ABSTRACT

FOX, THOMAS REECE. Studies on the *in vitro* Propagation of Freshwater Mussels with Implications for Improving Juvenile Health. (Under the direction of Dr. Jay Levine).

In vitro propagation of freshwater mussels is a method of propagation that bypasses the mussels' need for an obligate host fish and transforms juveniles in an artificial media. This method of propagation has the potential to produce significantly more juveniles with less effort than traditional propagation methods that transform juveniles on their host fish. However, questions about the condition of the juveniles have caused concern about using this method to produce juveniles for population augmentation purposes. To investigate the problem of poor juvenile condition we conducted a long-term parallel study comparing the two methods of propagation. The objective was to determine how the survival and growth of *in vitro* propagated juveniles compares to juveniles propagated on host fish, over 10 weeks when grown in normal rearing conditions. We propagated *Lampsilis fasciola* using both propagation methods and measured the survival and growth of the juveniles every week for 10 weeks. We found that while juveniles propagated *in vitro* had a significantly higher transformation percentage, they also had significantly lower survival and growth when compared to the juveniles propagated. We also found that *in vitro* juvenile survival improved after 30 days and found the first 24 hours, 10 days, and 30 days, most critical times to improve survival and growth.

To improve the early survival and growth we added fish cells to the media to provide additional nutrients during the transformation period. We added three different fish cell lines to the media with Lampsilis radiata. We found that there was no difference in any of the treatment groups when compared to a control of the normal media. Removing the serum

complement in the media through heat inactivation also did not improve the survival or growth of the juveniles produced.

Manipulation of environmental conditions post transformation proved to have greater success than changing the conditions of the media during transformation. We examined adding the bacteria, *Bacillus subtilis,* to the diet of three freshwater mussels, *Alasmidonta raveneliana, Lampsilis fasciola, and Villosa delumbis,* to determine if adding a probiotic to the diet would increase the early survival and growth. The addition of the bacteria to the diet of the anodontine, *Alasmidonta raveneliana*, significantly increased its growth, however there was no difference in survival or growth in the lampsiline mussels, *Lampsilis fasciola* and *Villosa delumbis.* It appears as if *Alasmidonta raveneliana* utilizes the bacteria as a food source and not as a probiotic. To improve the survival of *in vitro* propagated juveniles in the first 24 hours post transformation, a series of dilutions were applied to the media containing the juveniles. We observed that diluting the media with freshwater significantly increased the survival of the juveniles and that the slower the dilution was performed, the greater the survival of the juveniles was observed. Our studies indicate that simple measures to improve the survival and growth of *in vitro* propagated juveniles can be taken to increase the benefits of this propagation method and that bacteria should be added to the diet of certain species of freshwater mussels to increase their growth.

Studies on the *in vitro* Propagation of Freshwater Mussels with Implications for Improving Juvenile Health

by
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DEDICATION

I would like to dedicate this to my wife and my parents whose constant support and encouragement helped and inspired me to complete this journey

BIOGRAPHY

Thomas Reece Fox was born in Brooklyn, NY and was raised in Shrewsbury, NJ where he fell in love with the water during his frequent trips to the beach. He attended Red Bank Regional High School in Little Silver, NJ graduating in 2002. He continued his education at The George Washington University in Washington, DC to earn his Bachelor of Science degree in 2006, majoring in biology with a minor in psychology. He spent the next two years working with the North Carolina Wildlife Resources Commission in Raleigh, NC where he was first introduced to freshwater mussels. He continued to gain knowledge of freshwater mussels and learn propagation techniques when he joined the Aquatic Epidemiology and Conservation Laboratory at NC State University. After working there for a year he was offered a position to begin graduate school and pursue his own Master's thesis research.

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INTRODUCTION

Freshwater mussels are benthic bivalves that were once extremely abundant in the rivers and lakes of North America. They provide a large number of beneficial ecosystem services and serve as food for a variety of animals including crayfish, some fish, salamanders, turtles, birds, otter, mink, muskrats, and raccoons (Fuller 1974, Vogt 1981, Shively and Vidrine 1984, Waters 1995, Neves and Odum 1989). Perhaps the most important ecosystem service they provide is the filtering of particulates and some toxins from the water column (McMohan and Bogan 2001). Even at low densities, mussel beds can filter a large volume of water column and influence ecosystem processes such as community respiration, algal clearance rates, as well as ammonia, nitrate, and phosphorus concentrations (Vaughn et al. 2004). Despite their importance to the ecosystem, over the past century freshwater mussels have experienced a greater decline than most other wide ranging groups of organisms (Strayer et al. 2004). Nearly 300 species inhabit North America, but more than 90% reside in the Southeast. Unfortunately approximately 35% of the freshwater mussels in North America are listed as federally endangered or threatened, and approximately 50% of the remaining taxa are species of special concern which makes them among the most endangered taxa of animals on the continent (Williams et al 1993, Neves et al 1997).

The decline of freshwater mussels began over a century ago with pearl hunters and the pearl button industry exploiting mussel populations for their commercial value (Coker 1914a, Anthony and Downing 2001). Anthropogenic activities such as impoundments, channelization, construction of impervious surfaces, eutrophication, pollution discharge, and

sedimentation continue to destroy mussel habitat and diminish mussel populations (Williams, Bogan, and Garner 2008). Coker (1916) was an early activist for freshwater mussels and argued to fisheries managers that immediate action was needed to reverse the trend of declining populations and that captive propagation might be needed to restore populations in some streams. The first propagation efforts in the early 1900s were aimed at restoring populations to augment the pearl button industry (Coker 1921). With the increasing popularity and use of plastic buttons the commercial propagation of freshwater mussels diminished. After the introduction of the Endangered Species Act in 1973 23 freshwater mussels were placed on the list for federal protection in June 1976, each having its own recovery plan. Captive propagation was noted as one of the means for recovery (Jones and Neves 2002).

Population augmentation and reintroductions have become increasingly valuable tools for offsetting and mitigating the decline of mussel species (Layzer and Scott 2006). Captive propagation and culture of juvenile mussels in a laboratory and hatchery setting are the primary option available to produce the large number of juveniles needed for these conservation activities (Neves 2004). Artificial propagation provides a unique opportunity for researchers to identify life history requirements and survival mechanisms as well as document the morphological development of different species (Bishop, Hudon, and Farris 2007). Newly transformed juveniles are also needed to conduct laboratory experiments to study their basic biology and response to environmental variables, and toxicity testing. However, the complex life cycle and sensitive nature of the juveniles make propagating freshwater mussels a difficult, labor-intensive, expensive task.

Freshwater mussels have an interesting and complex life cycle, which makes propagation more of a challenge. In the wild, male mussels release sperm into the water column. The sperm is then taken in through the incurrent siphon of a female mussel and fertilizes the eggs located in tubes in the gills (MacMahon 1991). The fertilized eggs are brooded by the female in gill demibranchs, called a marsupia while the eggs develop into larvae, called glochidia (Tankersley and Dimock 1992). The glochidia need to attach to either the gills or the fins of a specific fish host or salamander for 7-112 days, depending on the species (Lefevre and Curtis 1912). So the importance of knowing the host fish species of each mussel species is essential to their effective propagation, conservation, and recovery. During this time of attachment the glochidia are parasitic and receive nutrients from the host fish to develop their major internal organs such as gills, a foot, and digestive tract. When the transformation is complete the glochidia, which are fully developed juveniles, drop off the host fish and settle to the bottom where they bury into the substrate and begin feeding as a juvenile freshwater mussel (MacMahon 1991).

Traditional mussel propagation techniques utilize a fish host, however the proper host for many species of mussels is still unknown (Bishop, Hudon, and Farris 2007). Researchers must collect host fish from the wild or purchase the appropriate fish, transport the fish to the propagation facility, then put the fish through a quarantine system to control the contamination of diseases and parasites. The fish are then exposed to the glochidia that are concentrated in a small tank to promote attachment to fish gills. The host fish with the glochidia attached to the gills are moved to recirculating tanks for the duration of the encystment and transformation period. Each day, when the juveniles begin to drop off of the

host fish the researcher must work under a dissecting microscope to sort through fish feces and uneaten food that accumulates at the bottom of the tank to remove the transformed juveniles and transfer them to a juvenile culture vessel. Fish survival can also be jeopardized by excessive glochidial infestation, which can cause fish mortality prior to the completion of juvenile transformation. In addition to the difficulty of dealing with the fish, the percentage of glochidia that transform into juveniles is typically a very small portion of the total larval brood (Bishop, Hudson, and Farris (2007).

An under utilized method of propagating juveniles, propagation *in vitro* with an artificial media, can simplify the traditional method of mussel propagation and be more efficient and cost effective. This method of propagation bypasses the need for an obligate fish host and lets the glochidia transform in a Petri dish containing an artificial media that is placed in an incubator (Isom and Hudson 1982). The glochidia derive the necessary nutrients from the artificial media and develop into juvenile mussels in 7-35 days and are then transferred to an appropriate culture vessel containing freshwater where they begin to grow (Hudson and Shelbourne 1990). This method of *in vitro* propagation eliminates the time, effort, and space needed to collect, transport, and house host fish. It also allows for the potential propagation of mussel species for which the host is currently unknown (Bishop, Hudson, and Farris 2007). In addition to eliminating the use of host fish, *in vitro* propagation has the added benefit of producing juveniles with an extremely high transformation percentage as high as 95% (Owen 2009, Uthaiwan et al. 2001, Lima et al. 2006).

No matter which method is used to propagate mussels, researchers face many challenges culturing juveniles post transformation. When juveniles complete their

transformation they are typically transferred to one of several different culture systems for grow-out, including downwellers, recirculating tanks, static tanks, raceways, and ponds (Bishop, Hudson, and Farris 2007). Since the needs of mussels vary by species and juvenile survival is typically low, there is no standard culture vessel used and researchers continue to adapt and improve upon existing systems. While a mixture of different algae are fed to freshwater mussels in laboratories, the diet of juvenile mussels is poorly understood (Nichols and Garling 2000). Bacteria in the digestive tract may aid in digestion, but little is known about the natural bacteria associated with mussels (Syvokie et al. 1987, 1988, Nochols and Garling 2000). A better understanding of the nutritional requirements and diet of juvenile freshwater mussels and the role that bacteria play in digestion and nutrition could potentially improve upon the poor survival of juveniles being cultured.

Despite the added benefit of not using host fish and increased transformation percentage, *in vitro* propagation of freshwater mussels does have some shortcomings. Juveniles propagated *in vitro* have been shown to have a higher rate of mortality and slower growth when compared to juveniles propagated on host fish (Fisher and Dimock 2006). Early comparisons of the toxic response of *in vitro* vs *in vivo* transformed juveniles indicated that those transformed in an artificial medium were more sensitive to copper than the fish transformed juveniles (Warren and Klaine 1994). If both methods of propagation produced healthy juveniles then *in vitro* propagation could be the preferred method for many researchers to produce large quantities of juveniles with limited space and resources.

Accordingly the objective of these studies was to adapt *in vitro* propagation techniques for the propagation of freshwater mussel species found in North Carolina and to

improve the survival and growth of juveniles. Our hypothesis is that minor changes in the artificial media can improve the survival and growth of *in vitro* propagated juveniles mussels. In addition we hypothesize that the survival and growth of *in vitro* propagated juveniles can be improved by adding bacteria to the normal diet and refining the protocols used to transition juveniles from the media to freshwater. To address this question we first conducted a long-term parallel experiment comparing juveniles propagated on host fish to juveniles propagated *in vitro*. From this experiment we identified three critical periods that were most imperative to improve upon the survival and growth of *in vitro* propagated juveniles. Next we attempted to improve the condition of the *in vitro* propagated juveniles by manipulating the artificial media. We added fish cells to the media to provide extra nutrition during glochidial transformation and removed serum complement through heat inactivation and measured the growth and survival of juveniles to determine any benefit of the media manipulation. Next specific variables that could potentially effect overall post *in vitro* transformation success, growth and survival were examined. Slow acclimation and dilution of the media to freshwater was applied to the juveniles to increase the survival in the first 24 hours post transformation. Then because the cultured juveniles are reared in sterile media the role of bacteria in the diet of juveniles was studied to assess their potential benefit as probiotics. *Bacillus subtilis* was added to the normal diet of juvenile mussels to determine if it would increase the early survival and growth of *in vitro* propagated juveniles.

CHAPTER 1: A Comparison of Freshwater Mussel Propagation Methods

Introduction

With the continual decline of freshwater mussels in North America, the Endangered Species Act (ESA) of 1973 currently has 88 species of freshwater mussels listed as either threatened or endangered. Each species listed under federal protection is required to have a recovery plan that includes protective measures to prevent further population declines, all of which outline specific guidelines for the use of propagation as an essential tool for recovery and conservation of the declining population (Bishop, Hudson, and Faris 2007). Propagation efforts have generated viable juveniles for toxicity testing, *in situ* monitoring, and reintroduction efforts into recovering streams (Jacobson et al. 1993, Yeager, Cherry, and Neves 1994, Morgan, Welker, and Layzer 1997). In natural populations, the larval stage of freshwater mussels, glochidia, attach to the gills or fins of fish or other aquatic hosts. To date there are only two propagation techniques reported in the literature, one that includes the use of host fish, and another using artificial culture media (Lefevre and Curtis 1912, Isom and Hudson 1982). The vast majority of researchers choose to propagate mussels using the appropriate host fish, however, *in vitro* may offer some distinct advantages to using host fish to support the transformation of juveniles.

Ellis and Ellis (1926) reported the first successful transformation of glochidia to juveniles using an artificial media. However the details of their media solution were never published and the glochidia were excised from gill tissue so it is unclear if the artificial media

or the nutrients gained from the gill tissue prompted the transformation. This method of propagation was apparently forgotten until the 1980's when Isom and Hudson reported the first transformation of multiple species in a modified cell culture media without the use of fish (Isom and Hudson 1982). The major improvements made by Ellis and Ellis were the use of fish plasma as a source of protein, the use of a physiological salt solution as a growth medium, antibiotics, and a sodium bicarbonate buffer (Isom and Hudson 1982, 1984). Since fish plasma was not a readily available commercial product, studies were conducted to find a suitable serum replacement. Keller and Zam (1990) first addressed this problem and found horse serum to be the best serum replacement, however Hudson and Shelbourne (1990) obtained different results and reported that rabbit serum performed better than porcine, horse, sheep, chicken, and fetal bovine sera. Additional research investigating antibiotic cocktails, lipid supplements, and extraction protocols have refined the *in vitro* propagation process and improved its overall success (Tankersley 2000, Owen 2009,).

In vitro propagation has the great benefit of producing extremely large amounts of juveniles from a single brood. Transformation for some species has been reported as high as 95% (Uthaiwan et al. 2001, 2002). Since the host fish for many species of mussels are unknown, utilization of *in vitro* propagation may be the only way to augment populations of some species. While there are clear benefits of *in vitro* propagation, there are also concerns about the physiological health of the transformed juveniles. *In vitro* techniques are only of value if the large number of juveniles that are successfully transformed survive and grow to reproductive maturity. In one study, one day old *in vitro* transformed juveniles were found to be three times more sensitive to copper sulfate than juveniles propagated on host fish (Clem

1998). Only one published report has compared juveniles propagated *in vitro* with those propagated using the fish host method. In this report performed with *Utterbackia imbecillis*, early growth and survival of juveniles propagated *in vitro* was poor when compared to the juveniles propagated on fish hosts (Fisher and Dimock 2006). Protein, glycogen, and lipid levels of juveniles propagated *in vitro* was lower than juveniles propagated on fish hosts, suggesting they had lower energy reserves post transformation. Juveniles were not held in traditional culture vessels to optimize survival and none of the individuals survived longer than 22 days. Each mussel species, however, may react differently to the growth media, media formulation and holding conditions

If the *in vitro* propagation method is to be used to produce juveniles used for population augmentations then the juveniles produced must act the same and have similar growth and survival rates as juveniles that are propagated on fish hosts. Our goal of this experiment was to determine the duration of any diminished growth and survival in vitro propagated juveniles. Our hypothesis was that juveniles propagated with *in vitro* methods would have a higher mortality rate and not survive as long as juveniles propagated on host fish. To answer these questions we conducted a parallel experiment with *Lampsilis fasciola* and propagated juveniles using both methods and cultured them in systems with the same holding criteria to optimize growth and survival. Every week the growth and survival of juveniles was measured until the conclusion of the experiment at 90 days.

Methods

Standard in vitro Propagation Media

The basal media used was M199™ (Sigma-Aldrich, St. Lous, MO), which provided physiological salts, amino acids, glucose, vitamins, and buffers and equine serum was used as the main source of protein. A 3:1 ratio (vol:vol) of media:serum generated the best results in previous published results (Isom and Hudson 1982). Antibiotics were added to the media and consisted of 100 μg/mL rifampicin, 100 μg/mL gentamicin, and 1 μg/mL amphotericin B, which were added to the media prior to filter sterilization. A change in growth medium was performed under sterile conditions every other day until the juveniles were fully developed.

In Vitro Propagation Protocol

For long-term brooders that hold their glochidia in the gills, the exterior of the gravid mussel was rinsed with sterilized laboratory water and scrubbed with a mildly abrasive sponge for several minutes until the majority of the exogenous material was removed from the mussel. The mussel was then pried open enough to view the swollen demibranchs and sterilized laboratory water was sprayed inside the valves to remove any symbiotic parasites present and to reduce the chance of bacterial contamination in the *in vitro* culture. Working anterior to posterior, approximately 500 mL of sterile water was sprayed throughout the interior of the mussel, additional care was taken to thoroughly clean both sides of the foot and both demibranchs. A sterile 0.22 gauge needle was then used to puncture the distal end of the water tube to create a pore large enough to flush glochidia from the marsupium. Glochidia were flushed from the marsupium by holding the mussel over a sterile beaker filled

with MEM™ medium (Sigma-Aldrich, St. Louis, MO) and streaming sterile water into the demibranch until it no longer appeared swollen, indicating all glochidia had been removed. Physiological salts found in the MEM, stimulates glochidial valves to close, which makes them less susceptible to damage during the rinsing and sorting process.

Once extracted from the marsupium, the glochidia were rinsed and sorted to remove microbial contaminants, maternal tissue, and non-viable glochidia. A squirt bottle was filled with sterile MEM and used to agitate the beaker containing the glochidia. Approximately 250 mL MEM was added to the beaker in a steady stream, to suspend the glochidia. When the glochidia settled to the bottom of the beaker, the MEM containing parental tissue, fungus, and mites was decanted off and put through a 0.22 μm vacuum filter to sterilize and reuse the MEM. This process was repeated up to 10 times or until no parental tissue was visible in the MEM. The glochidia were then put through a sterile nitex mesh with a pore size equal to the hinge length. This allowed for viable glochidia with closed valves to pass through the mesh but keep out non viable glochidia that did not close its valves and glochidia that closed its valves on other glochidia, rendering both of them non viable. Further sorting to eliminate undeveloped or premature glochidia was accomplished gravimetrically. Undeveloped glochidia with less visceral mass settled to the bottom of the flask slower than viable glochidia. Sorting continued until all glochidia fell through the MEM and settle to the bottom at a constant rate. Working in a Class II vertical laminar flow biological safety cabinet, the glochidia were then transferred to a Petri dish containing the standard *in vitro* propagation media (described above). To reduce the chance of microbial contamination, a micropipette was used to collect a small amount of glochidia to transfer them into the Petri

dish, transferring a minimal amount of MEM into the complete *in vitro* propagation media. The culture vessels containing the media and glochidia were placed in an incubator with 3% CO_2 environment. A media change was performed under sterile conditions in a biological safety cabinet every other day until the juveniles were fully developed.

Fish host propagation

Four gravid *Lampsilis fasciola* were collected from the Pigeon River, NC, French Broad River basin in April, 2010. The mussels were transferred to the Aquatic Epidemiology and Conservation Laboratory (AECL) at the North Carolina State University College of Veterinary Medicine (NCSU CVM) and held in the laboratory for one week while they were slowly acclimated to the laboratory water chemistry. The glochidia from one gill from each of the mussels were extracted by puncturing the distal end of each water tube with a 0.22 gauge sterile syringe needle and flushing the gill with water. The hole created by the syringe was sufficiently large enough for the glochidia to be flushed out of the marsupium and into a sterile beaker held below the mussel. The gill was flushed with water until it no longer appeared swollen and the majority of the glochidia were removed. A sample of the extracted glochidia was tested for viability by using a saturated NaCl solution to stimulate the glochidia valves to close. Glochidia that failed to respond or had their valves closed prior to NaCl application were recorded as non-viable.

Largemouth Bass (*Micropterus salmoides*) purchased from Foster Lake and Pond Management, Garner, NC., approximately four inches in length, were used as host fish. Glochidia harvested from gravid females were placed in a five-gallon bucket filled with approximately four gallons of water at a concentration of 5600 glochidia/liter of water. A

large air stone was placed at the bottom of the bucket to vigorously aerate the water to keep the glochidia suspended in the water column and encourage host fish attachment. Forty-three largemouth bass were placed in the five-gallon bucket for 15 minutes to allow glochidia to attach to the gills of the fish. To get the most accurate estimate of the transformation percentage, fish were transferred to a 20-gallon tank for 10 minutes to allow improperly attached glochidia to fall off the fish body. Thirty-three fish were placed in a 50-gallon recirculating round fish tank at 21°C ± 1 °C to hold the fish during glochidia transformation. The tanks were constructed with a double stand pipe bottom draw design that collected juveniles and sloughed off glochidia into a 150 μm nitex filter bag, suspended in the reservoir. After attachment, ten fish were individually housed in two-gallon tanks, to get an estimate of glochidia transformation percentage. Each tank had a bottom draw double standpipe and drained to a separate 150 μm nitex filter bag.

Once each day the fish tanks were stirred to create a circular flow, which concentrated all glochidia and transformed juveniles to the center of the tank allowing the double standpipe to collect them into the filter bags. Filter bags were rinsed into 150 x 26mm Petri dish and examined under a dissecting microscope to count and separate the number of sloughed off glochidia and fully transformed juveniles. The percent that successfully transformed was calculated (transformation percent = # of fully transformed juveniles / # of fully transformed juveniles + # of sloughed off glochidia).

In vitro Propagation

The same four *Lampsilis fasciola* used for natural propagation also had glochidia extracted from one gill for *in vitro* propagation. The glochidia were processed using the *in*

vitro propagation protocol (described above) and then placed in the standard M199™ *in vitro* propagation media. To determine the transformation percentage, six replicates of approximately 500 viable glochidia were placed in a six well culture plate with 3 mL media. The rest of the viable glochidia were placed in Petri dishes with 10 mL media. Glochidia were cultured in the media at 22 °C with 3% CO_2 for 38 days. After 38 days the 6 well culture plate was examined under an inverted microscope to determine the number of glochidia that transformed into juveniles. Transformation percent = number of transformed juveniles / total number of viable glochidia. The transformed juveniles were taken out of the media and placed directly into six 1 L beakers filled with 21 °C freshwater and an air stone. Juveniles were examined under a dissecting microscope after 24 hours to determine the number of juveniles still alive.

Experimental Design

Juvenile survival and growth of juveniles propagated by both methods were compared at one week and at 70 days. The juveniles during the five peak days of the drop off period were collected to determine the one-week survival and growth of juveniles propagated on fish hosts. Each day the number of live juveniles was counted and were split into three equal treatment groups. Each replicate was placed in a 150 μm nitex mesh coupler and put into three different downwelling mucket buckets (Barnhart 2006). After one week, each replicate was emptied into a Petri dish and examined under a dissecting microscope to count the number of live juveniles. Thirty randomly selected juveniles were measured using a calibrated ocular micrometer. When all the juveniles were counted and measured they were placed in the same beaker then randomly distributed into three equal treatment groups of 500

juveniles. Each replicate was placed into a different downwelling mucket bucket (Barnhart 2006) for long term growth and survival measurements. Once a week, for 10 weeks, all the juveniles in each replicate were counted for survival and 30 random juveniles were measured.

After 38 days the juveniles were fully transformed *in vitro* and were placed directly into freshwater. The 6 replicates used to determine the transformation percentage were combined into 3 treatment groups of approximately 1000 juveniles. The juveniles were placed in a 150 μm nitex mesh coupler and put into the same three mucket buckets that contained the fish host propagated juveniles. At 24 hours and 1 week, juveniles from each replicate were examined under a dissecting microscope and the number of live juveniles was counted. Thirty randomly selected juveniles were measured using a calibrated ocular micrometer. After one week, all juveniles were combined and three equal treatment groups of 500 juveniles and were randomly distributed into the three mucket buckets containing the fish host propagated juveniles. Once a week, for 10 weeks, all the juveniles in each replicate were counted for survival and 30 randomly selected juveniles were measured.

The mucket buckets were filled with municipal water that had been treated with sodium thiosulfate and Ammo Lock (Aquarium Pharmaceuticals, Chalfont, Pennsylvania) to remove chlorine and chloramines. The lights in the building were controlled by a 12-hour on, 12-hour off cycle timer. Unless otherwise noted, water temperature for all tanks was maintained at 23°C. The commercial algae used in these experiments were a mixture of Nanno 3600 (*Nannochloropsis* spp.) and Shellfish diet (*Isochrysis* spp., *Pavlova* spp., *Tetraselmis* spp., and *Thalassiosira weissflogii*) both from Reed Mariculture Inc. (San Jose,

CA). Approximately 50,000 cells/mL of algae were fed to the appropriate treatments once per day and a 100% water change was performed once a week to maintain optimal water quality.

Statistical Analysis

An analysis of variance, ANOVA, was performed to assess the difference in growth and survival of juveniles transformed *in vitro* and juveniles transformed on fish hosts. Tukey's Honestly Significant Difference (HSD) was performed to identify differences between treatments. A P value of <0.05 was considered significant.

Results

The transformation percentage of *Lampsilis fasciola* glochidia attached to their fish host was relatively high, with a mean of 69% (fig. 1). However there was a significant ($p = 0.0002$) increase in transformation percentage when using the *in vitro* propagation method. With a mean of 92% transformed juveniles and very little variance, the *in vitro* method increased the transformation from glochidia to juveniles by 23% (fig. 1).

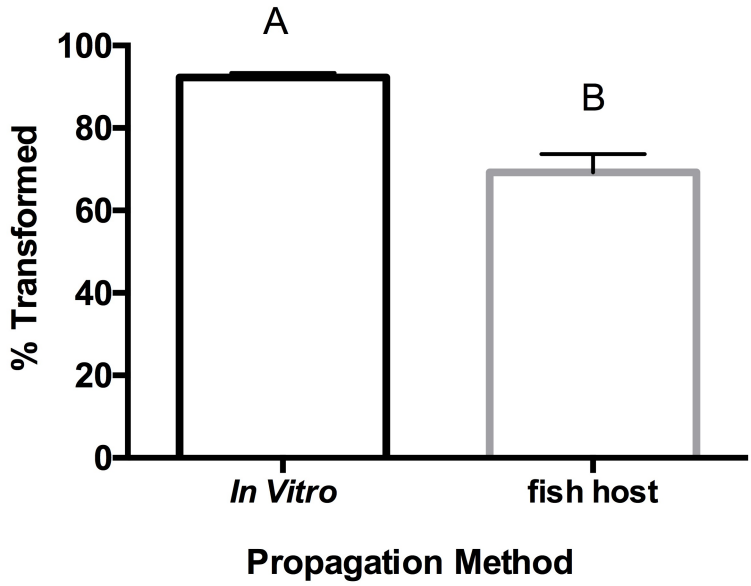

Figure 1. Mean transformation percentage (± SEM) of *Lampsilis fasciola* propagated with *in vitro* methods and on fish hosts. N = 500 for each of 6 replicates. Data was analyzed using the arcsine transformation of the proportion of glochidia that transformed into juveniles. Letters that are different were statistically significant (Tukey's HSD $p<0.05$)

After being cultured in a mucket bucket with 150 μm mesh for one week, the juveniles were measured using a calibrated ocular micrometer. The propagation technique had a significant effect on the growth of juvenile *Lampsilis fasciola* after one week ($p < 0.0012$). Juveniles that were propagated on fish hosts grew a mean of 51 μm more than juveniles that were propagated *in vitro* (fig. 2).

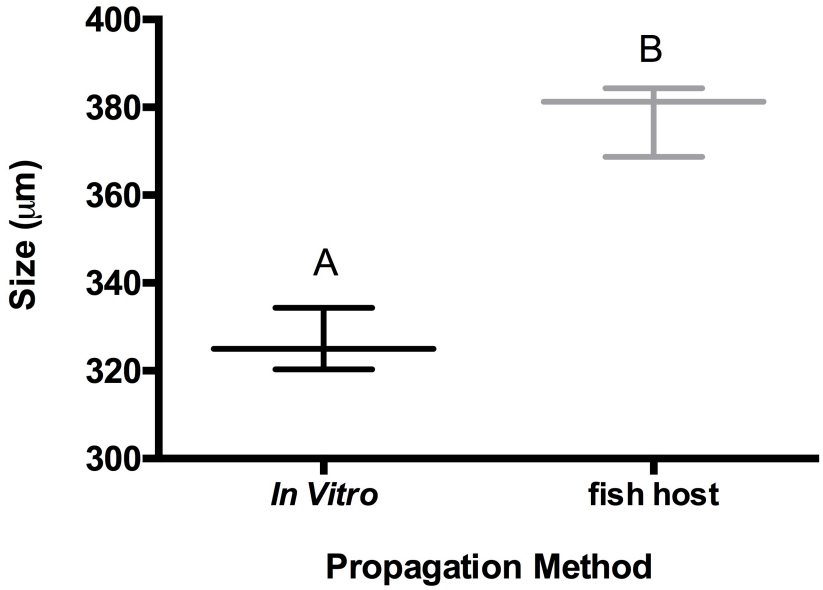

Figure 2. Mean size of *Lampsilis fasciola* juveniles one week post transformation propagated with *in vitro* methods and on fish hosts. N = 30 for each of 3 replicates. Letters that are different are statistically significant (Tukey's HSD $p<0.05$)

All juveniles in each replicate were counted to determine the number of live juveniles after one week. Propagation technique had a significant effect on the survival of juveniles after one week ($p = 0.0018$). Juveniles that were propagated on fish hosts had a higher percent of juveniles alive after one week than juveniles that were propagated *in vitro* (fig. 3). With 82% of the juveniles alive after one week, the fish host method of propagation exhibited a 37% increase in survival over *in vitro* propagated juveniles.

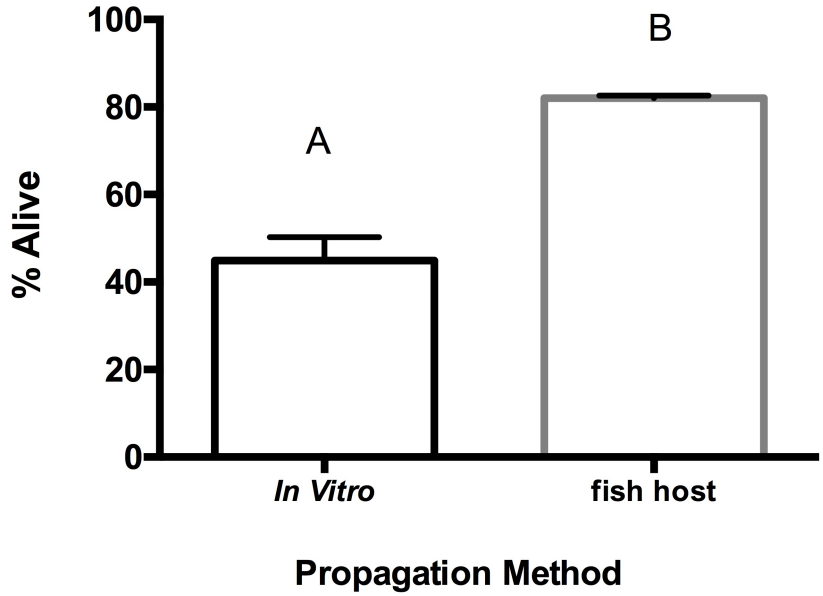

Figure 3. Mean percent (± SEM) of number of live *Lampsilis fasciola* juveniles one week post transformation. Juveniles were propagated using *in vitro* methods and using fish hosts. Initial N = 500 for each of 3 replicates. Data were analyzed using the arcsine transformation of the proportion of juveniles alive. Letters that are different were statistically significant (Tukey's HSD $p<0.05$).

When culturing the juveniles in mucket bucket grow out tanks for 70 days, there was a difference in growth between the two propagation methods. At every time point measured, the juveniles that were propagated on fish hosts had a higher mean size than the juveniles propagated *in vitro* (fig. 4). At 7 days post transformation the fish host propagated juveniles were larger than the *in vitro* propagated juveniles. In the subsequent weeks the *in vitro* propagated juveniles became closer to the size of the fish host propagated juveniles until the 32 days post transformation time period when the juveniles had the smallest difference in size at 17 μm (fig. 4). After the 32-day time period the fish host propagated juveniles grew much faster and the largest difference in size between treatment groups was observed at the conclusion of the experiment at 70 days

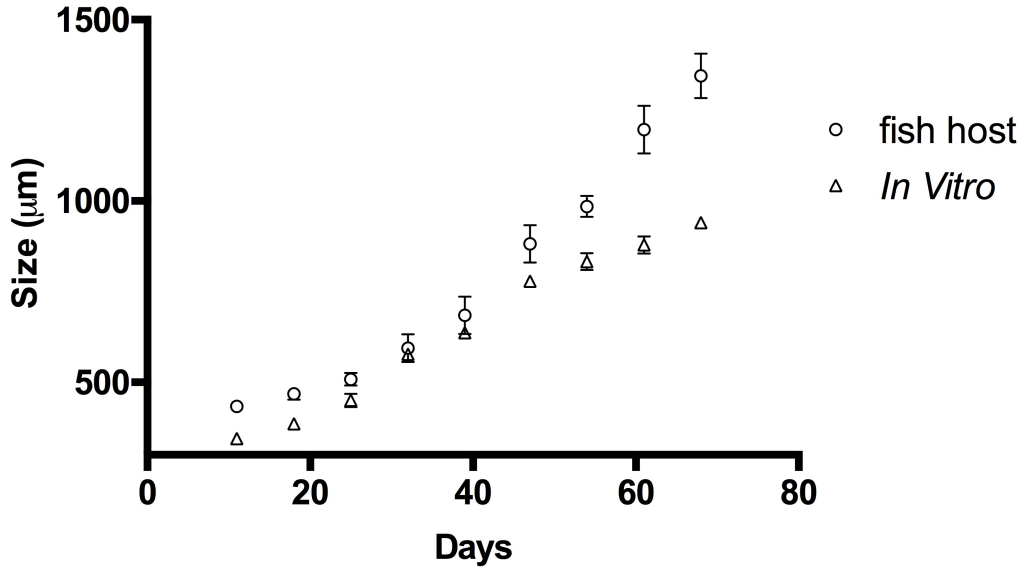

Figure 4. Mean size (± SEM) of *Lampsilis fasciola* from 10 to 70 days post transformation. Juveniles were propagated using *in vitro* methods and using fish hosts. N = 30 for each of 3 replicates.

The survival of the juveniles propagated from both of the methods was measured on a weekly basis. At every time period there was a higher percentage of juveniles alive that were propagated on fish hosts than there were of juveniles that were propagated *in vitro* (fig. 5). The highest percentage of *in vitro* propagated juveniles occurred in the first 4 weeks. After the fourth week, the percentage of juveniles that died each week was considerably reduced, losing less that four percent each subsequent week (table 1). The juveniles from both propagation methods died at nearly the same rate after the fourth week of the experiment.

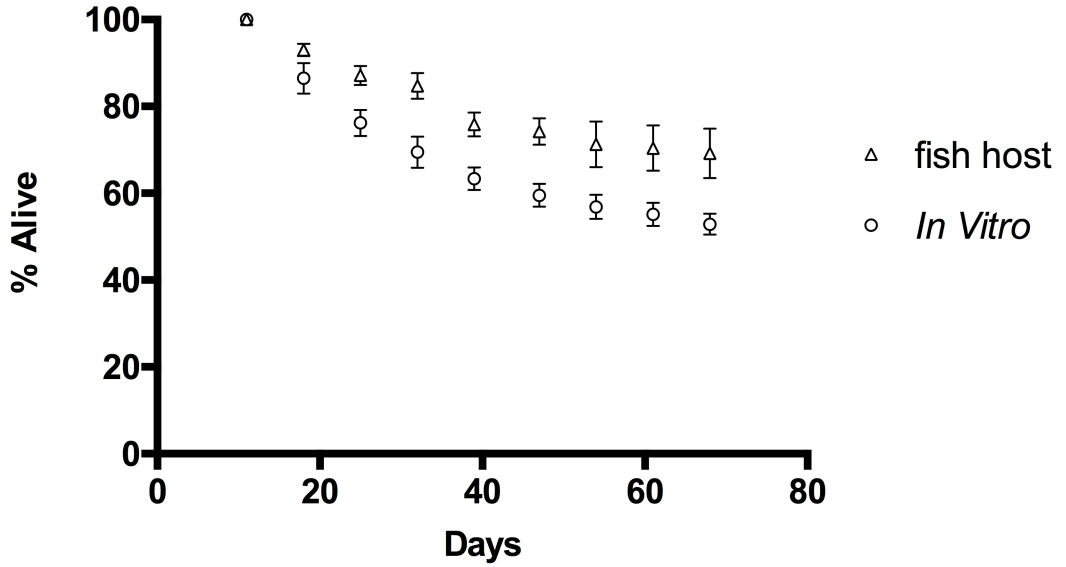

Figure 5. Mean percent (± SEM) of live juvenile *Lampsilis fasciola* from age 10 to 70 days post transformation. Juveniles were propagated using *in vitro* methods and on fish hosts. Initial N = 500 for each of 3 replicates from each propagation technique

Discussion

Lampsilis fasciola juveniles that were propagated *in vitro* exhibited a significantly higher transformation percentage than juveniles propagated on host fish. By counting the number of sloughed off glochidia and the number of transformed juveniles, this transformation percentage represents only the glochidia that were able to attach to the host fish. During the infestation process a large percentage of the glochidia will close in suspension and never attach to the gills of the fish, which will eliminate the opportunity to transform into a juveniles. The number of juveniles that transform from the total brood varies with each species but is typically only a small percentage due to failure of attachment, encystment, and avoidance of the host immune system (Kirk and Layzer 1997). With the *in vitro* process the glochidia closing in the media does not affect its transformation, therefore a

greater number of juveniles can be produced from each brood, with an observed mean of 92% transformation. This is particularly important when dealing with threatened and endangered species, or species that produce very small broods. In these cases every glochidia is extremely valuable and it is important to maximize the number of juveniles produced from each brood.

Consistent with the results of Fisher and Dimock (2006), the juveniles that were cultured *in vitro* exhibited a higher rate of mortality and less growth than juveniles propagated on fish hosts in the first week post transformation. Fisher and Dimock demonstrated that the *in vitro* propagated juveniles have significantly lower levels of triglycerides, cholesterol, glycogen, and protein one-day post transformation. Juveniles build up these energy reserves during their transformation period either in media or attached to host fish. It is these energy reserves that juveniles draw upon while they transition to eating and digesting food particles or during physiologically stressful events (Fisher and Dimock 2006). One such stressful event for the *in vitro* propagated juveniles is the dramatic change from the saline environment of the artificial media, to freshwater aquaria. It was observed that a large portion of the one-week *in vitro* juvenile mortality occurred within the first 24 hours after transferring the juveniles from the media to the freshwater. During this time the juvenile mussels undergo a large change in osmolarity, which is a considerable stressor at such a critical time in their lifecycle. Many of the *in vitro* propagated juveniles die, making the first 24 hours in freshwater a critical to their survival. Additional studies are needed to more fully define the physiologic changes that are occurring as the animals move from the

medium to freshwater, and what additional measures can be taken to mitigate this initial loss in transformed juveniles.

Fisher and Dimock attempted to culture the juveniles in 96-well microtiter plates with 300 μl water in each well. They experienced a 50% mortality for *in vitro* propagated juveniles at 10 days while that for the fish host propagated juveniles was 14 days. They did not have any juveniles survive past 16 and 22 days respectively. In contrast we attempted to growout the juvenile mussels for 70 days in normal freshwater mussel culture aquaria. At the conclusion of our experiment at 70 days the juveniles that were propagated *in vitro* exhibited the greatest mortality from the commencement of the experiment to day 30, with 30% of the juveniles dying during this time period. In contrast, the juveniles that were propagated on their host fish had a mortality rate that was half of what the *in vitro* propagated juveniles showed and only 15% of the juveniles died during the same 30-day period. However, from day 30-70, the mortality of the *in vitro* propagated juveniles was less than half when compared to the first 30 days, and we only lost 16% of the juveniles during this time period. The mortality of the juveniles propagated on host fish from day 30-70 remained consistent at 15%, which was nearly the same as the *in vitro* propagated juveniles over the same study period.

During the course of these studies we identified three critical time periods in which the survival and growth of *in vitro* propagated juveniles needs improvement. The first critical period identified is the 24 hours after moving juveniles from the artificial media to freshwater. The suspected cause for the observed high mortality, osmotic shock, needs to be addressed in future studies by testing different acclimation rates for juveniles transitioning to

freshwater. The first week after transformation has traditionally been a period when substantial mortality is noted in juveniles transformed on fish, and we observed similar losses during this period in *in vitro* transformed animals. It is difficult to compare survival of mussels among different studies because of the great difference in species variability, culture system, and diet fed. However Henley and coworkers (2001) reported a similar depression in the survival of *Lampsilis fasciola* that were propagated on host fish with a 27% survival after two weeks when grown in raceways. The third critical time period we identified is the first 30 days of being cultured in freshwater. After this 30-day window, the juveniles propagated *in vitro* display nearly the same survival as the juveniles propagated on host fish. Previous studies (Fisher and Dimock 2006) suggest that *in vitro* propagated juveniles have lower energy reserves immediately following transformation when compared to juveniles propagated on fish hosts. These energy reserves are drawn upon during the transition to filter feeding and other stressful events, so any small environmental change such as temperature, pH, ammonia level, or changes in food availability can cause physiological stress on the juvenile and cause premature death (Fisher and Dimock 2006). It was after 30 days that the juveniles from both propagation methods had the same survival rate so it is possible that it takes the *in vitro* propagated juveniles 30 days of feeding to build up enough energy reserves to combat potentially deadly environmental stressors that would have killed them earlier. If a better source of nutrition was provided to the juveniles either during the transformation period or immediately following transformation, *in vitro* propagated juveniles could be healthier and have better survival and growth during these early critical time periods. Increasing the early growth and survival of *in vitro* propagated juveniles could make this

technique of propagating freshwater mussels even more efficient when compared to propagation on host fish.

CHAPTER 2: Improving *in vitro* Propagation Success of Juveniles During Transformation

Introduction

Freshwater mussels have a complex life cycle, which makes their propagation more of a challenge. The larvae of freshwater mussels (glochidia) need to attach to the gills or the fins of an obligate fish host to complete their life cycle (MacMahon 1991). When the glochidia are attached to the fish they receive nutrients while they develop their major internal organs and transform into juveniles (Arey 1932). The fish cells form a cyst around the developing glochidia, which can last anywhere from several days to several months. Encysted glochidia transform into juveniles and then drop off the fish and settle into the substrate (Coker et al. 1921, Arey 1932a, Waller and Mitchell 1989). Traditional freshwater mussel propagation techniques try to mimic environmental conditions by exposing the glochidia to only the known host fish for that species of mussel. Fish are then held in aquaria until the glochidia transform into juveniles and the researcher can collect the juveniles from the bottom of the aquaria and place them into culture units (Bishop, Hudson, and Farris 2007).

As an alternative to propagating freshwater mussels with fish hosts, the glochidia can be held in artificial media *in vitro* until they transform into juveniles. This method of propagation by-passes the need for an obligate host fish. Forty-two species of mussels have been successfully propagated *in vitro* (Lima et al. 2012). A large proportion of glochidia

successfully transform when propagated *in vitro,* however the overall health of the juveniles produced is variable (Clem 1998). Juveniles produced by *in vitro* propagation have shown slower growth and increased mortality when compared to juveniles transformed on host fish (Fox et al. in preparation, Fisher and Dimock 2002). The majority of the mortality has occurred in the first 10 days and 30 days, after 30 days juveniles from both propagation methods exhibited the same survival rates (Fox et al. in preparation). Fisher and Dimock (2006) demonstrated that immediately following transformation, juveniles propagated *in vitro* had lower levels of energy reserves than juveniles propagated on host fish. Uthwaiwan and coworkers (2002, 2003) investigated using the plasma of four fish species (common carp, nile tilapia, hybrid catfish, and striped catfish) in the artificial media for *in vitro* propagation of the freshwater mussel *Hyriopsis myersiana*. They observed a significant increase in the survival of juveniles that were propagated using plasma from the host fish, the common carp, which suggests that the nutrient uptake from the gills of the host might be contributing to the transformation of glochidia to juveniles (Uthwaiwan et al. 2002). They postulated that amino acids may be the nutrients that determine the survival rates of glochidia and found LEU, PRO, THR, ALA, CIT, and GLX in significantly higher concentration in common carp serum when it was compared with other fish treatments (Uthwaiwan et al. 2002, 2003).

There are several ways in which the glochidia may receive nutrition during the transformation process. Sources of nutrition include absorption through fish blood and plasma, dissolution of the larval adductor muscle, digestion of the atrophying mushroom body, and digestion of host tissue trapped between the valves of the glochidia (Arey 1932b).

Juveniles that are propagated *in vitro* have the availability of the first three sources of nutrition since the artificial media acts to mimic the environment on host fish. However since the glochidia are transformed in an artificial media and never utilize a host fish, they do not have the availability of gill tissue to digest during transformation. Gill cells of fish species may provide nutrients and varying amounts of essential amino acids to the glochidia during encystment. The objective for this study was to determine if the addition of fish cells to the medium could enhance the survival and growth of *in vitro* propagated juveniles. Glochidia were forced to snap shut on gill cells derived from three different fish species. After transformation the juveniles were cultured in downwelling bucket systems and their survival and growth were measured for 30 days after transformation.

Methods

Addition of Fish Cells

Fish cells were added to the juvenile culture media to determine if their addition can increase the survival and growth of recently transformed juveniles.

Study Design

Four treatments were used with five replicates per treatment and 500 juveniles per replicate. The treatments included: epithelial cells from the skin of a fathead minnow (*Pimephales promelas*) (EPC), fibroblast cells from the fin of a koi (*Cyprinus carpio*) (KF1), gill cells from the gills of a walking catfish (*Clarias batrachus*) (G1B), and a control without fish cells.

Study Animals

Three gravid *Lampsilis radiata* were collected in late August 2013 from the Pee Dee River, NC part of the Yadkin-Pee Dee River basin. The mussels were transferred to the AECL at NCSU CVM where they were held for three days to acclimate to water temperature and chemistry. Using a sterile 24ga hypodermic needle filled with conditioned laboratory water that was run through a 0.22 μm filter, the glochidia from one gill of each of the mussels flushed out. A 500 mL beaker filled with 250 mL sterile mussel water was used to catch the glochidia as they were flushed from the gills. Extracted glochidia were washed to remove parental tissue, micro invertebrates, fungus, and bacteria that washed into the beaker while flushing the parental gills. To wash the glochidia a sterile squirt bottle was filled with sterile mussel water and a steady stream of water was applied to the beaker to agitate the glochidia. Glochidia were allowed to settle to the bottom of the beaker while all other material was decanted off. This process was repeated several times until only the glochidia were apparent in the beaker.

Cell Lines

The cells used in these experiments were purchased from the American Type Culture Collection (Manassas, VA) (ATCC) and Health Protection Agency (London, England) (HPA) as established cell lines from different fish species. The EPC *(Epithelioma Papulosum Cyprini, ATCC #CRL-2872)* cell line is an adherent epithelial cell that was derived from the Fathead Minnow *(Pimephales promelas)*. Cells were cultured using 25 cm^2 – 175 cm^2 corning cell culture flasks held at 25 °C in a 5% CO_2 environment. The cell culture media used was Eagles Minimum Essential Media (Sigma-Aldrich, St. Louis, MO)

with 10% fetal bovine serum. The KF1 (HPA #10072801) cell line is an adherent fibroblast cell derived from the fin of a Koi Carp *(Cyprinus carpio)*. Cells were cultured in cell culture flasks and held at 25°C in a 5% CO_2 environment. The cell culture media used was Eagles Minimum Essential Media with 10% fetal bovine serum. The G1B (ATCC #CRL-2536) cell line is an adherent pleomorphic cell that was derived from normal gill tissue taken from a walking catfish *(Clarias batachus)*. Cells were cultured in cell culture flasks and held at 25 °C in a 5% CO_2 environment. The cell culture media used was F-12K medium with 10% fetal bovine serum.

To capture the same amount of cellular material between the valves of the glochidia, a standard cell volume of $4.3X10^{10}$ μm^3/mL media was used for all cell lines. A cell sorter (Cellometer™, Nexelon Inc,. Lawrence, MA) was used to determine the diameter of the cells in each cell line. A 10 μL sample of each cell line was loaded into the counting chamber and inserted into the machine. The Cellometer™ measured the diameter of each cell in the counting chamber. Once detached from the substrate each cell was assumed to be spherical and the mean diameter was used to calculate an average volume per cell type.

When the cell culture flasks reached approximately 100% confluence, each flask was treated with the appropriate volume of trypsin to detach the cells from the substrate. The cells from multiple flasks containing the same cell line were pooled together and spun in a centrifuge at 3500 rpm for 5 minutes. The media containing the trypsin was decanted and cells were resuspended in 40 mL media. A hemocytometer was used to determine the cell concentration and total number of cells present in each cell line. An aliquot from each cell line containing the appropriate number of cells was pipetted into a 15 mL conical tube and

spun at 3500 rpm for 5 minutes. Each cell line was resuspended in 10 mL media to give a final concentration of $4.3X10^{10}$ μm^3/mL media.

Four petri dishes were filled with 10 mL media; three petri dishes contained $4.3X10^{10}$ μm^3/mL of each cell type and one petri dish contained media without any cells. The previously washed glochidia were divided into four approximately equal groups and concentrated into four 50 mL beakers. As much water as possible was decanted off the top of the 50 mL beaker. A Pasteur pipette was used to transfer the glochidia from the sterile water to the appropriate media treatment group containing cells or no cells. Once the glochidia entered the media, the physiological salts in the media triggered the open glochidia to snap shut. Because the cells were highly concentrated in the media the glochidia trapped the fish cells between their valves. To avoid glochidia closing their valves on other glochidia, they were slowly added drop wise to the media in a clockwise spiral fashion.

After all the glochidia in the media fully closed their valves, the media containing the cells was decanted. Media (MEM) that did not contain any cells was added to each treatment and the glochidia from each treatment were sorted and washed according to the standard protocol (Fox et al. in preparation) in to remove any leftover cells and to separate the viable glochidia from the nonviable glochidia. When the glochidia were washed and sorted, glochidia from each treatment were transferred to a 6-well culture plate to determine the percent transformation. Approximately 500 viable glochidia were transferred to each well containing three milliliters of the juvenile culture media. Two additional petri dishes for each treatment containing 10 mL juvenile culture media were used to transform additional glochidia needed for the experiment.

After 26 days in the media, the glochidia were finished transforming into juveniles. At this time each replicate was examined under a microscope to determine the proportion of glochidia (% transformation) in each treatment group that transformed into juveniles. The percent transformation was calculated as: % Transformation = # fully transformed juveniles / # viable glochidia in media. When the juveniles were acclimated to freshwater, they were separated into 20 equal treatment groups of 500 juveniles representing five replicates per treatment. Using a randomized complete block design, the juveniles were placed into downwelling mucket buckets (Barnhart 2006) with 150 μm mesh length. Each mucket bucket contained one replicate from each treatment; five mucket buckets were used in total. At the time periods of one week and one month, juveniles from each replicate were placed into a petri dish and examined under a dissecting microscope to determine the total number of live animals. At this time, 30 randomly selected juveniles were measured for total length using a calibrated ocular micrometer.

Conditioned municipal water was used to culture the juveniles at an average temperature of 23.2 °C. Water quality parameters such as pH, total ammonia, nitrates, dissolved oxygen, and hardness were taken at the time of each assessment and water temperature was recorded daily. The commercial algae fed in this experiments were a mixture of Nanno 3600 (*Nannochloropsis* spp.) and Shellfish diet (*Isochrysis* spp., *Pavlova* spp., *Tetraselmis* spp., and *Thalassiosira weissflogii*) both from Reed Mariculture Inc. (San Jose, CA). Approximately 50,000 cells/mL of algae were fed to the appropriate treatments once per day and a100% water change was performed once a week to maintain optimal water quality.

Heat Inactivation of Serum

An experiment was conducted to test the effect of the complement found in serum and heat-inactivated serum used in the juvenile growth media on *in vitro* glochidia survival and growth. Two treatments were tested, with three replicates per treatment and 1000 juveniles per replicate.

Study Animals

One gravid *Lampsilis fullerkati* was collected from Lake Waccamaw, NC, part of the Lumber River basin in early July 2010. The mussel was held at the AECL at NCSU CVM for one week and was slowly acclimated to the laboratory water chemistry.

Study Design

One week later the glochidia from both gills of the mussel were extracted and processed using the *in vitro* propagation protocol. When the glochidia were finished being processed they were randomly split into 12 equal treatment groups of 500 individuals.

The treatments were:

1. Heat Inactivated – equine serum that was heat inactivated
2. Control – equine serum that was not heat inactivated

Six replicates were placed in a six well plate containing three mL of the standard *in vitro* propagation media and six replicates were placed in three mL of the standard *in vitro* propagation media made with heat inactivated serum. Equine serum purchased from Invitrogen Life Technologies was divided into two bottles. To inactivate the serum, one of the bottles was placed in a 56 °C water bath for 30 minutes, then allowed to cool before being added to the rest of the standard *in vitro* propagation media components. The other

bottle was not inactivated and was used to make a second batch of standard *in vitro* propagation media. Glochidia were placed in an incubator at 23°C and 4% CO_2 environment and allowed to develop for 26 days until they were fully transformed.

After 26 days, all of the glochidia that successfully transformed into juveniles were combined into one beaker for each of the serum treatments. The juveniles were transitioned out of the media and into freshwater. Once the dilution was completed the juveniles were then randomly split into six equal treatment groups of 1000 individuals, representing three replicates per treatment. Using a randomized complete block design the juveniles were placed on 150 μm mesh in mucket buckets. One replicate per treatment was placed in each bucket making a total of 3 buckets used. At the time periods of one week and one month, juveniles from each replicate were placed into a petri dish and examined under a dissecting microscope to determine the total number of live animals. At this time, 30 randomly selected juveniles were measured for total length using a calibrated ocular micrometer.

Conditioned municipal water was used to culture the juveniles at an average temperature of 23.2 °C. Water quality parameters such as pH, total ammonia, nitrates, dissolved oxygen, and hardness were taken at the time of each assessment and water temperature was recorded daily. The commercial algae fed in this experiments were a mixture of Nanno 3600 (*Nannochloropsis* spp.) and Shellfish diet (Isochrysis spp., *Pavlova* spp., *Tetraselmis* spp., and *Thalassiosira weissflogii*) both from Reed Mariculture Inc. (San Jose, CA). Approximately 50,000 cells/mL of algae were fed to the appropriate treatments once per day and a 100% water change was performed once a week to maintain optimal water quality.

Statistical Analysis

An analysis of variance, ANOVA, was performed to assess the difference in survival and growth of juveniles transformed with different cell lines added to the media. Tukey's Honestly Significant Difference (HSD) was performed to identify differences between treatments. And an unpaired t test was performed to assess the difference in survival and growth of juveniles transformed with heat inactivated serum vs control serum. A P value of <0.05 was considered significant.

Results

Addition of Fish Cells

The transformation percentage across all treatment groups was very high. The treatment that had KF1 cells added to the media resulted in the highest transformation percentage with a mean of 98% ± 1% SEM, and the control group of adding no cells to the media gave a mean transformation of 92.7% ± 1% SEM (fig 1). There was no statistically significant difference in the percentage of transformed juveniles obtained with the three experimental treatments and the control (fig 1).

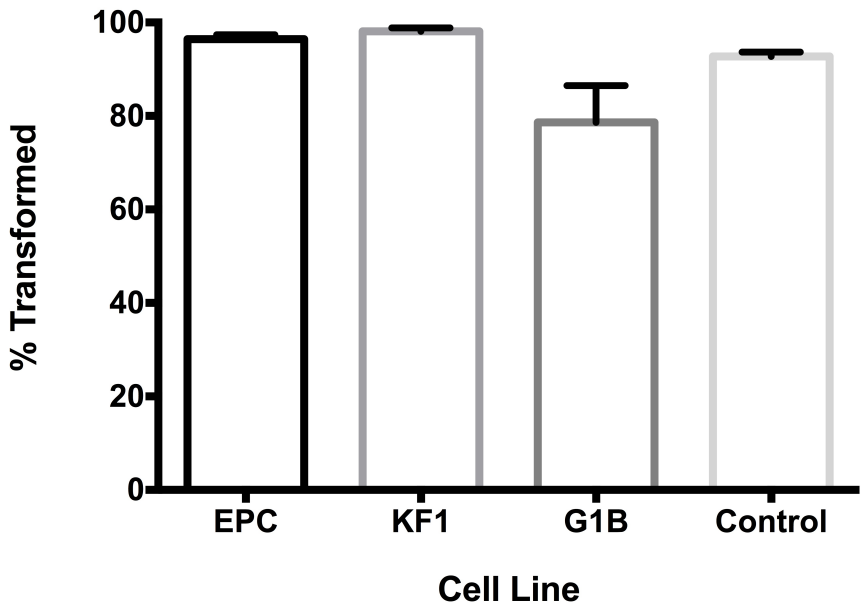

Figure 1. Mean transformation percentage (± SEM) of *Lampsilis radiata* propagated with different fish cell lines added to the artificial media. N = 500 for each of 5 replicates. Data was analyzed using the arcsine transformation of proportion of glochidia to transform into juveniles.

After being cultured in a mucket bucket for one week, the juveniles that were transformed with EPC cells in the media grew the largest with a mean of 306.1μm ± 3.1μm SEM. The control treatment that did not have any cells added to the media grew the least over one week, however it was not significantly different ($p = 0.823$) with a mean of 299.8μm ± 7.2μm SEM; slightly smaller than the juveniles with EPC cells (fig. 2). There was no significant difference in length observed between any of the treatment groups after 10 days. The same trend that was observed after 10 days was also observed after being cultured in a mucket bucket for 30 days. The juveniles that had KF1 cells added to the media were larger than those grown with the other cell-lines and without a cell line after 30 days with a mean length of 629.3μm ± 38.8μm SEM while the control group were the smallest with a

mean of 603.9μm ± 28.7μm SEM, however this difference in length was not statistically (p = 0.945) significant (fig.2). There was no significant difference in length observed between any of the treatment groups after 30 days.

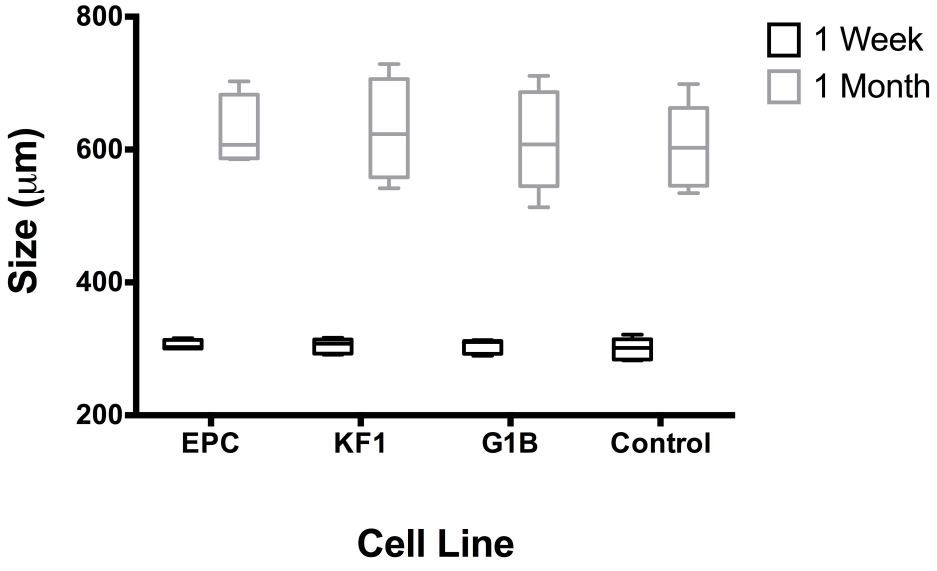

Figure 2. Mean length of *Lampsilis radiata* juveniles one week and one month post transformation. Glochidia were cultured with different fish cell lines in the culture media. N = 30 for each of 5 replicates.

Survival of *Lampsilis radiata* juveniles after one week being cultured in a mucket bucket was high among all the treatment groups. The juveniles that had KF1 cells added to the media exhibited the greatest survival over one week and one month had a mean of 88.7% ± 1% SEM and 55.5% ± 3% SEM respectively (fig.3). The control treatment had a mean survival of 83% ± 3% SEM and 48.5% ± 6% SEM at one week and one month respectively (fig.3). Neither the one-week survival, (p = 0.468) or the one month survival (p = 0.852) of the control group was statistically different from the KF1 treatment group.

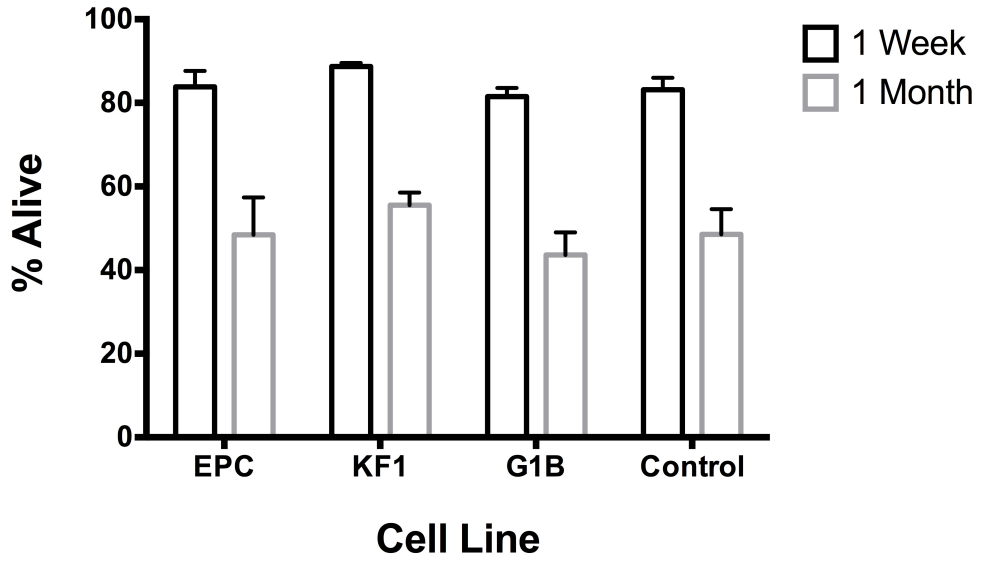

Figure 3. Mean percent (± SEM) of number of live *Lampsilis radiata* juveniles one week and one month post transformation. Glochidia were cultured with different fish cell lines in the culture media. Initial N = 500 for each of 5 replicates. Data were analyzed using the arcsine transformation of proportion of juveniles alive.

Heat Inactivation of Serum

After both one week and one month of being cultured in mucket buckets, *Lampsilis fullerkati* juveniles that were grown in media containing untreated horse serum grew only slightly larger than juveniles that were grown in media containing heat inactivated horse serum (fig. 4). This increase in length was negligible and was not statistically significant at one week ($p = 0.923$) or at one month ($p = 0.315$).

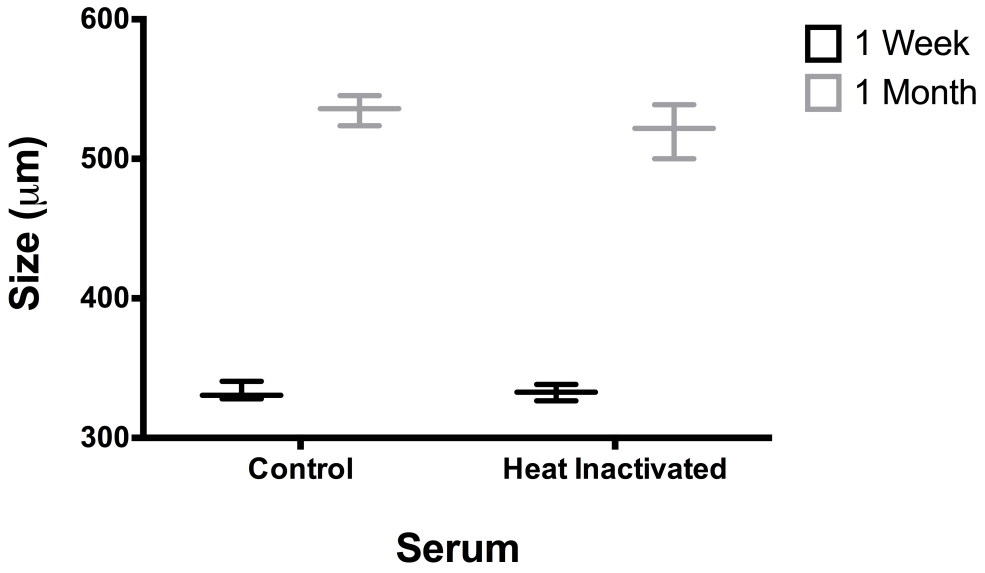

Figure 4. Mean length of *Lampsilis fullerkati* juveniles 10 days and 30 days post transformation. Artificial media contained horse serum that was either heat inactivated or left untreated. N = 30 for each of 3 replicates.

Survival of *Lampsilis fullerkati* juveniles after being cultured in mucket buckets for one week and for one month was the same for both treatment groups (fig. 5). There was no statistically significant different between the treatment groups at one week ($p = 0.263$) or at one month ($p = 0.501$).

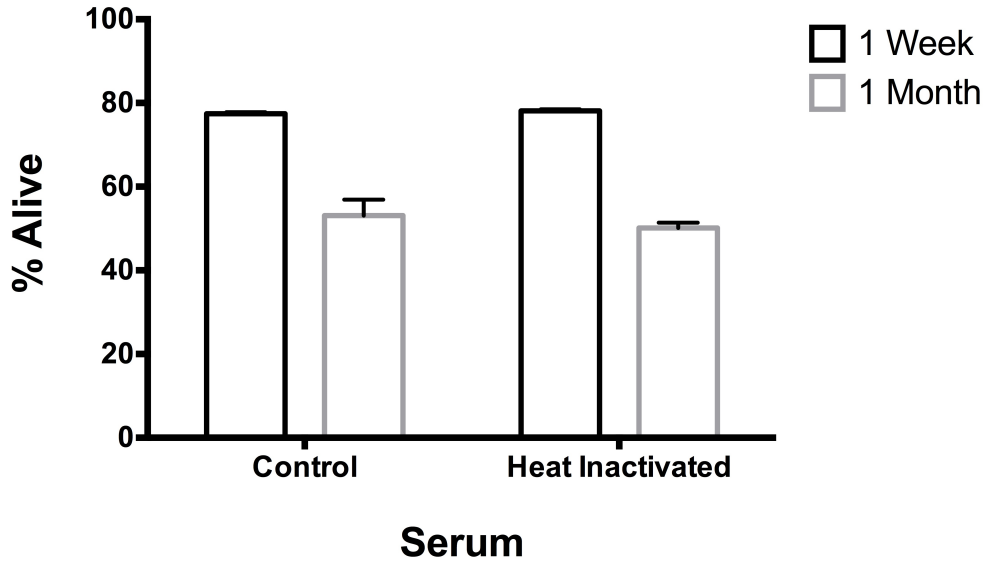

Figure 5. Mean percent (± SEM) of number of live *Lampsilis fullerkati* juveniles one week and one month post transformation. Glochidia were grown in media containing untreated horse serum or heat inactivated horse serum. Data were analyzed using the arcsine transformation of proportion of juveniles alive.

Discussion

Addition of Fish Cells

We hypothesized that the addition of fish cells would provide an additional source of nutrition that would boost the physiological energy reserves immediately following transformation and increase early survival and growth of transformed juveniles. It was visually observed through light microscopy that in each of the 3 treatments, fish cells were added to the media and the glochidia snapped shut, trapping fish cells between the valves. After only a few days, the fish cells were no longer observed existing between the valves of the glochidia. After encystment, the larval mantle cells become constricted basally and

enlarged distally, forming a clump of cells that resemble a mushroom (Arey 1932). Arey (1932b) suggested that this mushroom body was the site of phagocytosis of both the enclosed fish tissue and the larval adductor muscle, and Fukahara et al. (1990) suggested that larval mantle cells contain host tissues within their vacuoles. Fisher and Dimock studied the morphological development of the freshwater mussel *Utterbackia imbecillis* comparing both methods of propagation. By day 4 of development, the heterophagosomes in the larval mantle cells of the fish reared animals were more numerous than those of the *in vitro* reared animals and contained large amounts of ingested material, presumably host fish tissue. At the same time of development there was little evidence of lipids and glycogen in the larval mantel cells of the *in vitro* reared animals whereas the fish reared animals had numerous lipid droplets and glycogen granules in the basal portion of the cells, which indicates that fish reared animals may utilize both the larval adductor and fish tissue as sources of nutrition (Fisher and Dimock 2006). Our observations were consistent with that of Fisher and Dimock (2006) in that we observed the disappearance of fish cells added between the valves of the glochidia within the first few days, however when we cultured the juveniles to test for a change in growth or survival we observed no change between the juveniles that had fish cells added to the media when compared to the control treatment of no fish cells being added.

There are a number of reasons that the addition of fish cells to the media with the glochidia did not result in increased survival and growth. The first explanation for this could be that the gill tissue that the glochidia attach to while going through their transformation does not provide any nutritional value to the juveniles. While it has been observed by Fisher and Dimock (2006) that the tissue trapped between he valves of the glochidia is digested, this

digestion of tissue may occur only out of necessity to create room for the newly developing organs to fit between the valves. Another possibility is that the fish cells trapped between the valves of the glochidia were not in a high enough concentration to provide enough nutritional value to the juveniles to observe a difference in survival and growth. Cells in gill tissue are packed extremely close together and it is difficult to simulate the same density of cells when growing cells in suspension. The concentration of fish cells used in the experiment was very high and the cells were resuspended in the smallest amount of media that allowed for the immersion of glochidia, so using the current techniques it would be difficult to achieve a higher concentration of fish cells. A final explanation for the lack of increased survival and growth is that each mussel may need specific nutrients that are only provided by the cells of the exact host fish that the mussel utilizes for transformation in the wild. It is possible that the three different fish cell lines that were tested did not contain the necessary essential nutrients that its normal host fish provides to the mussel. It has been shown that some species of mussel exhibit a higher transformation percentage when the serum from the host fish is utilized in the growth media (Uthaiwan et al 2002). They showed that the serum in fish blood contains significantly different levels of amino acids, inorganic elements, protein, glucose, and triglycerides among the four different species of fish tested (Uthwaiwan et al 2002). It therefore follows that the gill cells of different fish species can contain distinctive levels of these nutrients, which may be more beneficial to certain species of mussels.

Heat Inactivation of Serum

While the serum in the media contains many beneficial components such as proteins, growth factors, hormones, amino acids, glucose and lipids, it also contains serum

complement (Mills and Taylaur 1971). With certain cell lines, serum complement can lead to complement mediated cell lysis (Noguchi 1906). For this reason many cell culturists will heat inactivate their serum to denature the possibly deleterious complement (Soltis et al. 1979). We conducted an experiment to determine if heat inactivation of the serum used in the media would result in increase survival and growth of the transformed juveniles. The results showed now difference in growth or survival after one week or one month suggesting that complement mediated cell lysis is not an issue in the transformation of glochidia in media. Since heat inactivating the serum provided no observable benefit to the juveniles, it is not included as part of the propagation protocol.

CHAPTER 3: Improving *in vitro* Propagation Success of Juveniles Post Transformation

Introduction

Freshwater mussels are one of the most endangered group of taxa in North America (Williams et al. 1993). Captive propagation of freshwater mussels and their release back into their native habitat is a conservation tool being used to attempt to mitigate the continued decline observed in freshwater mussel species (Neves 2004). Previous studies focused on the captive propagation and rearing of freshwater mussels have routinely documented difficulty in maintaining juveniles beyond four weeks of age. (Lefevre and Curtis 1910, Jones et al. 2005). While a mixture of different algae is fed to freshwater mussels in laboratories, the diet of juvenile mussels is poorly understood (Nichols and Garling 2000, Beck and Neves 2003, Christian et al. 2004). Both bacteria and algae have been found in the gastrointestinal tract of freshwater mussels harvested in the wild (Chittuck et al. 2001, Allen 1914, Gatenby et al. 1997, Christian et al. 2004). However their role in the diet of freshwater mussels and how they contribute to the overall nutrition of juveniles is not well known (Nichols and Garling 2000, Gatenby et al. 1996, 1997, Jones 2005). This limited understanding of unionid diets is likely contributing to the poor success of some species in propagation facilities.

It is possible that freshwater mussels either use bacteria as a food source and derive nutrients from the bacteria, or they may utilize the bacteria as a probiotic to aid in the digestion and assimilation of nutrients from algae and other sources. A probiotic was defined by Gatesoupe (1999) as "microbial cells that are administered in such as way as to enter the

gastrointestinal tract and to keep them alive, with the aim of improving health." The use of probiotics is a common practice in aquaculture and has been shown to be beneficial to marine bivalves (Douillet and Langdon 1993), shrimp (Garriquues and Wyban 1993), crabs (Nogami et al. 1997), and finfish (Olsson et al. 1992). Douillet and Langdon showed that pacific oyster (*Crassotrea gigas*) larvae that were fed the CA2 strain of bacteria had a 22% increase in survival and 16-20% increase in growth when compared to control cultured fed only algae (Douillet and Langdon 1993). With the rapid appearance of antibiotic resistant bacteria and their circulation through the ecosystem, the use of antibiotics to control disease in aquaculture is becoming less popular (Muroga et al 1989). More often bacteria are being used as a means of biocontrol of internal and external microbial environments (Murogoa et al 1989). A marine strain (BS107) of bacteria identified as Roseobacter species enhanced Chilean scallop (*Argopecten purpuratus*) larval survival by protecting it from infection of *Vibrio anguillarum* related larval pathogen (Riquelme et al. 1996).

While the use of probiotics is well documented in the field of marine aquaculture, there have been no studies investigating the use probiotics on freshwater mussel propagation. In one study, Chittick (2001) conducted bacteriological sampling of the gastrointestinal tract of the freshwater mussel *Elliptio complanata* and found 18 aerobic bacterial species, with the most predominant being *Aeromonas hydrophila, Enterobacter spp,* and *Bacillus spp* (Chittick et al. 2001). Resident bacteria in aquatic systems have been implicated as an important ingredient in the juvenile bivalve diet (Urban and Langdon 1984, Crosby et al. 1990, Baldwin and Newell 1991). Some studies have investigated adding a commercial bacteria

suspension for mussels to use as a food source, but showed no benefit (Gatenby, Neves, and Parker 1996).

The *in vitro* propagation of freshwater mussels is an underutilized technique of propagation that has the potential to greatly increase the efficiently of traditional mussel propagation protocols (Owen 2009). However in a previous parallel experiment, comparing the two techniques of propagation, we demonstrated that juveniles propagated *in vitro* have an increased mortality rate and decreased growth in the first 30 days post transformation (Fox et al, in preparation). The juveniles that are propagated *in vitro* are handled using aseptic technique and are transformed in sterile artificial media. They do not have the same exposure to environmental bacteria that juveniles propagated on host fish gain while the fish swims around in the water during the transformation period. It is therefore possible that the juveniles that transform on host fish gain exposure to probiotic strains of bacteria that colonize their gut and contribute to the observed increase in growth during the first days after transformation. Our objective for this study was to determine if the addition of *Bacillus subtilis*, a bacteria species previously found in the gut of freshwater mussels (Chittuck et al. 2001), to the diet of *in vitro* propagated juvenile mussels would increase early survival and growth. *Bacillus subtilis* was added in different concentrations to the normal algae diet and fed to three species of freshwater mussels that were propagated *in vitro*: *Alasmidonta raveneliana*, *Lampsilis fasciola,* and *Villosa delumbis*. Juveniles were cultured in downwelling tanks and had their survival and growth assessed at 10 and 30 days when possible

Methods

Using Bacteria as a Probiotic

To examine the potential benefits of probiotics in the captive rearing of *in vitro* propagated freshwater mussels, a laboratory experiment was designed to determine if freshwater mussels exhibit increased survival and growth with the addition of *Bacillus subtilis.*

Aquaculture System

One of the aquaria used in the following experiments to culture juvenile mussels was a modified downwelling mucket bucket (Barnhart 2006). Two 2.5 gallon buckets were nested into each other creating a small reservoir between the two buckets. A hole was drilled on the bottom of the upper bucket and a PVC chamber fit snuggly inside this hole. A small pump (mini pump model 404) was placed in the reservoir between the two buckets and was connected via flexible plastic tubing to a bulkhead that was inserted into the upper bucket. Water was pumped from the lower bucket reservoir up through the bulkhead to the upper bucket then down through the PVC chamber passing over the mussels and back to the lower reservoir, creating a recirculating downweller system with only one PVC mussel chamber. The PVC chamber was created by wedging a nitex screen with a mesh length of 150 μm between a two inch PVC coupler and two-inch PVC section of pipe. A second coupler with 150 μm nitex screen was placed on top of the first, creating a cylindrical chamber for the mussels that was enclosed on both ends by 150 μm nitex screen to prevent mussel from escaping from the chamber.

The other aquaria used in some of the experiments to grow juvenile mussels consisted of a static two gallon plastic bucket. The bottom of the aquaria was lined 50 mL of fine sediment (<200 μm), collected from New Hope Creek, NC, part of the Neuse River watershed. Sediment was sieved to <200 μm, placed in an autoclave for 2 hours and then a dry heat oven for 24 hours to sterilize and dry the collected sediment and well as to kill any unwanted parasites. The tanks were slowly filled with 6.5 L water, taking care not to disrupt the sediment and cause it to go into suspension in the water column and water was changed one a week.

Prior to the commencement of each experiment the tanks and PVC mussel chambers were sterilized by soaking them in a 5250 ppm sodium hypochlorite solution for 24 hours to kill any resident bacteria. The tanks were rinsed out with treated municipal water three times then allowed to air dry before being used. The tanks were filled with 6.5 liters of municipal water that had been treated with sodium thiosulfate and Ammo Lock (Aquarium Pharmaceuticals, Chalfont, Pennsylvania) to remove chlorine and chloramines. The lights in the building were controlled by a 12-hour on, 12-hour off cycle timer. Unless otherwise noted, water temperature for all tanks was maintained at 23°C. The commercial algae used in these experiments were a mixture of Nanno 3600 (*Nannochloropsis* spp.) and Shellfish diet (*Isochrysis* spp., *Pavlova* spp., *Tetraselmis* spp., and *Thalassiosira weissflogii*) both from Reed Mariculture Inc. (San Jose, CA). Approximately 50,000 cells/mL of algae were fed to the appropriate treatments once per day and a100% water change was performed once a week to maintain optimal water quality.

The bacteria strain used in these experiments was *Bacillus subtilis* (ATCC #6051) purchased from American Type Culture Collection (Manassas, VA). Bacteria were grown in a broth culture at 37 °C using Difco nutrient broth (BD #234000), until it reached a concentration of $5x10^8 – 5x10^9$ CFU/mL. When the desired concentration was reached it was then kept refrigerated at 10°C to slow bacterial growth and maintain a stock solution. Concentration of bacteria was determined by using 3M petrifilm (St. Paul, MN). Serial dilutions of the bacteria culture were created and plated onto the petrifilm where it was incubated at 37 °C for 24 hours. The number of colonies on the petrifilm was counted after the incubation period, which allowed the concentration of the bacteria in the broth to be calculated. Bacteria at the same approximate concentration were added once per day to each tank in the appropriate treatment.

Experiment 1

Study Animals

Three gravid *Alasmidonta raveneliana* were collected from the Tuckaseegee River, NC, part of the Little Tennessee River basin in late March 2012. The three individuals were held at the Aquatic Epidemiology and Conservation Laboratory (AECL) at the North Carolina State University College of Veterinary Medicine (NCSU CVM) in conditioned water cooled to 14 °C. Five days after the mussels were collected, the glochidia from one gill were extracted from each of the individual mussels. The glochidia were processed using the standard *in vitro* propagation protocol and placed in the standard *in vitro* propagation media. The glochidia were transformed in the incubator at 19 °C with a 3.5% CO_2 atmosphere.

Study Design

Experiment 1 commenced the last week of April 2012. *Alasmidonta raveneliana* was used as the experimental animal. Four treatments were set up with four replicates per treatment. The treatments were as follows:

1. Commercial algae only, no bacteria
2. Commercial algae and 10^5 CFU/mL *Bacillus subtilis*
3. Commercial algae and 10^4 CFU/mL *Bacillus subtilis*
4. 10^5 CFU/mL *Bacillus subtilis* only, no commercial algae

After 24 days in the media, the glochidia were transformed into juveniles and acclimated to fresh water. When the dilution time was completed the juveniles were split into equal treatment groups of 200 individuals and placed into their culture tanks. Both culture systems noted above were utilized during this experiment, a sediment free downwelling mini mucket bucket system that was previously described, and the static two gallon plastic buckets with fine sediment on the bottom. Each tank was fed their algae and/or bacteria treatment once per day. *Bacillus subtilis* was grown to a concentration of 5.43 X 10^8 CFU/mL in Difco nutrient broth (BD #234000), and dispensed into the appropriate tanks in volumes of 0.12 mL and 1.2 mL to achieve the desired concentration. Measurements of 30 randomly selected juveniles were taken on day 1, 10, and 30 of the experiment using a calibrated ocular micrometer. Survival was measured on day 10 and day 30 by sieving the sediment through a 200 μm sieve, and spraying the juveniles into a Petri dish to be examined under a dissecting microscope and counting to the total number of live juveniles from each replicate.

Water quality parameters (pH, total ammonia, nitrates, and hardness) were taken at the time of each assessment and water temperature was recorded daily. During each length and survival assessment a water sample from each tank was taken and applied to a 3m aerobic bacteria plate counter to determine the concentration of bacteria in each tank.

Experiment 2

Study Animals

Villosa delumbis were collected from the Little River, NC, part of the Yadkin-Pee Dee river basin. Gravid individuals were transported to the North Carolina Wildlife Resources Commission Conservation (NCWRC) Conservation Aquaculture Center (CAC) in Marion, NC until 5/3/12. Two individuals had their glochidia extracted and were processed using the *in vitro* propagation protocol and placed in the standard *in vitro* propagation media. The glochidia were transformed in the incubator at 22.5 °C with a 3.5% CO_2 atmosphere.

Study Design

A second experiment with *Villosa delumbis* was conducted in May 2012. The same four treatments as experiment 1 were set up with four replicates per treatment. The treatments were as follow:

1. Commercial algae only, no bacteria
2. Commercial algae and 10^5 CFU/mL *Bacillus subtilis*
3. Commercial algae and 10^4 CFU/mL *Bacillus subtilis*
4. 10^5 CFU/mL *Bacillus subtilis* only, no commercial algae.

After 20 days in the media, the glochidia were transformed into juveniles and acclimated to fresh water. After the dilution time was completed the juveniles were split into

equal treatment groups of 200 individuals and placed into their culture tanks. A two-gallon mini mucket bucket was used to culture the juveniles with 150 μm mesh length. The mini mucket bucket systems were held at room temperature and water temperature averaged 24 °C. Each tank was fed their algae and/or bacteria treatment once per day. *Bacillus subtilis* was grown to a concentration of 2.55 X 10^8 CFU/mL in a Difco nutrient broth and dispensed into the appropriate tanks in volumes of 0.12 mL and 1.2 mL to achieve the desired concentration. Measurements of 30 randomly selected juveniles from each replicate were taken on day 1 and 10 of the experiment using a calibrated ocular micrometer. Survival was measured on day 10 by placing the juveniles into a Petri dish to be examined under a dissecting microscope and counting the total number of live juveniles from each replicate. On day 20 of the experiment the pH of all tanks dropped to 3.4 - 4.3, which killed all the juveniles and did not allow for a 30-day measurement.

Experiment 3

Study Animals

One *Lampsilis fasciola* was collected from the Little Tennessee River, part of the Little Tennessee River basin, NC and held at the NCWRC CAC in Marion, NC. On 5/22/12 glochidia from both gills were extracted from the mussel and were processed using the *in vitro* propagation protocol and placed in the standard *in vitro* propagation media. The glochidia were transformed in the incubator at 22.5°C with a 3.5% CO_2 atmosphere.

Study Design

A third experiment was conducted with *Lampsilis fasciola* in June 2012. The same four treatments as experiment 1 and 2 were used with four replicates per treatment. The treatments were as follows:

1. Commercial algae only, no bacteria
2. Commercial algae and 10^5 CFU/mL *Bacillus subtilis*
3. Commercial algae and 10^4 CFU/mL *Bacillus subtilis*
4. 10^5 CFU/mL *Bacillus subtilis* only, no commercial algae.

After 37 days in the media, the glochidia were transformed into juveniles and acclimated to fresh water. After the dilution time was completed the juveniles were split into equal treatment groups of 200 individuals and placed into the mini mucket bucket system without sediment that was previously described. The mini mucket bucket systems were held at room temperature and water temperature averaged 24°C. Each tank was fed their algae and/or bacteria treatment once per day. *Bacillus subtilis* was grown to a concentration of $4.55 \text{ X } 10^8$ CFU/mL in Difco nutrient broth and dispensed into the appropriate tanks in volumes of 0.12 mL and 1.2 mL to achieve the desired concentration. Measurements of 30 randomly selected juveniles from each replicate were taken on day 1 and 10 of the experiment using a calibrated ocular micrometer. Survival was measured on day 10 by placing the juveniles into a Petri dish to be examined under a dissecting microscope and counting the total number of live juveniles from each replicate. On day 25 of the experiment the pH of all tanks dropped to 3.6 – 5.3, which killed all the juveniles and did not allow for a 30-day measurement.

Experiment 4:

In an effort to improve the early survival of *in vitro* propagated freshwater mussels, an experiment was conducted to test different methods of acclimating the newly transformed juveniles from the media to freshwater.

Study Animals

In April 2010, four *Lampsilis fasciola* were collected from the Pigeon River, NC part of the French Broad River basin and brought back to the AECL in Raleigh, NC. Ten days later, glochidia from one gill of each of the mussels were extracted and processed using the *in vitro* propagation protocol and placed in the standard *in vitro* propagation media. The glochidia were transformed in the incubator at 22.5 °C with a 3.5% CO_2 atmosphere. After 38 days in the media the glochidia were fully transformed into juveniles. Newly transformed juveniles were separated into equal treatment groups of 500 individuals. Three dilution methods were used with three replicates per treatment. They were as follows:

1. No Dilution – juveniles were placed directly from the media into freshwater
2. Fast Dilution – media was diluted with freshwater by 50% every hour for 6 hours
3. Slow Dilution – media was diluted with freshwater by 10% every hour for 10 hours

Conditioned municipal water being held at 23 °C that is used for culturing freshwater mussels in the AECL was used to dilute the culture media. When the dilution time for each treatment was completed the juveniles were transferred into a mucket bucket downwelling system for 24 hours. After being in 100% freshwater for 24 hour the juveniles were placed into a petri dish and examined under a dissecting microscope to count the total number of live juveniles from each replicate.

Statistical Analysis

An analysis of variance, ANOVA, was performed to assess the difference in survival and growth of juveniles that had different concentrations of bacteria added to the diet and also to compare the different methods of dilutions performed. Tukey's Honestly Significant Difference (HSD) was performed to identify differences between treatments. A P value of <0.05 was considered significant.

Results

Using Bacteria as a Probiotic

When *Alasmidonta raveneliana* juveniles were cultured in fine sediment, the juveniles that were fed a high concentration (10^5 CFU/mL) of *Bacillus subtilis* and algae grew the largest after 10 days (fig. 1). The juveniles fed a high concentration of *Bacillus subtilis* grew to a mean of 366.4 μm ± 2.23 SEM, which was statistically significantly larger than the length of juveniles fed algae only ($p = 0.0375$). They were also larger than juveniles fed a low concentration (10^4 CFU/mL) of *Bacillus subtilis* ($p = 0.0408$), which grew to 349.7 μm ± 0.8 SEM, and, 350.0 μm =/- 2.12 SEM respectively (fig. 1). Juveniles fed *Bacillus subtilis* with no algae showed no difference in growth when compared to the other three treatments.

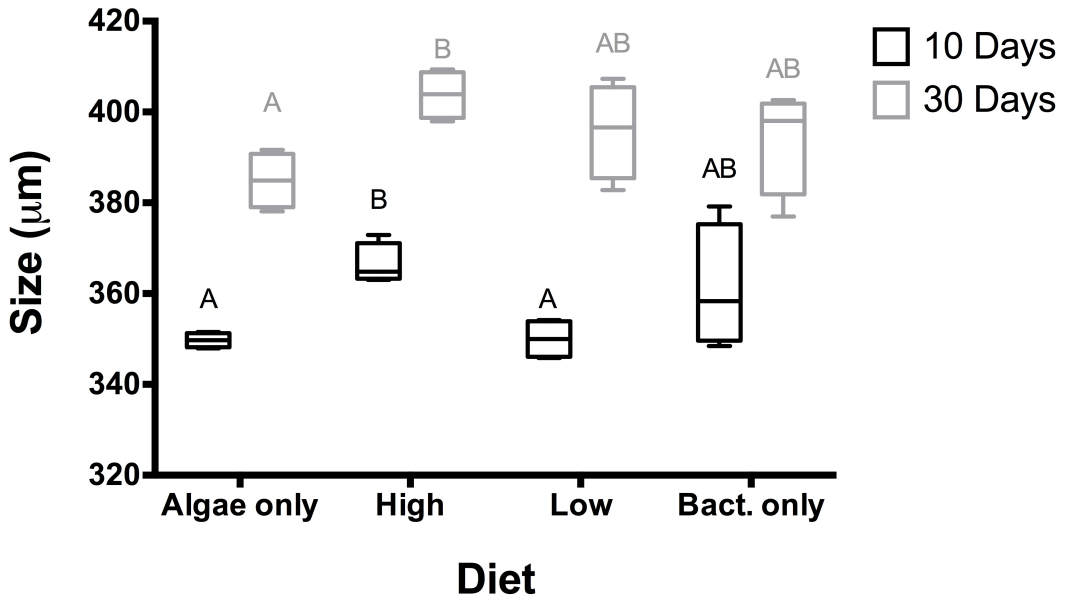

Figure 1. Mean length of *Alasmidonta raveneliana* juveniles 10 days and 30 days post transformation cultured in fine sediment. Juveniles were fed different algae and bacteria concentrations. N = 30 for each of 4 replicates. Letters that are different are statistically significant (Tukey's HSD $p<0.05$)

After being cultured in fine sediment for 30 days, the *Alasmidonta raveneliana* juveniles showed a similar trend in growth as they showed after 10 days. The juveniles that were fed a high concentration of *Bacillus subtilis* grew the largest with a mean length of 403.8 μm ± 2.61 SEM. They were also significantly larger ($p = 0.0436$) than juveniles fed algae only, which grew to a mean length of 384.9 μm ± 3.04 SEM (fig. 1). Juveniles fed a low concentration of *Bacillus subtilis* and juveniles fed only *Bacillus subtilis* with no algae showed no difference in growth when compared to the other two treatments (fig. 1).

The survival of juvenile *Alasmidonta raveneliana* being cultured in fine sediment for 10 days was very high across all treatments. There was no statistically significant difference in the percentage of juveniles alive after 10 days between the four treatment groups (fig. 2).

The proportion that survived 10 days ranged from a mean of 92.8% ± 1.2% SEM to 96.5% ± 1.5% SEM. The proportion of juveniles that survived 30 days in fine sediment was lower but not statistically significant and ranged from a mean of 40.3% ± 11.6% SEM to 59.6% ± 9.5% SEM. There was no statistically significant difference in the mean percent survival of the high *Bacillus subtilis* treatment and the algae only treatment returned at 30 days (P=0.7853), which was probably due to the large standard error observed among treatments (fig. 2).

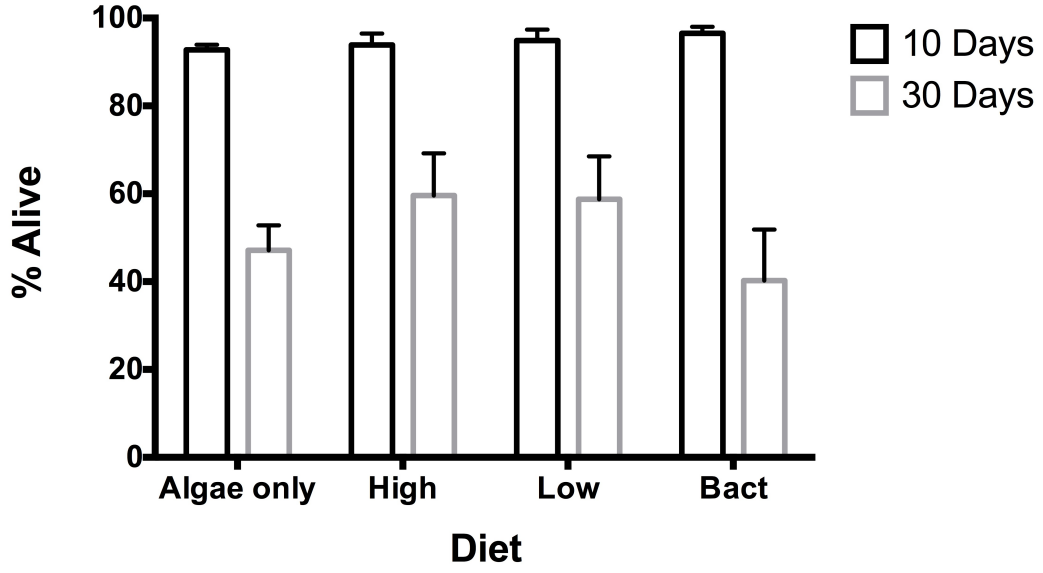

Figure 2. Mean percent (± SEM) of number of live *Alasmidonta raveneliana* juveniles 10 days and 30 days post transformation cultured on fine sediment. Juveniles were fed different algae and bacteria concentrations. Initial N = 200 for each of 4 replicates. Data were analyzed using the arcsine transformation of proportion of juveniles alive.

Slightly different results occurred when *Alasmidonta raveneliana* juveniles were cultured in a downweller instead of fine sediment. Juveniles that were fed a diet of only *Bacillus subtilis* grew the largest with a mean length of 416 μm ± 6.72 SEM, however there

was no significant difference among the four treatment groups (fig. 3). Juvenile mean length after 10 days ranged from 390 μm – 416 μm. Similarly there was no significant difference in *Alasmidonta raveneliana* juvenile survival among treatment groups after being cultured in downwellers for 10 days (fig 4.). Mean survival ranged from 73% - 96% with a large standard error within treatment groups being the reason for no significant difference (fig. 4).

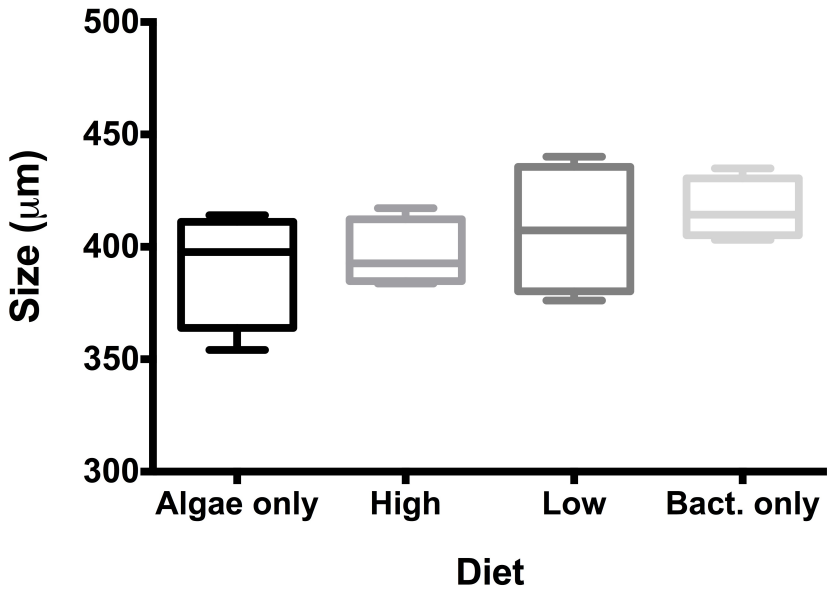

Figure 3. Mean length of *Alasmidonta raveneliana* juveniles 10 days post transformation cultured in downwellers. Juveniles were fed different algae and bacteria concentrations. N = 30 for each of 4 replicates.

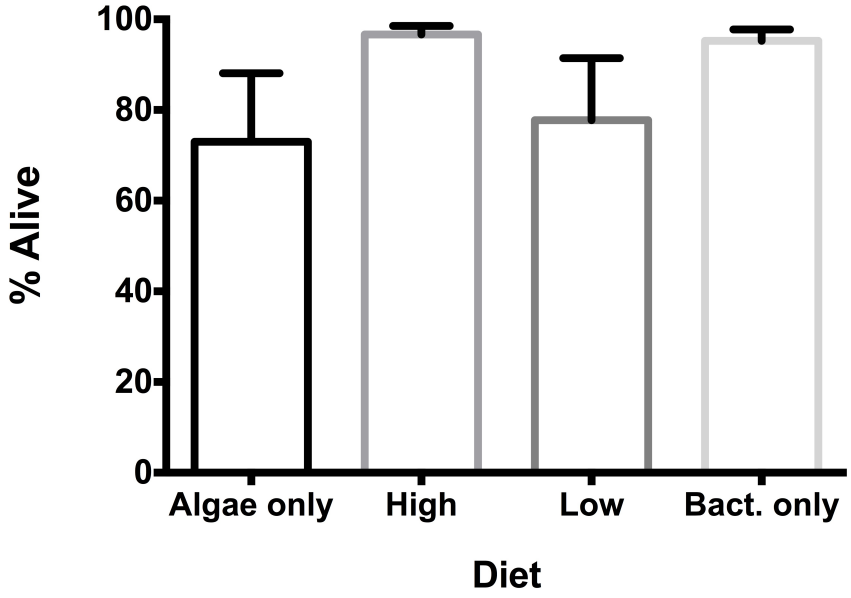

Figure 4. Mean percent (± SEM) of number of live *Alasmidonta raveneliana* juveniles 10 days post transformation cultured in downwellers. Juveniles were fed different algae and bacteria concentrations. Initial N = 200 for each of 4 replicates. Data were analyzed using the arcsine transformation of proportion of juveniles alive.

Markedly different results were obtained with *Lampsilis* than with *Alasmidonta raveneliana*. Juveniles that were fed a low concentration of *Bacillus subtilis* with algae grew the largest over 10 days with a mean length of 353 μm ± 8.52 SEM (fig. 5.) Although it grew the largest, the low bacteria concentration group was not significantly different from the algae only and high bacteria treatment group. The juveniles that were fed a diet of *Bacillus subtilis* only were the smallest with a mean length of 282.3 μm ± 4.54 SEM, and showed very little growth at all (fig. 5). Juveniles from bacteria only treatment group were significantly smaller than the algae only, high, and low bacteria concentration with p values of 0.0015, 0.0021, and <0.0001 respectively (fig. 5). Juvenile *Lampsilis fasciola* cultured in downwellers had high survival across all treatment groups and there was no significant

difference between any treatment groups (fig. 6). Survival after 10 days ranged from 89.5% - 91.63% (fig. 6).

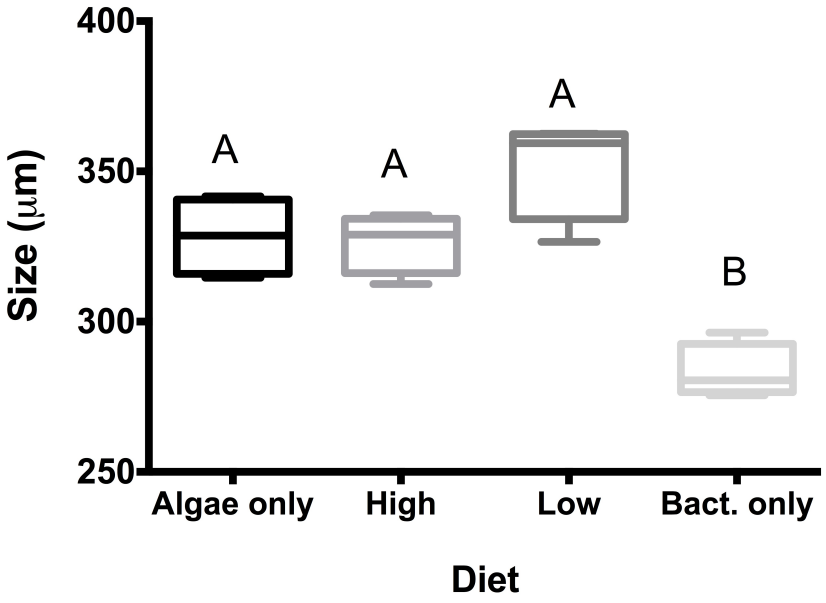

Figure 5. Mean length of *Lampsilis fasciola* juveniles 10 days post transformation cultured in downwellers. Juveniles were fed different algae and bacteria concentrations. N = 30 for each of 4 replicates. Letters that are different are statistically significant (Tukey's HSD $p<0.05$)

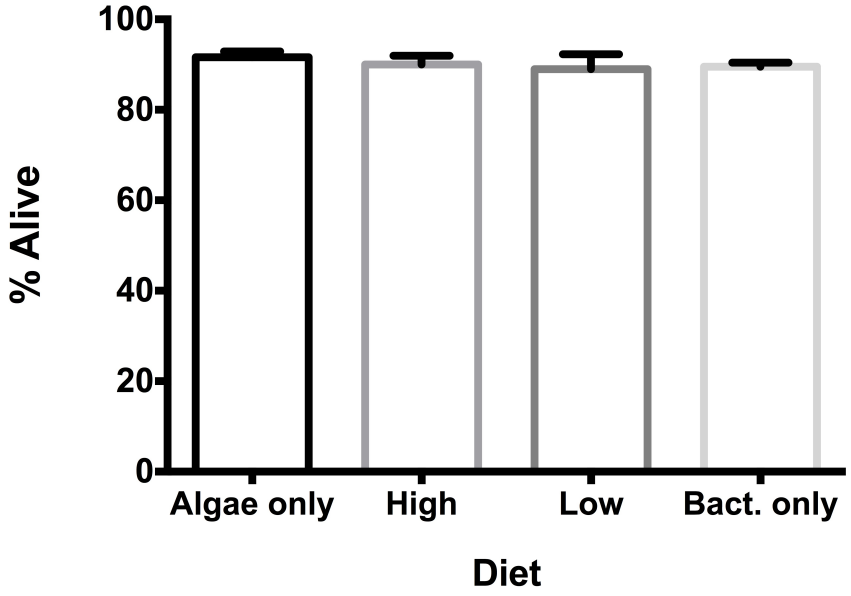

Figure 6. Mean percent (± SEM) of number of live *Lampsilis fasciola* juveniles 10 days post transformation cultured in downwellers. Juveniles were fed different algae and bacteria concentrations. Initial N = 200 for each of 4 replicates. Data were analyzed using the arcsine transformation of proportion of juveniles alive.

The growth of Villosa *delumbis* was similar to that observed with *Lampsilis fasciola.* The mean length ranged from 351.3 μm ± 8.5 SEM to 374.7 μm ± 4.7 SEM between the treatments that were fed some algae in the diet. The juveniles fed a diet of algae only grew the largest, however with a P value of 0.562 and 0.3367, this length was not significantly different than the length of juveniles fed a high or low concentration of *Bacillus subtilis* with algae (fig. 7). Juveniles that were fed only *Bacillus subtilis* exhibited very little visible growth and had a mean length of 295.1 μm ± 5.51 SEM. The length of the bacteria only treatment was significantly smaller than that of the algae only, high, and low bacteria concentration with p values of 0.0003, 0.0027, and 0.0056 respectively.

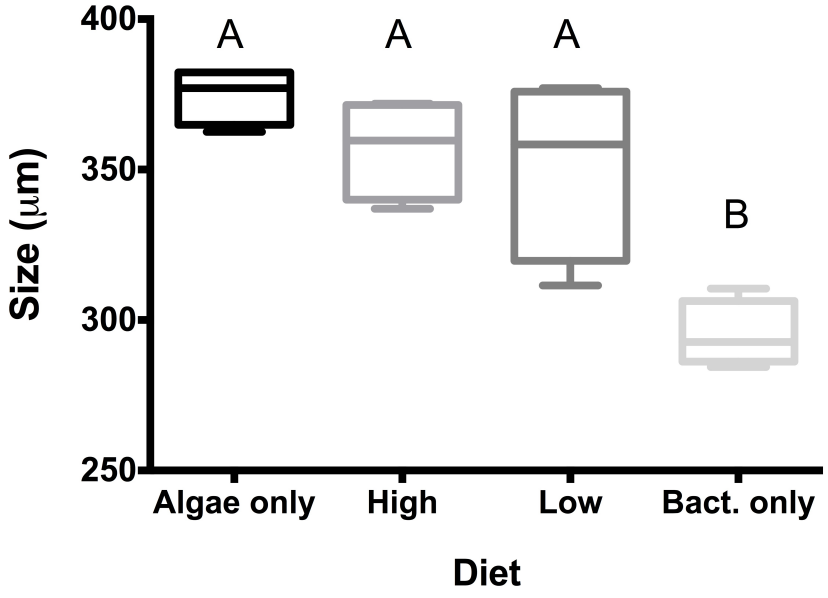

Figure 7. Mean length of *Villosa delumbis* juveniles 10 days post transformation cultured in downwellers. Juveniles were fed different algae and bacteria concentrations. N = 30 for each of 4 replicates. Letters that are different are statistically significant (Tukey's HSD $p<0.05$)

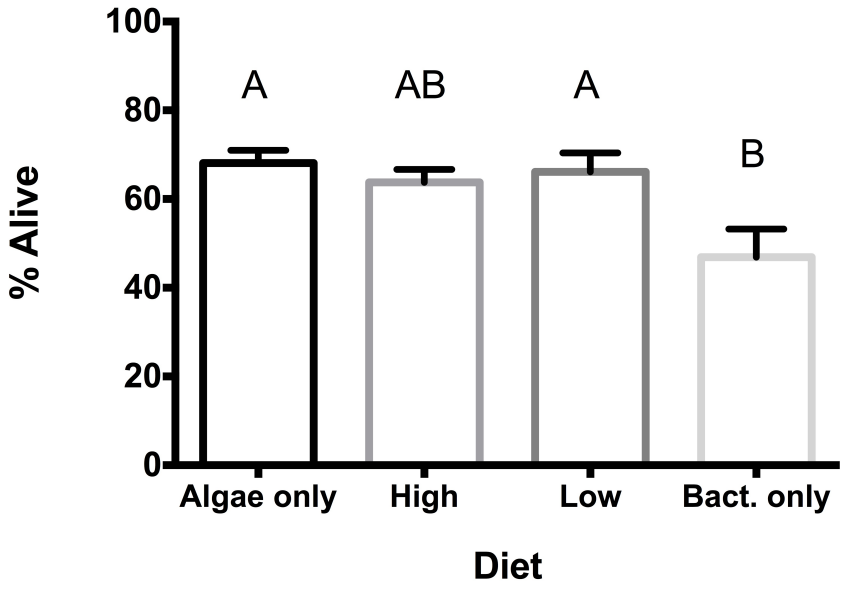

Figure 8. Mean percent (± SEM) of number of live *Villosa delumbis* juveniles 10 days post transformation cultured in downwellers. Juveniles were fed different algae and bacteria concentrations. Initial N = 200 for each of 4 replicates. Data were analyzed using the arcsine transformation of proportion of juveniles alive. Letters that are different are statistically significant (Tukey's HSD $p<0.05$)

The survival of *Villosa delumbis* in a downweller after 10 days reflected the results that were seen in the growth of *Villosa delumbis* after 10 days. The juveniles that were fed algae only exhibited the highest survival of 68% ± 2.86 SEM (fig 8). Juveniles that were fed *Bacillus subtilis* only exhibited the lowest survival with only 46.8% ± 6.35 SEM surviving after 10 days (fig. 8). The survival of the juveniles fed bacteria only was significantly less than those fed algae only, and a low concentration of bacteria with p values of 0.021 and 0.0372 respectively. With a p value of 0.0725 the treatment fed a high concentration of bacteria was larger than the bacteria only treatment but it was not statistically significant (fig. 8).

Dilution of Media

When being transitioned from the artificial media to freshwater, *Lampsilis fasciola* juveniles reacted differently to each acclimation schedule tested. Placing the juveniles directly into freshwater from the media yielded the worst results with only 33.5% ± 4% SEM of the juveniles surviving in the freshwater after 24 hours (fig. 9). The fast method of diluting the media by 50% every hour with freshwater produced a significantly higher percentage ($p = 0.0071$) of juveniles alive after 24 hours (fig 9). The fast dilution method produced a mean of 55.2% ± 3% SEM juveniles alive after 24 hours in freshwater, which was significantly less ($p = 0.0004$) than the slow method of diluting the media by 10% with freshwater every hour. The greatest results were observed when the slow method of dilution was used, which produced a 24 hour survival of 93.5% ± 2% SEM (fig 9).

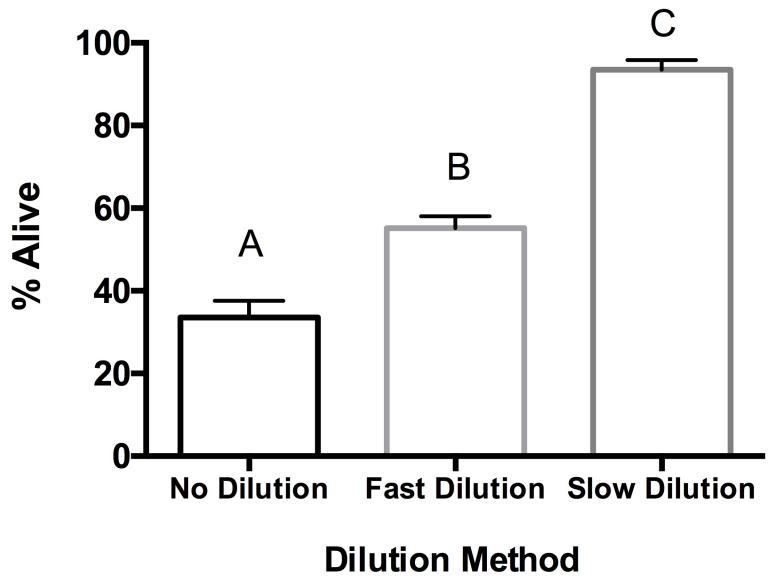

Figure 9. Mean percent (± SEM) of live *Lampsilis fasciola* juveniles 24 hours post transformation. Different methods of transitioning juveniles from artificial media to the freshwater were tested. Initial N = 500 for each of 3 replicates. Data were analyzed using the arcsine transformation of proportion of juveniles alive. Letters that are different are statistically significant (Tukey's HSD $p<0.05$)

Discussion

Using Bacteria as a Probiotic

The Appalachian Elktoe (*Alasmidonta raveneliana*), part of the Anodontini tribe, is a federally endangered freshwater mussel that is located in the western parts of North Carolina and eastern Tennessee. Propagation and culture of this species for population augmentation and reintroductions is a critical part of its recovery plan (Fridell 1996). As shown in experiment 1, juvenile *Alasmidonta raveneliana* exhibited the greatest growth when they were fed a high concentration of *Bacillus subtilis* and algae. These juveniles were significantly larger at both 10 and 30 days than juveniles fed only algae and no bacteria. This suggests that the bacteria are playing a role in juveniles nutrition that supports their growth.

The significant increase in growth was only observed when the juveniles were cultured in fine sediment. In experiment 2 the juveniles were cultured in a downwelling mucket bucket without any sediment. During this experiment there was no difference in growth observed between the treatment groups. If the mussels use *Bacillus subtilis* as a probiotic to help digest the algae, one would expect to see the greatest growth in the treatments with algae and bacteria and the least amount of growth in the treatment fed only bacteria. We observed this result when the juveniles were cultured on fine sediment, however we observed no difference in growth across treatments when cultured in a mucket bucket with no sediment. In fact, while not significantly different, the treatment that was fed only *Bacillus subtilis* and no algae yielded the largest mean length among the treatments in the mucket bucket. Since there was no other source of food available to the juveniles this suggests that the *Bacillus subtilis* are being utilized as a source of nutrition and support early survival and growth just as much as an algal diet.

Experiment 3 and 4 used *Lampsilis fasciola* and *Villosa delumbis* as the test subject. Both of these species are from the tribe lampsilini and exhibited very similar results in survival and growth. In both of these experiments there was no significant difference in the length of the juveniles between the three treatments that were fed algae as part of their diet. The addition of *Bacillus subtilis* to the algae in either a high or low concentration did not effect the growth or survival of the juvenile *Lampsilis fasciola or Villosa delumbis*. However, unlike the *Alasmidonta raveneliana* both of these species displayed no visible growth and were significantly smaller in the treatment that was fed bacteria only and no algae. Additionally, the *Villosa delumbis* that were fed only bacteria exhibited a significant

decrease in survival over 10 days when compared to the treatment that was fed only algae. This leads us to believe that for *Villosa delumbis*, Bacillus *subtilis* neither acts as a probiotic to help in digestion nor acts as a source of nutrition capable of producing the same survival and growth of an algal diet.

Different species of freshwater mussels are found in a wide variety of habitats, ranging from a lotic environment with gravel and cobble to a lentic environment with sand and silt (Williams et al. 1993). It therefore makes sense that difference species of mussels can have different dietary requirements for optimal survival and growth (Nichols and Garling 2000). Carbon stable-isotope ratios show that mussels feed heavily on bacteria and phytoplankton in small temperate streams (Nichols and Garling 2000, Raikow and Himilton 2001, Christian et al. 2004), whereas they feed almost exclusively on phytoplankton in large productive rivers such as the Mississippi (Thorp et al. 1998). Our study suggests that *Alasmidonta raveneliana*, part of the tribe anodontini, utilizes *Bacillus subtilis* differently than *Lampsilis fasciola* and *Villosa delumbis,* which are both part of the tribe lampsilini. It appears as if *Alasmidonta raveneliana* use *Bacillus subtilis* and potentially other bacteria as a food source and can derive nutritional value to sustain their survival and growth. In contrast, *Lampsilis fasciola* and *Villosa delumbis* do not receive the same benefit from the bacteria and exhibited no growth after 10 days when fed only bacteria. Our results are consistent with what Nichols and Garling (2000) observed when their stable isotope data showed that *Pyganadon grandis* have different feeding habits from the other species they tested. *Pyganadon grandis* was the only species of mussel they tested that belongs to the same anodontini tribe of mussels as *Alasmidonta raveneliana.* The $\delta^{13}C$ ratio in *Pyganadon*

grandis was the most depleted and the $\delta^{15}N$ ratio was the highest, both of which indicate that the assimilation of bacterially derived compounds into the soft tissue (Nichols and Garling 2000). Algae has been found to be able to pass through the digestive tract of freshwater mussels and still be intact (Christian et al. 2004) so it is possible that species in the tribe anodontini assimilate nutrients from bacteria and algae differently than other species.

Slightly different results were observed when *Alasmidonta raveneliana* juveniles were cultured in fine sediment rather than on a screen in a mucket bucket. One explanation for the difference is that the fine sediment may act as a substrate with a large surface area for the bacteria to attach to and proliferate (Gatenby et al. 1996). Since juveniles pedal feed (use their foot to feed) immediately following transformation it may be easier for the juveniles to assimilate the bacteria that has settled into the fine sediment than try to pull it out of the water column when they are cultured on mesh screens (Yeager et al. 1994). Some studies have demonstrated that other juveniles prefer to be cultured in fine sediment where they exhibit increased survival and growth (Gatenby et al. 1996, 1997). It is postulated that the juveniles may ingest some of the particles of the fine sediment and may draw some nutritional value from the sediment in which they are cultured (Gatenby et al. 1996, 1997). Perhaps it is the bacteria adhered to the sediment that has nutritional value for juvenile mussels.

Dilution of Media

There was a statistically significant difference in survival after 24 hours observed when comparing different methods used to move the transformed juveniles from the media to freshwater. The slowest dilutions of the media with freshwater gave the greatest survival

while direct transfer to freshwater gave the poorest survival. The basal media that the juveniles transform in is M199, which contains the inorganic salts: $CaCl_2$, $Fe(NO_3)_3$, $MgSO_4$, KCl, KH_2PO_4, $NaHCO_3$,, NaCl, Na_2HPO_4 (Sigma-Aldrich Formulation) The resulting osmolarity of the solution is 270-310 mOsm/kg H_2O (Sigma-Aldrich Formulation). Once transformation is complete the juveniles need to make the transition from the saline environment of the media to the hypoosmotic environment of freshwater. When the juveniles are placed directly into the freshwater they experience an osmotic shock causing an influx of water to cross the cell membrane and enter the cytoplasm of the cells (Kilburn 1976). At such a young age the juveniles are extremely susceptible to environmental stressors and rely upon the energy reserves they gained during transformation to get them through stressful events like osmotic shock. However juveniles that have been transformed *in vitro* have lower reserves of lipids, carbohydrates, and glycogen immediately following transformation and are therefore less tolerant to stress (Fisher and Dimock 2006). The juveniles that were placed directly into the freshwater have a difficult time regulating the large influx of water into the cells and over 50% of them do not survive after 24 hours. By adding a small amount of water over time and slowly diluting the media, it is slowly lowing the osmolarity of the media that the juveniles are in and slowing the influx of water into the cells to a rate that the juveniles can handle and regulate. When the saltwater blue mussel (*Mytilis edulis*) was transferred from 600 mOsm to 300 mOsm its weight increased by 10% until was able to regulate the influx of water and return to its original weight after 12 hours (Deaton 2008). We observed that by slowly diluting the media by 10% every hour for 10 hours, 93% of the juveniles survived this stressful event after 24 hours.

Previous studies (Fox et al. in preparation, Fisher and Dimock 2006, Clem 1998) have shown that juveniles propagated *in vitro* are more sensitive and prone to early mortality that juveniles propagated on host fish. Improving the early survival and growth of *in vitro* propagated juveniles would make this method of propagation more appealing to researchers who want a cost effective method to produce large number of juveniles. It appears that improving the handling and feeding of the juveniles after they have transformed are two ways to enhance the early critical period. We have shown that adding *Bacillus subtilis* to diet of some species of mussels will increase their early growth and that slowly transitioning juveniles from the artificial media to freshwater greatly increases their survival. Continued research on other strains of bacteria and how the mussels assimilate the nutrients could improve results of future mussel propagation efforts.

CONCLUSIONS

Juvenile freshwater mussels, *Lampsilis fasciola*, that were propagated *in vitro* demonstrated a significantly greater transformation percentage from glochidia to the juvenile stage when compared to juveniles that were transformed on their host fish. However, the juveniles that were propagated *in vitro* exhibited less growth and increased mortality though the duration of the experiment. The days immediately following transformation were responsible for a large portion of the overall mortality and we identified three critical time periods that warranted further investigation to improve survival and growth. After 30 days, mortality in the *in vitro* propagated juveniles decreased by 50%, and was the same as the host fish propagated juveniles for the duration of the experiment to its conclusion at 70 days.

We attempted to improve the condition of *in vitro* propagated juveniles by manipulating variables in the growth media and adding an extra source of nutrition. The addition of fish cells to the artificial media of *in vitro* propagated juveniles did not increase their survival and growth post transformation. While the fish cells were observed to be trapped between the valves and digested by the glochidia, it did not increase their early survival and growth, and we saw no difference between any of the treatment groups. It is possible that cells were not provided in an adequate volume, that glochidia need host specific cells, or that the cells do not provide a significant amount of nutrition to the developing glochidia. Heat inactivating the serum used in the growth media exhibited no difference in growth or survival of the developed juveniles. There was no observed benefit from added

fish cells or removing the complement from the serum so we would not suggest adding these measures to the *in vitro* propagation protocol.

To improve their overall condition we tried to manipulate environmental variables of the *in vitro* propagated juveniles immediately following transformation. Since the *in vitro* propagated juveniles are held in a sterile environment they are not exposed to potentially beneficial environmental bacteria that juveniles that are propagated on host fish are. We added *Bacillus subtilis* to the diet of newly propagated juveniles and observed an increase in growth of anodontine juveniles (*Alasmidonta raveneliana*) however we did not observe a difference in growth among lampsiline juveniles (*Lampsilis fasciola* and *Villosa delumbis).* Since *Alasmidonta raveneliana* displayed survival and growth comparable to that of the algae diet when fed only *Bacillus subtilis* it appears as if *Alasmidonta raveneliana* utilizes the bacteria as a food source and not a probiotic. By slowly transitioning the juveniles from the growth media to the freshwater we increased the proportion that survived during the first 24 hour from under 40% to over 90% survival. Juveniles at this life stage are extremely sensitive to any environmental stressors and the slow acclimation most likely stress of osmotic shock and allowed a large percentage of the juveniles to survive the first 24 hours post transformation.

Future Research

Additional work is necessary to continue to improve the health of *in vitro* propagated juveniles. A long term study that tracks survival and growth of *in vitro* propagated animals over several years until the mussels reach adulthood should be conducted in a hatchery setting where the mussels receive a natural supply of food. The addition of fish cells may be

beneficial to some species of mussel that are more difficult to propagate *in vitro*. There has been documented troubles (Owen 2009) with getting juveniles that grow during the encystment period to transform *in vitro*. Fish cells may provide the additional nutrients that are needed to grow during their transformation period. To test this, other lines of fish cells should be tested, as well as excising the glochidia from host fish gills the placing them in the growth media to see if the actual gill tissue makes a difference.

The use of bacteria in the diet of *in vitro* propagated anodontine juveniles showed the most promise to improve their early survival and growth. More research needs to be conducted on whether the bacteria are acting as a probiotic aiding digestion or if the bacteria are acting as a source of nutrition for the juveniles. Since the presence of bacteria or algae in the gut does not necessarily mean they are digesting this material, a stable isotope analysis can help to determine if the mussels are assimilating the bacteria into the tissue of different species of mussels from different tribes. Additional studies with other species are needed to assess the contribution of bacteria to their survival and growth. Different strains of bacteria can give adverse, neutral, and beneficial effects so no generalization about the beneficial effects of specific bacterial strains can be made, each strain must be tested again with each new target (Doullet and Langdon 1994). It is also possible that a combination of different species of algae would provide a wider array of nutrition to the juveniles instead of a single strain added to the diet. Studies focused on the actual nutritional benefits of bacterial feeding, and of alternative species of bacteria would further our understanding of the dietary needs of *in vitro* propagated juveniles in captivity.

If *in vitro* propagated juveniles are to be used for propagation reintroduction and augmentation purposes we must understand how these animals would behave in the wild. Placing mussels in a cage in the river is a good way to determine if they maintain the same survival and growth as wild mussels. Long-term observation of the mussels is also important to determine if they become gravid, have the same sex ratio, and if they produce functional lures. A host trial must be conducted to determine if the *in vitro* transformed mussels retain the same host range and/or specificity as the mussels that are propagated on host fish. *In vitro* propagated mussels that have become gravid must also be tested to see if the proportion of their glochidia that transform on host fish is similar or different than that observed when they feed on host fish. Additional studies are needed to assess if the juveniles produced grow from these 2nd and 3rd generation animals display the same fecundity, survival and growth as juveniles that are progeny of natural propagation. Determining how these mussels and their offspring would behave in the wild is a critical question that must be answered before *in vitro* propagation methods are used for population augmentation.

Application of Research

Freshwater mussel propagation facilities across the country may be interested in using *in vitro* propagation techniques as another method to produce large quantities of juveniles. *In vitro* propagation has many advantages over the traditional host fish method including: elimination of host fish, cost and time efficiency, reduced space requirements, and easy manipulation of environmental and culture variables. This method of propagation could appeal to researchers that only have a small lab and do not have the space or specialized equipment to hold and collect host fish species. Since the juveniles transform in a Petri dish

and not on the gills of fish, this method may also appeal to researchers who are interested in studying morphological of glochidia as they transform into juveniles.

In vitro propagation has the potential to produce many more juveniles from a single brood than host fish methods. It could greatly benefit the propagation of threatened and endangered species, where the goal is to make as many juveniles as possible for population reintroductions and augmentations. It would be particularly helpful in the propagation of small species of mussels that produce undersized broods where each glochidia is extremely valuable. The host fish for many species of mussels are unknown, so *in vitro* propagation may be the only way for these species to avoid extinction. Most researchers would agree that propagating mussels on host fish is an extremely labor intensive process in which many different things can diminish the number of juveniles that are produced. *In vitro* propagation is a method that would greatly reduce the amount of labor required and increase the number of individuals produced. Fisheries biologists still have many questions about the viability of *in vitro* culture for mussel propagation. *In vitro* propagation may be more widely accepted in the field of freshwater mussel propagation as the survival and growth of *in vitro* propagated juveniles improves, and questions about the fecundity, and fish host range of propagated juveniles are answered.

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