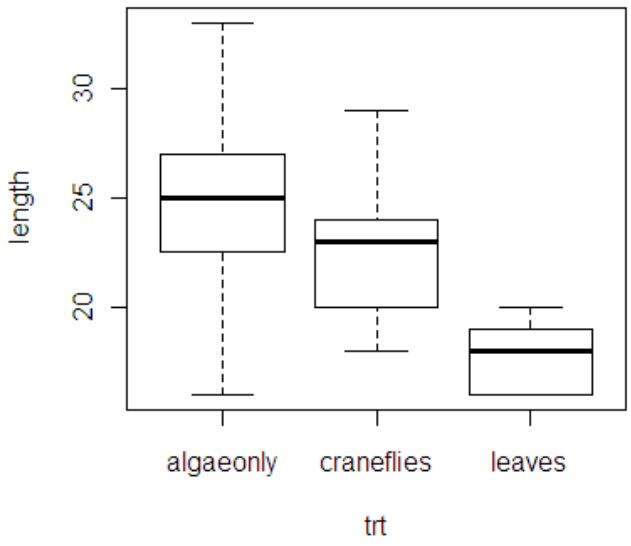

Figure 5-8. Length of *Elliptio lanceolata* held either with or without craneflies at 130 days (in mm x 10^1).

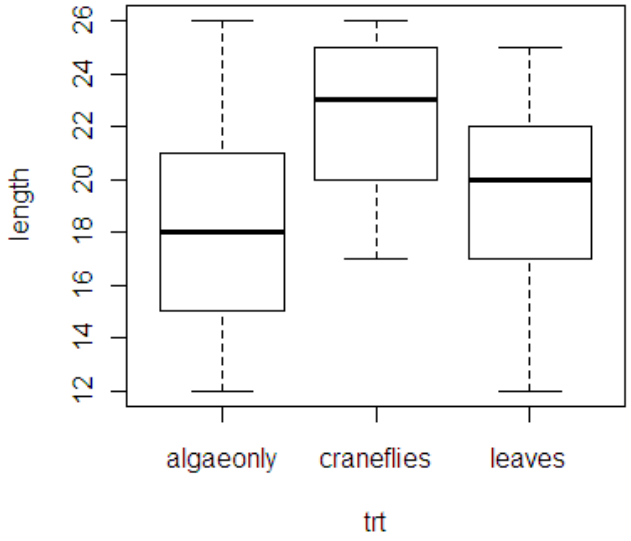

Figure 5-9. Length of *Lampsilis fasciola* held either with or without craneflies at 130 days (in mm x 10^1).

Table 6-6. Length of *Elliptio lanceolata* and *Lampsilis fasciola* held with and without craneflies at 130 days (in mm x 10^1).

	Elliptio lanceolata			***Lampsilis fasciola***		
	average	stdev	TukeyHSD	average	stdev	TukeyHSD
algae only	24.4	4.9	A	18.3	4.1	A
craneflies	22.6	3.7	AB	22.4	3.2	B
leaves	17.8	1.7	B	19.5	3.7	AB
ANOVA p-value	0.01			5.49E-03		

Survival was best in the algae only treatment for *Lampsilis fasciola* (Table 6-7). There were no significant differences in survival of *Elliptio lanceolata* in the different treatments (Table 6-8).

Table 6-7. Percent survival of *Elliptio lanceolata* and *Lampsilis fasciola* held with or without crane flies.

Elliptio lanceolata (n=50/trt)		***Lampsillis fasciola*** (n=150/trt)	
algaeonly	22%	algaeonly	28%
craneflies	14%	craneflies	9%
leaves	12%	leaves	14%

Table 6-8. Pearson's Chi-Square test results comparing the survival of *Elliptio lancelolata* and *Lampsilis fasciola* held with or without craneflies.

	Elliptio lanceolata		***Lampsilis fasciola***	
	X-Squared	*p-value*	*X-Squared*	*p-value*
2 degrees of freedom	2.0833	0.3529	21.3095	2.36E-05
1 degree of freedom (removed craneflies trt)	-	-	8.037	4.58E-03

Dissolve organic carbon

In both experiments tanks with craneflies exhibited higher DOC values than tanks without (6-9 and 6-10). DOC values are higher in tanks with leafpack than control tanks.

Table 6-9. Dissolved organic carbon concentrations observed in the first trial comparing the growth and survival of mussels held with or without craneflies (mg/L) at 50 and 210 days.

Experiment 1

	Lampsilis fasciola		***Villosa delumbis***	
	50 days	210 days	50 days	210 days
craneflies and algae	6.24	4.53	6.73	3.87
craneflies no algae	7.38	3.85	7.74	3.88
leaves no algae	6.49	3.24	5.98	3.10
algae only	3.71	2.69	3.65	2.24

Table 6-10. Dissolved organic carbon concentrations observed in the second trial comparing the growth and survival of mussels held with or without craneflies (mg/L) at 130 days.

Experiment 2

	Lampsilis fasciola		***Elliptio lancelolata***	
	replicate 1	replicate 2	replicate 1	replicate 2
algae only	2.22	1.65	2.71	2.55
leaves	3.81	3.57	3.43	3.23
craneflies	4.37	3.68	4.18	5.02

Table 6-11. Dissolved organic concentrations of city water, laboratory water laboratory water with Ammolock a compound used to eliminate ammonia from treatment tanks).

Controls

	replicate 1	replicate 2
city water (experiment 1, at 50 days)	7.17	ND
lab water	2.40	2.38
lab water with ammolock	5.50	6.06

ND=Not done

Experiment two was shorter than experiment one by 1.5 months, so the effect of the different treatments on growth in experiment two is likely decreased (Table 6-12). In addition, the presence of craneflies in the control leaf tank for a portion of the study made the comparison between animals with and without craneflies a more conservative and less likely to show a statistically significant difference between treatments and the control leaf tank.

Table 6-12. Summary of the two experiments comparing the growth and survival of juvenile freshwater mussels held with or without craneflies.

Species	**Treatment with best growth rate**	**Treatment with best survival**	**Treatment with highest DOC**
Villosa delumbis	craneflies and commercial algae	no difference	craneflies without algae
Lampsilis fasciola[exp1]	no difference	no difference	craneflies w&w/o algae
Elliptio lanceolata	no difference	no difference	craneflies and algae
Lampsilis fasciola[exp2]	craneflies and commercial algae	algae only control	craneflies and algae

Discussion

Lampsiline juvenile mussels reared in the presence of shredding craneflies demonstrated increased growth over mussels fed commercial diets alone. The increased growth demonstrated by *Villosa delumbis* and *Lampsilis fasciola* in the presence of craneflies indicates that craneflies somehow contribute to an increase in food quality or abundance for some species of mussels. Since the best growth was seen in treatments that were also fed commercial algae, as previously recognized, algae do appear to play an important role in the diet of freshwater mussels. The commercial algae diet may provide an immediate energy source or serve as a vehicle for bacteria or other small particles generated by the insects to be gathered by the gills while juvenile mussels feed. Juvenile mussel diets in freshwater mussel propagation facilities may benefit from including both cranefly and algae in the diet. In this manner both the nutritional value and physical presence of algal particles will be supplemented with particles (FPOM) and dissolved organic protein and other nutrients generated by the craneflies during feeding. Filtered water from separate *Tipula* tanks could be added to juvenile mussel tanks as food on a daily basis. Studies building on this work have been initiated to examine how various filtrates of the products of shredding insects can be used to supplement juvenile mussel diets. Leafpack and shredding *Tipula* could be added to separate chambers of mussel rearing containers to provide a continual dietary supplement. Until we determine the specific elements of the material *Tipula* produce that contribute to mussel growth, some species of juvenile mussels may benefit from adding *Tipula* to propagation grow-out systems.

Elliptio lanceolata showed no difference in growth between the craneflies treatment and the algae only control, however the algae only control showed better growth than the leaves control. This *Pleurobemine* species may use algae as a bigger component of their diet for growth than the other *Lampsiline* species tested. Nichols and Garling (2000) found that some species of mussels are more herbivorous than others, and retain a higher fraction of algal carbons in their tissue stores. They found that *Lamspilis* species specifically had a lower percentage of algal carbons in their body stores and attribute this difference possibly to this animals' different digestive capabilities (Nichols and Garling 2000).

Tipula create more FPOM and DOM than the others species tested. Additionally, we found that *Tipula* treatments produced more complex DOM molecules and increased levels of microbially derived protein than were observed with the other species tested. *Tipula* have a highly efficient protein-digesting system (Martin *et al* 1980). Although it is accepted that most the gastrointestinal tracts of shredding insects house bacteria that aid in digestion and energy extraction of detritus (Klug and Kotarski 1980), Sinsabaugh and coworkers found that only *Tipula* (of *Tipula*, *Pycnopsyche* and *Pteronarcys*) have endosymbiotic bacteria in their gut (1985). These bacteria have been found to be cellulose-degrading bacteria. Other shredding insects obtain the necessary bacteria needed for digestion from their diet. Also, *Tipula* have unique hindgut morphology with an extra fermentation chamber (Sinsabaugh *et al* 1985) that houses a diverse community of epimural and loose bacteria (Klug and Kotarski 1980). The region just anterior of the rectum is the densest with bacteria (Klug and Kotarski 1980). It follows that *Tipula* probably excrete these bacteria in feces to some degree. With the abundance and ubiquity of *Tipula* in streams, it is possible that *Tipula* mediated bacteria production contributes to the diet of freshwater mussels (and probably other aquatic animals) in nature.

Craneflies appear to increase DOC concentration in the tanks. The unaided breakdown of leaves also increases DOC concentration in the water (as demonstrated by the leaves only control), but not as much as with shredding craneflies present. Adding AmmoLock raises DOC values to levels present in cranefly treatments. AmmoLock was added with the weekly water changes, but after the DOC samples were taken from the experiment tanks. It is likely that either the DOC from the AmmoLock breaks down within a week or it is getting used by the mussels and then not replenished until the weekly water change. In saltwater oysters, soluble organic nutrients are directly absorbed and used for growth and metabolism (Kennedy *et al* 1996). Roditi and coworkers (2000) found that zebra mussels get up to 50% of their carbon needs from DOC. A study designed to determine DOC uptake from Unionids would be useful.

Future studies

This study documented that the presence of other macroinvertebrates may contribute to the growth and survival of freshwater mussels. Additional studies are needed to understand the manner in which *Tipula* contribute to the diet of juvenile and adult freshwater mussels. Stable isotope analysis of mussel tissue and cranefly produced particulates would determine if the mussels assimilate cranefly mediated material in their body stores. A nutrient analysis on the cranefly produced particulates (such as percent carbohydrates, protein, lipids, cholesterol, etc), in addition to research on the dietary needs of freshwater mussels, would help further understanding of the potential need for *Tipula* in freshwater mussel habitats and the role their co-culture can play in mussel propagation facilities.

Population augmentation, streamside infestations and translocation efforts may want to examine the proposed sites for shredding insects. Increased survival and growth of some species of juvenile mussels in the presence of *Tipula* in our lab indicate the possibility of the same effects in the field. Until this is tested, it seems justified to choose sites with *Tipula* in close proximity when considering the augmentation of remaining populations with mussels propagated in captivity.

OVERALL CONCLUSIONS

These studies were initiated to enhance the opportunity to rear endangered species of freshwater mussels in captivity for reintroduction or augmentation of remaining populations. During the course of these studies we have refined propagation facility protocols, enhanced and improved our understanding of differences in the rearing requirements of the tested species that need to be addressed when attempting their propagation. The studies have prompted the following series of recommendations.

RECOMMENDATIONS FOR MUSSEL PROPAGATION FACILITY MANAGEMENT

1. Have specific standard operating practice protocols for all phases of facility operation.
2. Minimize handling, transport and acclimation stress to fish prior to their use in the laboratory as fish hosts.

   Specific steps:
   - use seines rather than electrofishing to collect fish in streams
   - collect and transport fish during cooler weather
   - transport fish in volumes of appropriate for the species transported e.g. some species of shiners require a larger body mass to water ratio
   - to reduce transport stress keep fish cool during transport
   - use 2 mg/l salt, and a slim coat protectant (e.g Stress Coat®) during transport

3. Quarantine fish prior to transport for at least 21 days prior to use as fish hosts in the laboratory
4. Plan propagation activities based on this quarantine period
5. Prevent cross-contamination of nets and other equipment used in quarantine tanks, and other tanks.
6. Treat fish after arrival with 20 mg/l formalin for 5 days, followed by a 3-day waiting period and another 5 day treatment at 20 mg/l. This is to remove gill parasites and prevent introduction of predacious flatworms into the culture environment.
7. Monitor fish planned for fish host work daily for clinical signs that reflect health problems.
8. Maintain a consistent ambient temperature within the facility that mirrors the requirements of the species being propagated. Monitor temperature daily.
9. Cover culture systems with fine mesh to prevent Chironomidae from laying eggs in your chambers.
10. Closed laboratory systems used to rear newly transformed juveniles should be protected from contamination of pond or river water that may contain zooplankton, such as Daphnia or Copepods

RECOMMENDATIONS TO ENHANCE THE PROPAGATION OF ENDANGERED UNIONIDS

1. The following host fish are recommended for captive propagation of the studies species.

Endangered mussels		*Recommended fish hosts*	
Appalachian elktoe	*Alasmidonta raveneliana*	mottled sculpin	*Cottus bairdi*
Carolina heelsplitter	*Lasmigona decorata*	bluehead chub golden shiner	*Nocomis leptocephalus* *Notemigonus chrysoleucas*
Dwaf wedge mussel	*Alasmidonta heterodon*	fantail darter	*Etheostoma flabellare*
Tar spinymussel	*Elliptio steinstansana*	White shiner	*Luxilus albeolus*

2.Conduct additional studies to determine the species-specific nutritional requirements of each species and how appropriate diets can be provided in captivity.

3. Continue studies focused on assessing the role shredding insects such as *Tipula* can play in mussel nutritional health.

4. A successful mussel hatchery facility has already been established at the Marion Fish Hatchery in Marion, NC, a similar facility with sufficient full-time staff is needed in the Piedmont.

5. Captive propagation using fish hosts is labor intensive. In vitro techniques in development should be explored to culture species for which it proves to be a viable alternative means of producing juveniles for potential augmentation.

6. Captive propagation is generally focused on the broods of a relatively small number of gravid adult female mussels. The consequences of releasing juveniles spawned from this limited pool of genetic material and the ability of mussels propagated in captivity to produce new generations of young mussels in the field needs additional genetic study.

7. Conduct a feasibility assessment and the needed modeling to identify specific species for which propagation and augmentation is a viable alternative to mitigate population declines.

8. Conduct a requirements assessment for individual species identified to be viable candidates for population augmentation.

9. Consider captive propagation of freshwater mussels during crossing structure planning include appropriate levels of funds for propagation in the basic cost of construction. Remove adult females from the site prior to construction. Propagate juveniles of those species during construction, and release propagated juveniles back into the stream at the same site a minimum of one year after construction.

IMPLEMENTATION AND TECHNOLOGY TRANSFER

During this study, we have identified host fish that can be used to successfully support the captive propagation of 4 endangered species of fresh water mussel. Additional studies supporting the captive propagation efforts focused on temperature and housing conditions, release of glochidia and the transfer of glochidia to host fish, and role shredding insects could play in enhancing food resource availability for captive freshwater mussel diets. The host fish studies and the techniques deviced and refined during the course of these studies are already being used to support further captive mussel propagation research. In addition, the information has already been transferred and adopted by the Marion hatchery to support the propagation of species found in western NC. Results of these studies have been presented at the biannual freshwater mollusk conservation society meeting, Louisville Kentucky, April 2011 and shared through active discussion and consultation with other propagation facilities. The techniques have also been adapted for the culture of juveniles currently being used in effluent toxicity testing. Selected studies that were performed will also be submitted for review for publication in peer-reviewed journals to ensure that the information is readily available to other researchers and freshwater mussel biologists.

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