ABSTRACT

WALTER, MICHAEL JOHN. *In-vitro* Propagation of the Federally Endangered Dwarf Wedgemussel (*Alasmidonta heterodon*) and Identification of Bacterial Communities of *In-vitro* and *In-vivo* Propagated Freshwater Mussels. (Under the direction of Dr. Jay F. Levine).

Captive propagation of freshwater mussels has proven to be a valuable tool for conservationists. Freshwater mussels have been propagated *in-vivo* under controlled laboratory conditions by introducing the parasitic mussel larvae, called glochidia, to host species of fish housed in aquaria. The process is often labor and cost intensive but has been a dependable propagation method for many years. *In-vitro* propagation is a promising alternative to these traditional methods that removes the host fish component of the mussel's life cycle. Instead, glochidia are incubated under sterile conditions in a nutrient rich medium. *In-vitro* methodologies are less expensive, less labor intensive and potentially a more efficient use of a female mussel's larval brood making them ideal for the propagation of rare species.

Previous efforts in our laboratory to propagate the federally endangered Dwarf Wedgemussel (*Alasmidonta heterodon*) using *in-vitro* methods failed to produce any transformed juveniles. To optimize the *in-vitro* process for the federally endangered Dwarf Wedgemussel, an experiment was designed to test the effects of both temperature and media serum component type on the transformation percentage of *in-vitro* propagated juvenile mussels.

Earlier work has identified substantial differences in long-term growth and survival of *in-vivo* and *in-vitro* propagated juveniles. This longitudinal study was designed to investigate differences in the associated microbiomes of *in-vitro* and *in-vivo* propagated juvenile mussels. These results will aid in our understanding of observed discrepancies in long term growth and survival between *in-vitro* and *in-vivo* propagated juveniles.

In-vitro propagated Dwarf Wedgemussel showed no significant differences in transformation percentage between the two temperature treatments of 18°C and 23°C. However, glochidia reared at 23°C transformed two days earlier than those reared at 18°C. Juveniles propagated using rabbit serum-based media transformed in significantly higher percentages than those propagated using equine serum-based media. These results indicate that the animal species sourced for the serum can dramatically affect the outcomes of *in-vitro* propagation

Analysis of the microbiome of *in-vitro* and *in-vivo* propagated juveniles indicated that the microbial communities of *in-vitro* propagated juveniles were less abundant and less diverse than the microbial communities of *in-vivo* propagated juveniles. Our results also show that the diversity of gastrointestinal bacteria in *in-vitro* and *in-vivo* propagated juveniles, though initially very different, converges to a similar composition after 30 days in the grow out environment. This research also encourages further optimization of the *in-vitro* process by identifying potential probiotic bacteria species and the need for additional studies focused on the use of antibiotics in the growth media.

The primary goal of these trials is to improve outcomes of *in-vitro* propagation by advancing our understanding of the resulting juveniles and refining the *in-vitro* propagation process. With this knowledge, conservationists can tailor their methods before and after transformation to optimize long-term growth and survival of *in-vitro* propagated juvenile mussels.

In-vitro Propagation of the Federally Endangered Dwarf Wedgemussel (*Alasmidonta heterodon*) and Identification of Bacterial Communities of *In-vitro* and *In-vivo* Propagated Freshwater Mussels.

by
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DEDICATION

This thesis is dedicated to my partner Brianna Smith-Overman who continues to support me in countless ways as I pursue my passions. Thank you for your patience through weekend feedings, unexpected late nights in the lab, the stress of exams and hours of mussel talk.

BIOGRAPHY

Mike Walter was born in Stillwater, Oklahoma in 1988 to John and Candy Walter. The family moved several times around the country before landing in Chapel Hill, North Carolina and he has lived in the Triangle ever since. His love for the natural world was fostered throughout childhood and ultimately led him to pursue his Bachelor of Sciences in Ecology/Environmental Biology from Appalachian State University in 2010. Following graduation, Mike worked a variety of jobs from retail to landscaping to research technician before attending Graduate School at North Carolina State University.

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Introduction

Among imperiled taxa in North America, few are more threatened than Unionid freshwater mussels (Bivalvia: Unionidae) (Bogan 2008). More than 70% of the nearly 300 species that occur in North America are considered vulnerable, imperiled, critically imperiled or presumed extinct (Stein et al. 2000). Freshwater mussels are directly impacted by degrading habitat quality and availability. As filter feeders, freshwater mussels provide numerous ecosystem services ranging from toxin sequestration, removal of organic debris and sediment biodeposition (Vaughn 2018). Freshwater mussels are also an important food resource for predators such as muskrats (Neves and Odom 1989), racoons and turtles (Vogt 1981). Their shells provide valuable habitat and a source of calcium carbonate for benthic communities (Gutierrez et al. 2003). Freshwater bivalves often comprise 50% of the total benthic biomass (Strayer et al. 1994) and in dense populations, freshwater mussels can increase substrate stability (Zimmerman and Szalay 2007).

The chronic plight of freshwater mussels is largely the result of human influences on their habitats. Anthropogenic disruptions to flow affect freshwater mussel reproduction (Galbraith and Vaughn 2011), limit host fish access (Roy et al. 2005) and alter water temperature profiles that increase mussel mortality (Williams et al. 1993). Increased erosion as a result of deforestation, agricultural practices and destruction of riparian zones degrades habitat quality by increasing the benthic sediment load, reducing the amount of suitable substrate available for mussel habitation (Box and Mossa 1999). Historically, human harvesting of mussels for the button trade resulted in gross overharvesting of North American mussels and decimation of previously robust populations (Anthony and Downing 2001). In addition, the introduction of invasive aquatic species, such as the Asian Clam (*Corbicula fluminea*) and the Zebra Mussel (*Dreissena*

polymorpha) in North America, has also impacted populations worldwide. Invasive species compete for food resources, suitable habitat (Haag 2012; Williams et al. 1993) and have been associated with diminished physiological condition and growth rates of sympatric native mussel populations (Ferreira-Rodríguez et al. 2018).

Given the plight of freshwater mussel populations, the efforts of conservationists have become as diverse as the problems being addressed. In 1997, a "National Strategy" was developed to guide freshwater mussel conservation efforts in North America (NNMCC, 1997) by identifying 10 key "problems" faced by conservationists. One important conservation problem identified by the committee was the preservation and restoration of suitable habitat. Habitat loss is widely considered the most common threat to freshwater mussels and the protection of existing habitats will continue to be a crucial part of mussel conservation. Another conservation method used by conservationists and resource managers is the relocation of threatened populations (Hamilton et al. 1997). However, relocation has produced mixed results. Successful relocation efforts ideally require identification of the precise habitat requirements of the species being moved and long-term monitoring (Cope and Waller 1995). Other conservation goals identified include increasing public awareness, investigating the basic biology and ecology of freshwater bivalves, developing a better understanding of how stream perturbations impact mussels and managing populations in the presence of invasive species introduction. In addition, the National Strategy identifies the need to "Develop, evaluate, and use the technology necessary to propagate and reintroduce juvenile mussels on a large scale".

Captive propagation of freshwater mussels has been a longstanding goal of both freshwater mussel conservationists and those who historically utilized the resource for commercial purposes. Originally proposed to replenish overharvested stocks (Lefevre and Curtis

1910), propagation in captivity is used now almost exclusively for conservation. As with any captive propagation efforts, there are concerns about loss of genetic variation, mixing of unique genetic stocks and the effects of hatchery selection on the resulting juveniles (Haag and Williams 2014). However, guidelines are in place at the Federal level to account for and minimize the genetic impacts of captive propagation of federally listed species (USFWS and NMFS 2000). Currently, propagation is one of the primary tools conservationists depend on for the recovery of species that are near extinction or extirpation from their native range.

Reproduction of most freshwater mussel species is dependent on the successful attachment of their parasitic larvae (glochidia) to the gills or fins of a host fish. A specific host fish is often required to fulfil this obligate parasitic requirement of larval mussels. When attempting artificial propagation in the laboratory, host fish are exposed to larvae at artificially high concentrations and glochidia attach to the gills and fins of the host and become encysted. Encystment can last from 1-6 weeks, during which time the larvae derive nutrients from their host and develop internal organs (Kat 1984). After transformation, newly transformed juveniles break out of their encystment and fall from the gills and fins of the host fish to the substrate where they continue to grow as free-living mussels. In the hatchery, transformed larvae are collected by filtering the outflow from the holding tanks of infested fish with mesh baskets. Juveniles are then moved to a grow out system where they are reared to sufficient size for release into their native streams.

Transformation of glochidia can also be achieved in the laboratory using techniques adapted from *in-vitro* cell culture. Glochidia are placed in sterile growth media containing vitamin and nutrient rich basal media, blood serum and a combination of antibiotics and antimycotics (Isom and Hudson 1982). Glochidia are then held in an incubator at a stable

temperature infused with a stable concentration of 3% carbon dioxide. The glochidia are monitored and receive regular media exchanges until transformation is complete.

The *in-vitro* propagation process was first attempted in 1926 (Ellis and Ellis 1926) but went largely undeveloped until 1982 when Isom and Hudson published specific methods for *in-vitro* propagation (Isom and Hudson 1982). Modern protocols (Owen 2009) have built upon early methods through the use of different serum sources, novel antibiotic/antimycotic mixtures and regular media changes. Following transformation, media is slowly diluted with water and juveniles are transferred to the grow out system where they are fed and monitored until they reach suitable size for restocking.

In-vitro propagation has many advantages over *in-vivo* (fish host based) propagation. Transformation percentages are often higher using *in-vitro* methods. *In-vitro* transformation eliminates both the need to identify a mussel species' specific host fish as well as the need to collect and house host fish (Lima et al. 2012). *In-vitro* propagation requires a laboratory setting that provides the opportunity for sterile preparation and processing of growth media and temperature controlled incubation of the developing glochidia. Although the initial startup cost is high, long-term equipment and personnel costs are less using *in-vitro* propagation. *In-vivo* propagation is more labor intensive than *in-vitro* propagation and the use of host fish requires strict adherence to the Institutional Animal Care and Use Committee's guidelines for vertebrates. Propagation using *in-vitro* methods is often the most efficient use of mussel larvae in terms of transformation success, potentially making *in-vitro* propagation methods ideal for rare species of freshwater mussels.

Differences in post-transformation survival and growth rates between *in-vitro* and *in-vivo* propagated have also been observed. Fox and coworkers (2014) documented survival and growth

rates between *in-vivo* and *in-vitro* propagated juveniles for one week post-transformation and found *in-vitro* propagated juveniles survived and grew at a significantly lower rate compared to *in-vivo* propagated juveniles. Their work also recorded long-term survival and growth of *in-vitro* and *in-vivo* propagated juveniles for 70 days after transformation. *In-vitro* propagated juveniles were nearly 0.5 mm shorter (total length) and had nearly 20% lower survival rate than *in-vivo* propagated juveniles. Lima and coworkers (2006) comment that *in-vitro* propagated juveniles appear to be less healthy than those produced by infestation. Furthermore, they suggested that future studies of *in-vitro* propagation should include comparisons of *in-vitro* and *in-vivo* propagated juveniles.

Here, we theorize that the observed differences in survival and growth may be a result of being reared in a sterile environment. Juveniles transformed using *in-vitro* methods may have a sterile or reduced intestinal microbiome and are therefore less able to fully utilize food resources. Previous investigations in the Aquatic Conservation and Epidemiology Laboratory at NC State University attempted to address this issue of poor post-transformation growth and survival performance by culturing and feeding bacteria that had been previously isolated from swabs of mussel digestive systems (Chittick et al. 2001, Fox, unpublished) with mixed results.

The following research aimed to improve our understanding of the health of newly transformed juvenile freshwater mussels and improve the outcomes of *in-vitro* propagation. By documenting differences in bacterial communities associated with *in-vivo* and *in-vitro* propagated juveniles, we will be able to identify what bacteria are missing from the gastrointestinal tracts of *in-vitro* propagated individuals and how to potentially supplement their diets to enhance survival and growth. Documenting disparities in bacterial communities between *in-vitro* and *in-vivo* juvenile mussels will help define the studies needed to address these

differences and optimize the *in-vitro* process. As long as captive propagation and population augmentation remain key freshwater mussel conservation management tools, the refinement of *in-vitro* and *in-vivo* propagation techniques will remain critical to the preservation of freshwater mussel populations.

In addition to this overview of freshwater mussel conservation efforts and methods, we will first present the results of the first successful propagation of the federally endangered Dwarf Wedgemussel (*Alasmidonta heterodon*). This work highlights both the value of *in-vitro* methods for the propagation of rare species and the need for refinement of *in-vitro* methods as we explore the role of different serum components on transformation success. We also present the results of an experiment designed to compare the microbiomes associated with juveniles propagated using *in-vitro* and *in-vivo* propagation methods using a metagenomics approach. The documented differences in microbiome composition offer insight into physiological differences of mussels propagated using *in-vitro* and *in-vivo* methods. Finally, we will reflect on the importance and implications of these findings and offer recommendations for future research and propagation efforts.

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Chapter 1: *In-vitro* propagation of the Dwarf Wedgemussel (*Alasmidonta heterodon*, Lea 1829)

Introduction

Unionid freshwater mussels are among the most imperiled animals in North America where approximately 70% of the nearly 300 species are listed as vulnerable, imperiled, critically imperiled or presumed extinct (Stein et al. 2000). Freshwater mussels have historically comprised up to 50% of the total benthic biomass in river systems (Strayer et al. 1994) but have extensively been subjected to the negative effects of human land use practices that have led to their sharp decline. Habitat degradation in the form of impoundments, wastewater discharge, increased sediment loading and competition from invasive species threaten nearly every species of freshwater mussel (Haag 2012). Fortunately, propagation and population augmentation efforts have proven to be effective tools in the rehabilitation of waning populations and are included as priority items in many recovery plans (NNMCC, 1997; NCWRC, 2015)

Freshwater mussels have been propagated using *in-vivo* methods in a hatchery setting. *In-vivo* propagation involves identification of the host fish species necessary for successful transformation, collection, quarantine and holding of the host fish and the artificial infestation of the host fish with the parasitic larvae of the mussel being propagated (Haag and Williams 2014). Once transformed, juvenile mussels are collected as they drop off of the host fish and are moved to grow out systems until they reach suitable size for release into the wild. *In-vivo* propagation efforts have proven critical in the conservation of waning populations and, with continued refinement, will continue to play an integral role in recovery plans (Haag and Williams 2014).

In-vitro propagation techniques, pioneered in 1926 (Ellis and Ellis 1926), rely on sterile artificial media formulations in place of a host fish to provide the necessary conditions for glochidial transformation. Media formulations vary but broadly consist of basal media, blood serum, antibiotics and antimycotics (Isom and Hudson 1982). Glochidia are suspended in sterile media inside a sterile container and receive regular media exchanges until transformation occurs. Juveniles are transitioned to freshwater when transformation is observed, then moved to a grow out system. When compared to *in-vivo* methods, *in-vitro* methodologies offer several benefits; elimination of the need for studies to identify host fish and collection and maintenance of host fish, higher transformation percentages and lower cost per propagated juvenile (Lima et al. 2012). These benefits make *in-vitro* propagation an appealing option for the propagation of endangered mussel species and species whose host fish is unknown.

The Dwarf Wedgemussel (*Alasmidonta heterodon*) is a federally endangered species of freshwater mussel (Unionidae) found along the Atlantic slope drainage in habitats with moderate flow and patches of fine sediment (Michaelson and Neves 1995; Strayer and Ralley 1993; USFWS 1993). Historically, the range of the Dwarf Wedgemussel extended from New Brunswick, Canada to the Neuse River in North Carolina (NCWRC 2018) forming sparse populations both naturally and because of human impacts (Strayer et al. 1996). The Dwarf Wedgemussel is found in at least 70 locations in 11 states and one Canadian province (USFWS 1993). In North Carolina, extant populations are limited to the upper Tar and Neuse River basins where populations continue to decline (NCWRC). Recent *in-vivo* propagation efforts at the Aquatic Conservation and Epidemiology Laboratory at North Carolina State University have resulted in the successful transformation of Dwarf Wedgemussel glochidia, using Johnny Darters (*Etheostoma nigrum*) as a viable host fish (Eads personal communication).

Prior *in-vivo* studies offer hope for future propagation and population augmentation efforts in North Carolina for this declining species. We hypothesize that the serum source and incubation temperature affect the rate of glochidia transformation.

In these studies, we describe the first successful *in-vitro* propagation of *A. heterodon* and explore the effects of various serum components and incubation temperatures on transformation percentage.

Methods

Treatment Structure

Experimental treatments were organized in a 2x2x2 factorial design (Table 1) where two broods were tested using two serum types (rabbit and equine), at two temperatures (18°C and 23°C). Two replicate culture flasks were included at each experimental level.

Table 1 - Experimental design to test the effect of two serum types (rabbit and equine) and two incubation temperatures (18°C and 23°C)

		Serum Component Type	
		Rabbit Serum	Equine Serum
Brood 1	18° C	2 Replicates	2 Replicates
	23° C	2 Replicates	2 Replicates
Brood 2	18° C	2 Replicates	2 Replicates
	23° C	2 Replicates	2 Replicates

Media Formulation

Growth medias consisting of equine or rabbit serum components were tested during this experiment. Media consisting of 74.3% sterile M199 basal media (Caisson Labs Inc., Smithfield, UT), 25% filter sterilized equine or rabbit serum (Gibco Labs, Gaithersburg, MD), 2% gentamicin sulfate (50 mg/mL; Genesee Scientific Corp., El Cajon, CA), 4% amphotericin B (0.25 mg/mL; Fisher Scientific, Fair Lawn, NJ) and 1% rifampin diluted in 1 mL DMSO (100 mg/mL DMSO; Alfa Aesar, Ward Hill, MA) was buffered to pH 7.3 and filter sterilized using a 0.2 µm vacuum driven bottle top filter (Corning Inc, Corning, NY). Sterile media was stored in sterile 0.2 µm filter cap 75 cm^2 cell culture flasks (Corning Inc, Corning, NY) inside a CO_2 infused incubator at a concentration of 3% CO_2 at either 18°C or 23°C. Holding temperatures corresponded to experimental treatment.

Glochidia Extraction, Sorting and Flask Inoculation

Two gravid *Alasmidonta heterodon* were collected from Little Shocco Creek (Warren County, NC) on 13 March 2019 measuring 41 mm and 47 mm in length. The gravid individuals were held without additional food for one day at 10°C in dechlorinated and buffered hatchery holding water (City of Raleigh, NC) prior to harvesting their glochidia. To avoid triggering an early brood release, the temperature of the holding water was gradually increased to 18°C over a period of 24 hours.

Once at 18°C, the adult mussels' outer shell surfaces were thoroughly cleaned to remove excess dirt and potential contaminates to the culture medium. Next, the inside of the mussel was rinsed with ~500 mL of 0.2 filtered sterile filtered dechlorinated and buffered holding water (City of Raleigh, NC). The glochidia were flushed from the gills using an 18-gauge needle and

sterile syringe filled with 0.2 µm sterile filtered dechlorinated and buffered holding water (City of Raleigh, NC). Glochidia were flushed directly into buffered sterile MEM media (Gibco Laboratories, Gaithersburg, MD) triggering their closure. The closed glochidia were then cleaned by agitation with sterile MEM media and sorted both gravimetrically and with 300 µm Nitex mesh (Sefar Inc., Buffalo, NY) sieves to remove unclosed (non-viable) and underdeveloped glochidia as well as those that had closed on another glochidia following extraction. Inside of a Class 2 Type A2 Biological Safety Cabinet (NuAire, Plymouth, MN), glochidia were transferred to a sterile 0.2 µm filter capped 25 cm^2 cell culture flask (Corning Inc, Corning, NY) filled with 5 mL filter sterilized complete M199 media formulation according to the treatment regime noted above. Care was taken to minimize the amount of MEM wash media introduced to the cell culture flask. A target flask load of 1000-1500 glochidia per 5 mL of growth media was established, a glochidia allocation that had proven successful in previous *in-vitro* efforts.

Rearing and Dilution

Glochidia were held in 3% CO_2 at their assigned treatment temperature and received 60% (3 mL) media exchange every 48 hours. Daily observations to monitor media contamination and developmental progress were made using an inverted microscope at 4x, 10x and 40x magnification. Upon observation of juvenile foot movement outside of the shell in any individual, the media holding the transformed juveniles was diluted to 100% freshwater using 0.2 µm filtered buffered and dechlorinated holding water at a rate of 10% per hour. During dilution, the animals being held at 18°C were raised to 23°C at a rate of 0.5°C per hour to match the temperature of the grow out system. Following dilution, viable juveniles were counted and transferred to grow out tanks with a layer of sediment sifted to 200 µm

Assessment

Following the 10-hour dilution, the mussels were counted under 10x magnification. A grid was drawn on the bottom of the flask to help with the counting process. Juveniles were considered transformed at the time of assessment upon observation of foot movement, heartbeat, mantle contraction or opening and closing of the valves.

Statistical Analysis

Statistical analysis was done using SAS Statistical Software Version 9.4. An "arcsine" transformation was applied to the data to account for the binomial nature of proportional data. A multi-factor Analysis of Variance (ANOVA) was used to detect differences in transformation percentage between temperature and serum type treatments and to test for an interaction between the treatments. A Student's t-test was used to compare the simple effects at each treatment level. A p-value ≤ 0.05 was considered statistically significant. This analysis was done using the "ESTIMATE" function in the "PROC GLM" procedure in SAS.

Results

Brood Size

Two adult Dwarf Wedgemussels were collected for this trial. Brood 1 was extracted from an adult measuring 41mm. After extraction, washing and sorting, brood 1 contained 5,456 viable glochidia. Brood 2, extracted from an adult measuring 47mm, contained 14,242 viable glochidia after extraction, washing and sorting.

Glochidia Condition

During incubation, a noticeable difference in the condition of the glochidia and culture media was observed between individuals held in media prepared with rabbit and equine serum. Glochidia held in equine serum exhibited partially gaped valves and a buildup of tissue at the shell margin (Figure 1) and floating in the culture media.

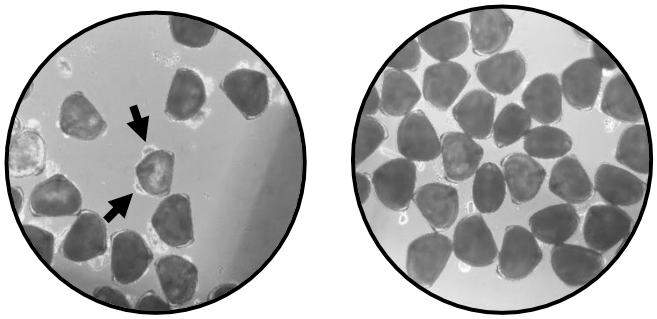

Figure 1 - Glochidia in media with equine serum (left) showing contamination at shell margin compared to glochidia held in media with rabbit serum (right)

The tissue on the shell margin appears to have been expelled from the visceral mass of the mussel because of the observed loose closure of the valves. Although glochidia showing tissue at the shell margin appeared to be alive, the body of the mussel appears less dense suggesting diminishment of the physiological condition of the mussel. This tissue, once expelled, appeared to grow freely in the media as evidenced by the presence of floating masses within the media with the same general appearance of the tissue on the glochidia shell margins. These observations were consistent across all eight cell culture flasks in the equine serum-based media treatment. Similar observations were made during a previous attempt to propagate the Dwarf Wedgemussel using equine serum-based media in April/May of 2018 that resulted in no transformed individuals. Tissue expulsion was much less prevalent and the glochidia remained

tightly closed throughout the incubation period in all eight treatment flasks containing rabbit serum-based media

Incubation Period

Successful transformation was indicated by the clear presence of adductor mussels, steady cilia movement on the foot and/or foot movement inside and outside of the valves. Counts of transformed juveniles were done immediately following the 10 hour dilution to freshwater. Nonviable glochidia were easily distinguished as they were typically completely gaped open (shell valves open) with no movement observed.

The amount of incubation time required to achieve transformation of glochidia differed between temperature treatments but not between serum treatments. Glochidia held at 18°C transformed in 24 days compared to a 22 day incubation period for glochidia held at 23°C.

Statistical Analysis of Transformation Percentages

A significant interaction was detected between the incubation temperature and serum component variables of the. Using a Student's t-test, we conducted individual hypothesis testing to detect differences in transformation percentage between the two levels of one treatment at a specific level of the other factor.

There was no statistically significant effect of brood origin on transformation percentage ($p = 0.9217$). However, *Alasmidonta heterodon* glochidia reared in rabbit serum-based media transformed at statistically significant higher rates than those reared in equine serum-based media (Figure 2) at both 18°C (estimate = 34.32%; s.e. = 0.006; $p < 0.0001$) and 23°C (estimate = 64.13%; s.e. = 0.006; $p < 0.0001$). Additionally, temperature was found to significantly

increase transformation percentage only in samples reared in rabbit serum-based media. Samples reared in rabbit serum-based media at 23°C transformed at a higher rate (estimate = 5.2%; s.e. =0.006; p = 0.0096) than those reared at 18°C. Temperature did not have a significant effect on glochidia reared in equine serum-based media (p = 0.356).

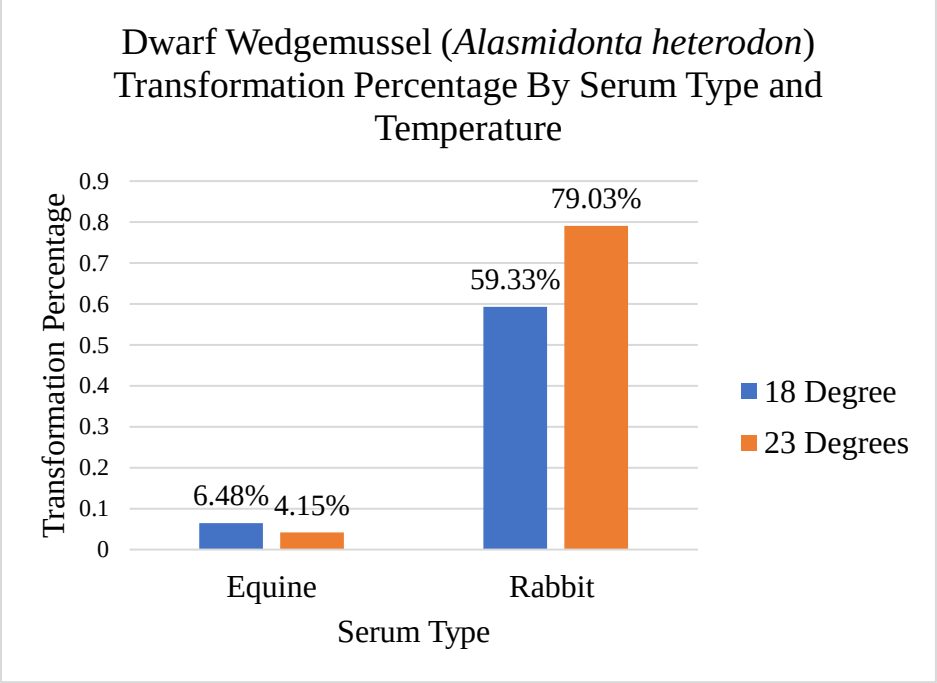

Figure 2 - Transformation percentage of A. heterodon reared in rabbit and equine serum-based media at both 18°C and 23°C (2 replicates per treatment)

Long Term Survival and Growth

Of juveniles reared at 23°C in rabbit serum-based media, 5.3% survived from brood 1 and 16.5% survived from brood 2 after 24 days in the sediment bucket grow out system. Only 4.8% of all juveniles (both broods) reared at 23 °C in equine serum-based media survived after 24 days. After 50 days of grow out post-transformation, 1.9% (brood 1) and 4.6% (brood 2) of juveniles reared using rabbit serum-based media at 23°C survived while no juveniles propagated

at 23°C using equine serum-based media survived. At 85 days after transformation, both broods of the 23°C, rabbit serum treatments were combined and together the survival rate was 3.5%.

Survival of juveniles propagated at 18°C was very low. At 48 days post-transformation, no individuals survived from the brood 1 rabbit serum treatment, 3.5% survived from the brood 2 rabbit serum treatment and 1% survived from both broods of the equine serum treatment. By the time survival counts were taken at 83 days after transformation, no individuals from any of the 18°C treatments survived.

Alasmidonta heterodon from brood 1 propagated using rabbit serum-based media at 23°C averaged 0.466 mm (± 0.07) after 24 days in the grow out system and brood 2 of the same temperature and media treatment averaged 0.500 mm ± (0.05). Both broods reared in equine serum-based media at 23°C averaged 0.500 mm (± 0) after 24 days in the grow out system. After 50 days in the grow out system, brood 1 juveniles from the 23°C rabbit serum treatment averaged 0.536 mm (± 0.08) while juveniles from brood 2 of the same treatment averaged 0.596 mm (± 0.08). No juveniles from the 23°C equine serum-based media treatment survived at this timepoint. At the last measurement taken 85 days after transformation, both broods of juveniles propagated using 23°C rabbit serum-based media had been combined and averaged 0.922 mm (± 0.14).

Discussion

These results represent the first successful transformation of *Alasmidonta heterodon* using *in-vitro* techniques. *In-vitro* methodologies are constantly evolving and we have adapted our techniques in several ways to improve success.

In-vitro cultures are often incubated and maintained in cell culture dishes or 6-well plates with lids that cover the culture while allowing free exchange of gases. These lids do not completely prevent, with any physical barrier, the introduction of airborne bacteria or fungal spores present in the laboratory or incubator environments. Given the nutrient rich nature of the culture medium, the risk of contamination is very high and threatens to compromise the growing environment of the glochidia. As a further step towards preventing the introduction of contaminants to the culture medium, we cultured these experimental treatments in 25 cm^2 cell culture flasks equipped with 0.2 µm filter caps (Corning Inc, Corning, NY). The 0.2 µm filter installed into the caps still allow for free gas exchange and pH regulation while providing a physical barrier to ambient contaminants.

The regulation of buffered stock media pH is vital to maintain culture environment consistency for the glochidia. However, in previous *in-vitro* propagation efforts, we observed fluctuations in the pH of stock media solutions stored at 10°C in 500 mL media storage bottles (Corning Inc, Corning, NY). The effects of temperature on pH are well known. Media was initially buffered to pH 7.3 but was observed to raise to pH 8.6 in refrigerated storage. We observed negative effects on previous cultures of glochidia when media changes were performed with media stored in refrigeration with fluctuating pH. To correct this storage issue, stock media was aliquoted into 75 cm^2 cell culture flasks with 0.2 µm mesh filter caps and stored inside of the CO_2 incubator. This change in storage condition allowed for the sodium bicarbonate buffering system present in the media to operate in the presence of 3% ambient CO_2 and fluctuations of pH were no longer observed. An additional benefit of storing stock media inside of the incubator is that stock media temperature is perfectly matched to the glochidia culture flasks. This eliminated

both the need to warm refrigerated stock media prior to media changes and negates the risk of thermal shock to the glochidia during media changes.

Since *A. heterodon* are commonly found to be gravid in late winter and early spring when water temperatures are low, 18°C was chosen for previous *in-vivo* propagation efforts to minimize the risk of early brood release during holding while reducing thermal shock to glochidia and to facilitate accelerated infestation times (Taeubert et al. 2014; Eads, C. personal communication). We used the *in-vivo* temperature protocol as a starting point for our *in-vitro* trials with *A. heterodon*. However, CO_2 incubators capable of sub-ambient temperature incubation are expensive and not widely available. For this reason, we were interested in testing the effectiveness of incubation at above-ambient temperature and tested incubation at 18°C and 23°C. The results of our incubation temperature trials also suggest a shorter incubation time for glochidia held at 23°C (22 days) than those held at 18°C (24 days). Shorter incubation time reduces overall cost of *in-vitro* propagation and reduces the risk of contamination exposure to the culture. In addition, glochidia cultured in rabbit serum-based media transformed at statistically significant higher percentages at 23°C than those reared at 18°C. Our results also suggest that a larger proportion of juveniles survived when incubated at 23°C than when incubated at 18°C. There were no apparent negative effects of culturing at the higher temperature.

Our observations of higher transformation percentages of *Alasmidonta heterodon* cultured in rabbit serum-based media than in equine serum-based media confirm the work of several prior studies that have documented the importance of serum component choice in *in-vitro* propagation methods (Excobar-Calderón et al. 2019; Uthaiwan et al. 2001; Keller et al. 1990, Lima et al. 2012). Many of these studies focused on finding alternatives to fish serum, which is often used as the serum component in many *in-vitro* methodologies (Uthaiwan et al. 2002) but

requires significant time and effort to procure since it is rarely commercially available. In most cases, serum choice does have a significant effect on transformation percent across a variety of species and serum types.

Investigations into the compositional differences of the rabbit and equine sera specifically used in this study were limited by the availability of information from the manufacturer. The Certificates of Analysis (COA) for the specific lots from which our sera were sourced provided little insight. Osmolality was reported for both sera and was thought to be a possible cause of the cell lysis and expulsion seen in equine serum treatments but the recorded values for these sera were within 6 mOsm/kg (rabbit – 296 mOsm/kg; equine – 289 mOsm/kg). This small difference in osmolality is unlikely to have an effect since the acceptable range for the osmolality of the serum is 270-310. Hemoglobin levels in this lot of rabbit serum were noticeably higher than this lot of equine serum (rabbit – 12.7 mg%; equine – 8.3 mg%) though the effects of this protein in animal cell cultures are not well known. More research into the compositional differences between the two serum types revealed protein levels to be consistently higher in rabbit serum. Albumin content in particular was noticeably higher in rabbit serum than in equine serum across all literature found on the subject.

Albumin has been found to comprise ~50% of total serum protein content (Haschek et al. 2013), bind many physiologically important nutrients including calcium, magnesium, zinc and copper (Mustafa et al. 2012; Majorek et al. 2012) and low albumin protein content is commonly associated with malnutrition and protein absorption dysfunction in humans (Mustafa et al. 2012). Comparative studies of serum protein content have consistently reported that albumin represents a 20-30% greater proportion of total serum proteins in rabbit serum than in equine serum (Brooksby 1947; Hewitt 1938; Robertson 1912). Albumin concentrations have been found to

vary significantly by fish species (Januar et al. 2015) raising questions regarding the role of albumin content levels in host fish suitability for *in-vivo* propagation. Future investigations into the role of albumin concentration on *in-vitro* freshwater mussel propagation success are recommended.

Serum is widely regarded to be an inconsistent reagent for *in-vitro* media formulation. The composition of the serum depends heavily on the extraction protocols and the source animal's location and physiological condition (Freshney 2010). Serum testing is often recommended for animal cell culture applications because of this variation. Serum free medium testing has delivered mixed results when culturing animal cells and our results suggest that the development of serum-free media could benefit *in-vitro* freshwater mussel propagation. Elimination of the serum variation and selection process would be invaluable when working with severely imperiled species.

In-vitro propagation has proven to be an efficient method for propagating the broods of rare species as shown by the high transformation percentages observed in this study. However, our results for long term rearing of the transformed juveniles were mixed and dependent on culture techniques. Additional research is needed to understand and optimize the post-transformation rearing of *in-vitro* propagated juveniles.

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Chapter 2: Bacterial communities present in juvenile freshwater mussels propagated using *in-vitro* and *in-vivo* methods

Introduction

Unionid freshwater mussels are among the most imperiled taxa in North America (Stein et al. 2000). Threats to mussel populations include degradation of water quality, flow restriction, sedimentation and invasive species introduction among many others (Bogan 2008). The efforts of conservation biologists are more vital than ever as we seek to protect and recover increasingly dwindling populations (Haag and Williams 2014). Laboratory propagation and population augmentation activities have been identified as key components in many mussel recovery plans and are crucial to mussel conservation efforts (Bishop et al. 2007; USFWS).

Two methods are used to propagate freshwater mussels in captivity, *in-vitro* and *in-vivo*. Most freshwater mussel species undergo a parasitic life history phase during which the larvae (glochidia) must attach to the gills and/or fins of a host fish. Often, a mussel species can only successfully complete its life cycle through attachment to a specific host fish species. For captive propagation efforts, this process is replicated in a hatchery setting with wild caught host fish. The host fish, once quarantined, are exposed to the glochidia of the mussel being propagated at high concentrations. Following exposure and attachment of the glochidia, the host fish are then moved to holding tanks while the attached glochidia undergo a complete metamorphosis into juvenile mussels. After transformation, they fall from the fish and are captured as the outflow from the tanks is filtered into mesh baskets. From there they are moved into a long-term grow out system and fed with cultured algae and/or commercial diets or with water from natural sources (e.g. ponds).

Transformation of glochidia can also be achieved using techniques adapted from *in-vitro* cell culture. Larvae are held under sterile conditions in sterile media consisting of basal media, blood serum and a cocktail of antibiotics and antimycotics (Isom and Hudson 1982). This technique was first attempted in 1926 (Ellis and Ellis, 1926) and is continually being refined. Often, higher rates of transformation are achieved with *in-vitro* propagation methods when compared to *in-vivo* methods (Lima et al. 2012). Although initial costs of establishing an *in-vitro* propagation laboratory are high, substantial long-term cost savings can be achieved using *in-vitro* methodologies. The process eliminates the person-hours required to collect and maintain host fish and circumvents the need for trials to identify the appropriate host fish for *in-vivo* propagation.

Although both *in-vivo* and *in-vitro* propagation methods produce viable juveniles, very little is known about the factors contributing to the post-transformation success of *in-vitro* propagated juvenile mussels. When comparing the long-term growth and survival of mussels produced under *in-vitro* and *in-vivo* methods, animals produced under *in-vitro* protocol often show reduced initial survival and growth when compared to *in-vivo* propagated individuals (Fox, T.R. unpublished, Lima et al. 2006).

Gastrointestinal flora play a key role in the digestion of algae and other food resources in the diet of many aquatic invertebrates (Harris 1993). One possible explanation for the contrasting survival and growth of juveniles propagated using *in-vivo* and *in-vitro* methods may lie in the bacterial communities present in and on the juveniles produced by each method. In one study, Fox (2014) used *Bacillis subtillis* as a probiotic and supplemented the feed of *in-vitro* propagated mussels with the bacteria and noted improved growth of *in-vitro* propagated juveniles. Aceves and coworkers (2018) documented significant differences in gut microbiome composition

between hatchery reared and wild caught freshwater mussels, supporting the need for investigations into propagation method's effects on microbiome composition. Accordingly, we hypothesized that the method of propagation (*in-vivo* vs. *in-vitro*) has an effect on overall bacterial diversity and abundance in recently transformed juveniles. In these studies we documented the presence and abundance the microbiome present following transformation in both *in-vivo* and *in-vitro* propagated juvenile *Lampsillis cariosa*.

Methods

Study Design

The experiment was designed as a longitudinal study to track bacterial diversity and abundance over time (Table 1). To achieve this, three broods were propagated using both *in-vivo* and *in-vitro* methods. The resulting juveniles were then raised in separate long term grow out systems and samples of 100 juveniles were taken immediately after transformation as well as 15 and 30 days after transformation.

Table 1 - Experimental design for longitudinal microbiome assessment at 3 timepoints (0, 15 and 30 days after transformation) and for 2 propagation methods (in-vitro and in-vivo).

Factorial Experimental Design		
	In-vitro Propagated	*In-vivo* Propagated
0 Days After Transformation	3 Reps (Broods)	3 Reps (Broods)
15 Days After Transformation	3 Reps (Broods)	3 Reps (Broods)
30 Days After Transformation	3 Reps (Broods)	3 Reps (Broods)

Animal Collection

Gravid adult *Lampsillis cariosa* measuring 49 mm (brood 1), 51 mm (brood 2) and 50 mm (brood 3) were provided by the Virginia Fisheries and Aquatic Wildlife Center (Charles City, VA) and held at 10° C until extraction. The adult female mussels received 1 mL of Nano 3600 (Reed Mariculture, Campbell, CA), 1 mL of Shellfish Diet 1800 (Reed Mariculture, Campbell, CA), 2 mL Amquel (Kordon LLC, Hayward, CA) and 5 gallons of buffered water daily via drip feeding. Thirty Largemouth Bass (*Micropterus salmoides*) approximately four inches in length were purchased from Foster's Pond and Lake Management (Garner, NC). The Largemouth Bass were quarantined in a 500 gallon tank and received a formalin treatment (Paracide F, Argent Chemical, Redmond, Washington) to eliminate gill parasites. The bass were fed daily using pelletized fish feed and water quality (NO_2^-, NH_4^+, pH) was monitored every other day. The fish were examined daily to assess their behavior and health (Institutional Animal Care and Use Protocol: 19-025-0).

In-vitro Culture

Glochidia were extracted from the left gill of each gravid mussel using a 22-gauge needle and 10 mL syringe filled with dechlorinated, buffered and sterile filtered water (City of Raleigh, NC). Glochidia were flushed into 250 mL of 0.2 µm filter sterilized and pH adjusted (pH 7.3) Minimum Essential Medium MEM "wash" medium (Caisson Labs Inc., Smithfield, UT). Once in the MEM medium solution, the glochidia were triggered to close by the biologic salts present in the media. Following closure, glochidia were washed and sorted by gravimetric sorting and agitated using a stream of MEM medium. Non-viable glochidia and contaminants were decanted off until all remaining glochidia sank at a consistent rate, indicating consistent visceral mass of

the brood. The remaining glochidia were passed through a 250 µm Nitex mesh (Sefar Inc. Buffalo, NY) sieve to remove non-viable open glochidia and glochidia that has closed on each other making them non-viable. The cleaned and sorted glochidia from each brood were then cultured using *in-vitro* using filter sterilized (0.2 µm), buffered (pH 7.3) media consisting of 74.3% sterile M199 basal media (Caisson Labs Inc., Smithfield, UT), 25% sterile equine serum (Life Technologies Corp, Grand Island, NY), 2% gentamicin sulfate (50 mg/mL; Genesee Scientific Corp., El Cajon, CA), 4% amphotericin B (0.25 µg/mL; Fisher Scientific, Fair Lawn, NJ) and 1% rifampin in DMSO (100 mg/mL; Alfa Aesar, Ward Hill, MA). Glochidia and media were held in 0.2 µm ventilated cell culture flasks inside a CO_2 controlled cell culture incubator at 23° C in 3% CO_2. Flasks were checked daily for media color, clarity, contamination and glochidia progression using an inverted microscope (Olympus Corp. Tokyo, Japan) at 4x, 10x, 20x and 40x magnification. Each flask received a 60% (3 mL) media change every other day until transformation was complete. Following transformation, glochidia were transitioned to the grow out environment by diluting the media at a rate of 10% per hour.

In-vivo Culture

Glochidia were extracted from the right gill of each mussel using an 18-gauge needle and 10 mL syringe filled with dechlorinated and buffered water (City of Raleigh, NC) into a petri dish. The extracted glochidia were then transferred to an infestation chamber with 1 L dechlorinated and buffered water and an air stone supplying vigorous aeration to prevent settling of the glochidia. Brood size was assessed by taking a 100 mL sample from the infestation chamber and counting the number of glochidia per 100 mL. The number of viable glochidia in a brood were assessed by adding less than 1 gram of iodized salt to the 100 mL water sample and

counting the number of glochidia that close to determine the percentage of viable glochidia in the brood. Estimated brood size was used to determine concentration of glochidia in the infestation chamber with a target concentration of 4300 glochidia per liter. Ten Largemouth Bass (*Micropterus salmoides*) were added to the incubation chamber and the duration of exposure to the glochidia determined by the concentration of glochidia in the infestation chamber. Transformed glochidia were then collected as they dropped off the host fish by filtration of outflow from the AHAB (Aquatic Ecosystems Inc.) tanks holding the infested fish.

Post-transformation Rearing

Following transformation, broods propagated using both *in-vitro* and *in-vivo* methods were held in separate 5 cell "mucket buckets" (Barnhart, 2005) in cells covered in 150 μm Nitex (Sefar Inc. Buffalo, NY) mesh. Fifty percent of the water was changed daily by drip feeding 4 drops of Nano 3600® (Reed Mariculture, Campbell, CA), 6 drops of Shellfish Diet 1800® (Reed Mariculture, Campbell, CA), 1 mL Amquel (Kordon LLC, Hayward, CA) in 2.5 gallons of buffered water.

Sample Collection

One hundred juveniles were randomly collected at each timepoint (0, 15 and 30 days post-transformation) from each propagation treatment. These juveniles were immediately frozen at -20°C in ~0.5 mL holding water. A 4 mL sample of the water in the grow out system was collected and immediately frozen at -20°C at each sampling timepoint for use as "background" in downstream analysis. A 4 mL sample of the *in-vitro* culture media was also taken and

immediately frozen at -20°C. All samples were collected in sterile containers and remained frozen until DNA extraction.

DNA Extraction

Frozen animal and water samples were thawed immediately before DNA extraction. Extraction was done using the DNeasy PowerSoil Pro Kit (Qiagen; Venlo, NE). Following the manufacturer's recommendation, a Protease K digestion was done for 10 min at 65 °C before bead beating to aid in tissue breakdown. Following extraction, DNA quantification was done using a NanoDrop (Thermo Fisher Scientific; Waltham, MA) and extracted DNA was stored at -20°C until PCR amplification.

PCR Amplification

Extracted DNA was amplified using DreamTaq Green PCR Mastermix – 2x (Thermo Fisher Scientific; Waltham, MA). Primer 341F (5'– ACCTACGGGNGGCWGCAG –3') and 806R (modified) (5'– TGGACTCANVGGGTWTCTAAT -3') (Walters et al. 2015) were used to target the V3-V4 regions of the 16S rRNA gene. Common sequence (CS) linkers (CS1 – ACACTGACGACATGGTTCTACA; CS2 – TACGGTAGCAGAGACTTGGTCT) were included with the primers to aid with library preparation before sequencing. The PCR program (Table 1) used to amplify the V3-V4 region was optimized over several rounds of trial amplifications. After each trial, the PCR products were run on an electrophoresis gel to assess the product length. The final PCR program (Table 2) was determined to provide a clear signal of the expected fragment size.

Table 2 - The PCR program used to amplify the V3-V4 region of the 16S rRNA subunit.

PCR Temperature and Time Program	
	16S
Denaturing	95°C – 1 min
Denaturing	95°C – 30 sec (35x)
Annealing	53°C – 1 min (35x)
Extension	72°C – 1 min (35x)
Extension	72°C – 5 min
Hold	4°C - ∞

RNA Library Preparation and Sequencing

Upon completion of PCR amplification, the amplified product was quantified using the Qubit 4 Flourometer (ThermoFisher Scientific, Waltham, MA) following the protocols for the "dsDNA: High Sensitivity" assay. Ten µL of amplified PCR product were loaded into 1.5 mL microcentrifuge tubes, labeled by sample number and frozen before the samples were shipped overnight on dry ice. Library preparation and sequencing were performed at the at the University of Illinois at Chicago (UIC) in the DNA Services (DNAS) facility within the Research Resources Center (RRC). Library preparation was done using Common Sequence (CS) linkers (CS1 – ACACTGACGACATGGTTCTACA; CS2 – TACGGTAGCAGAGACTTGGTCT) following the protocols of Naqib and colleagues (2018). Sequencing was performed on the Illumina MiSeq platform (Illumina Inc. San Diego, CA) to a read length of 2 x 300 bp.

Data Analysis

Initial analysis of sequencing data was performed using the "dada2" (Version 1.14) R package (Callahan et al. 2016). After reviewing sequence read quality profiles, the primer sequences were removed from the reads and read lengths were truncated to 270 base pairs (bp) for the forward reads and 230 bp for the reverse reads. Following denoising using error rates, the paired end reads were merged and reads of the desired read length (~460 bp) were target for further downstream processing. Finally, chimeric amplicon sequence variants (ASV's) were removed from the samples resulting in the final sequence table to be used in assigning taxonomy.

The SILVA v132 ribosomal RNA gene database (Quast et al. 2013) was used as the reference database in assigning taxonomy. After inspecting the taxonomy assignment and ensuring that most reads were identified taxonomically to at least the Family level, the taxonomy table was used for analysis in the "phyloseq" (Version 1.30.0) R package (McMurdie and Holmes 2013).

Once the taxonomy table and sample metadata files were combined into a "phyloseq" object, the "decontam" (Version 1.6.0) R package (Davis et al. 2018) workflow was run using two sequenced negative PCR control samples as the basis for contaminant identification using the prevalence method outlined in the "decontam" workflow. Taxa identified as contaminants were then removed from both the animal and water/media samples. Next, the "decontam" workflow was run again (using prevalence method), this time using the water/media samples as the basis for contaminant identification. Then the taxa identified as contaminants were removed from the animal samples. These steps were performed to ensure meaningful downstream analysis of the data through removal of background bacteria in the animal samples. By removing the

contaminants present in the rearing environment from the animal samples, we are able to investigate only the microbial communities associated with the juveniles.

Mitochondrial BLAST

To understand the degree to which our 16S rRNA primer sets would amplify mitochondrial DNA from the juvenile cells, both forward and reverse 16S rRNA primer sequences were submitted to NCBI's (National Center for Biotechnology Information) Nucleotide BLAST (Basic Local Alignment Search Tool) program. The "Nucleotide collection" database was searched using the "Organism" option with taxa ID:293138 (Lampsillinae) as the organism input.

Statistical Analysis

Statistical analysis was performed using the "vegan" (Version 2.5-6) R package (Oksanen et al. 2019). Permutational multivariate analysis of variance was performed using the "adonis" function on Bray-Curtis (Bray and Curtis 1957) distance matrices to determine the statistical significance of diversities between propagation method.

Results

Propagation

Brood 1 consisted of an estimated 27,904 glochidia. Emersion in saltwater was used to test the viability of the glochidia. Healthy animals respond by rapid closure of their valves. A subsample of 63 glochidia from brood 1 were tested. All 63 closed in testing indicating high brood viability. Brood 2 was estimated to have consisted of 38,800 glochidia and were 100%

viable with 71/71 glochidia closing during salt testing. Brood 3 contained an estimated 19,304 glochidia, 51/51 were found to be viable during salt testing.

In-vivo propagated *L. cariosa* began to drop off of the host fish after 14 days and were collected over 3 days. *In-vitro* propagated juveniles transformed after 16 days in the media. After 164 days, 207 *in-vivo* propagated juveniles were alive averaging 4.39 mm (± 0.39 mm) in length and 1,412 *in-vitro* propagated juveniles were alive averaging 6.34 mm (± 1.55 mm) in length.

Mitochondrial BLAST

The NCBI BLAST search results using the forward primer matched *Leptodea leptodon* mtDNA as the top alignment result with a max score of 19.0 and a total score of 38.1. The reverse primer sequence nucleotide BLAST search returned *Lampsilis cardium* mtDNA as the top alignment result with a max score of 17.5, total score of 32.8, 8/18 primer match, E score 88 and 44% coverage. *Lampsilis cardium* is a mussel from the same genus as our study species (*Lampsilis cariosa*) however, the low scores give us confidence that we would not be targeting mtDNA from our juvenile mussel species.

Microbiome Composition

Illumina sequencing resulted in 4,030,365 reads before preprocessing. After preprocessing with the "dada2" R package, 850,371 reads were used for downstream processing resulting in 2492 OTU's in 18 samples. In total, 24 bacterial phyla were discovered in association with juvenile *Lampsillis cariosa* propagated using both *in-vitro* and *in-vivo* methods (Figure 1). Of these, the three most abundant Phyla were *Proteobacteria, Firmicutes* and

Chlamydiae. At the family level, *Burkholderiaceae* (Phylum *Proteobacteria*) was the most dominant taxa at all timepoints among both *in-vitro* and *in-vivo* propagated samples.

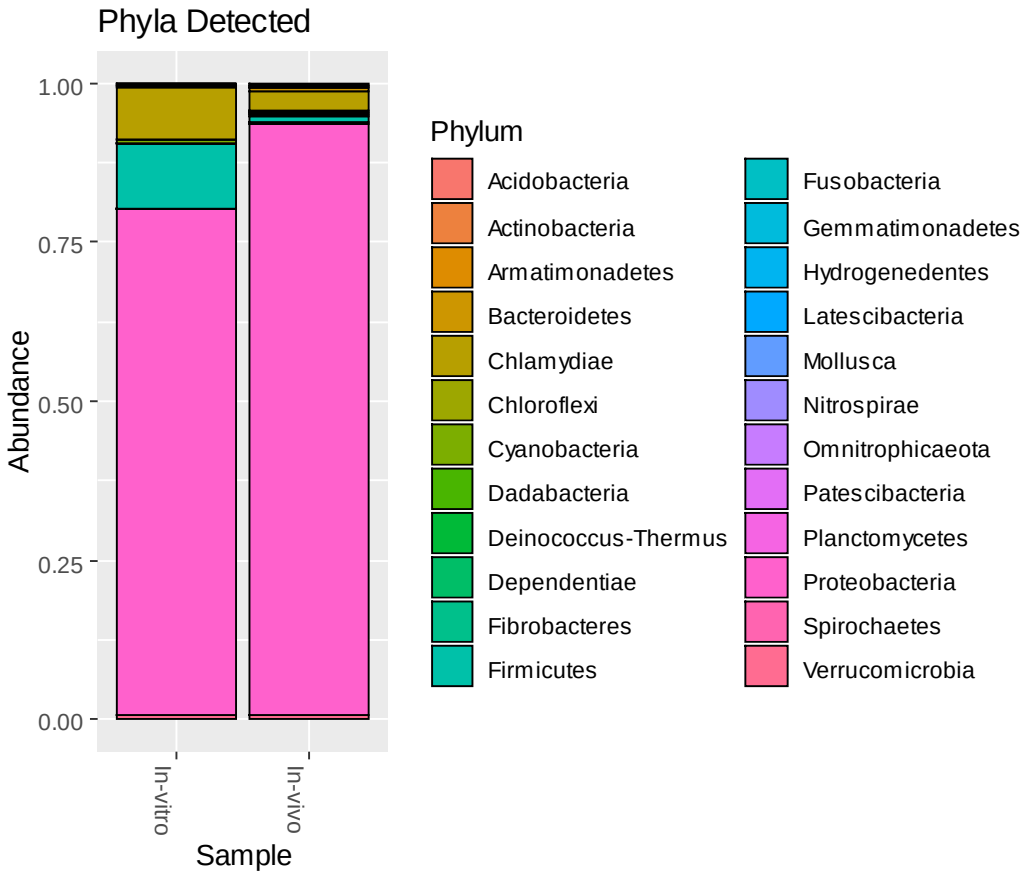

Figure 1 - Abundance of Phyla detected in in-vitro and in-vivo propagated juveniles

However, newly transformed (day 0) *in-vivo* propagated juveniles had several bacterial Families that were not present in the newly transformed (day 0) *in-vitro* propagated juveniles, notably *Moraxellaceae*, *Pseudomonaceae* and *Xanthomonadaceae* (Figure 2).

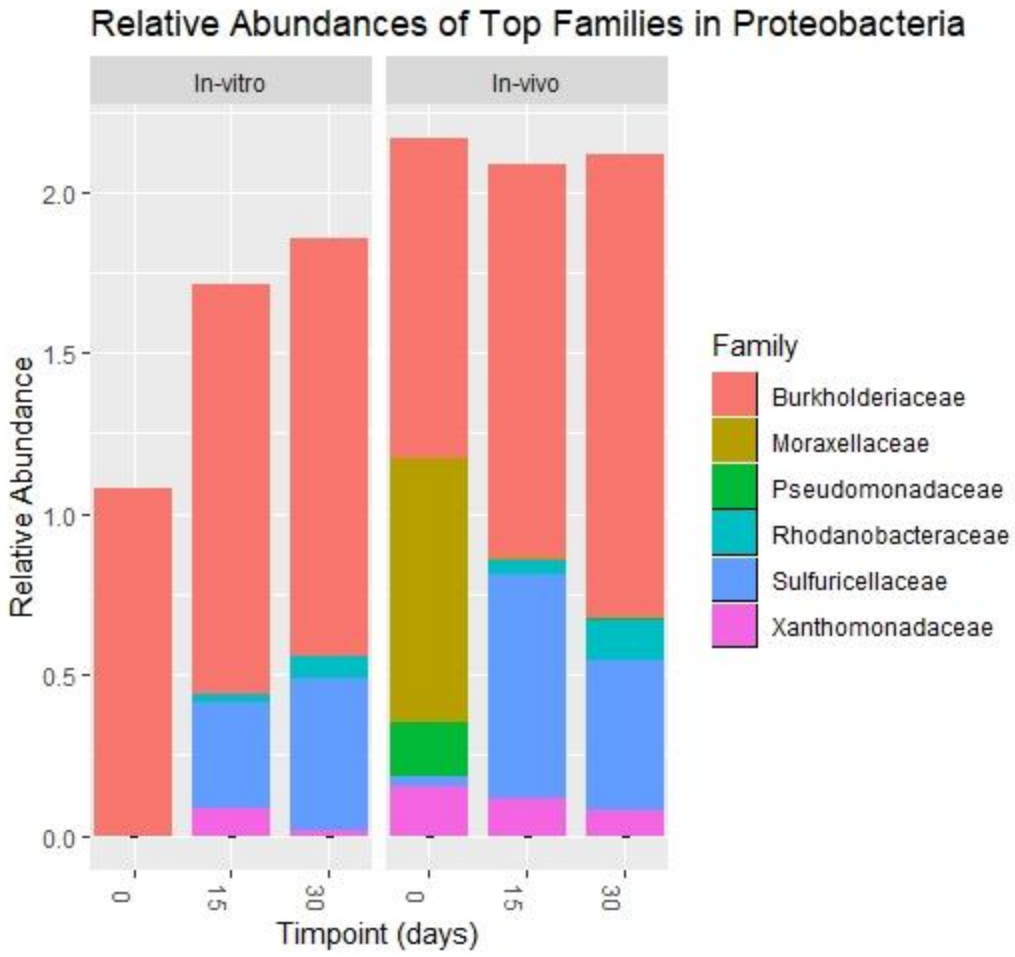

Figure 2 - Top bacterial Families identified in in-vitro and in-vivo propagated juveniles at 0, 15 and 30 days after transformation

Table 3 - The most abundant Genera in in-vitro and in-vivo propagated juveniles at 0,15 and 30 days after transformation.

	Top Genera Identified	
Propagation Method	**Days After Transformation**	**Most Abundant Genus**
In-vitro	0	*Acinetobacter, Curvibacter, Delftia, Methylibium*
In-vitro	15	*Bacillus, Methylibium, Neochlamydia, Sulfuriferula*
In-vitro	30	*Methylibium, Rubrivivax and Sulfuriferula*
In-vivo	0	*Acidovorax, Acinetobacter, Hydrogenophaga*
In-vivo	15	*AAP99, Leptothrix, Lysobacter, Methylibium, Sulfuriferula*
In-vivo	30	*AAP99, Methylibium, Rhizobacter, Sulfuriferula*

Microbiome Diversity

In-vitro propagated juveniles exhibited a significantly reduced microbiome diversity (Figure 3) of bacterial taxa comparted to *in-vivo* propagated juveniles (p = 0.015; Permutational Multivariate ANOVA) across all timepoints. Bray-Curtis Principle Coordinate Analysis (PCoA) plot shows that, at 0 days after transformation, diversity measures of both *in-vivo* and *in-vitro* propagated juveniles microbiome compositions are very dissimilar as indicated by their distant positions on the plot. As time after transformation progressed, microbiome compositions of the *in-vivo* and *in-vitro* propagated juveniles began to converge 15 days after transformation and ultimately becoming most similar at day 30 (Figure 3).

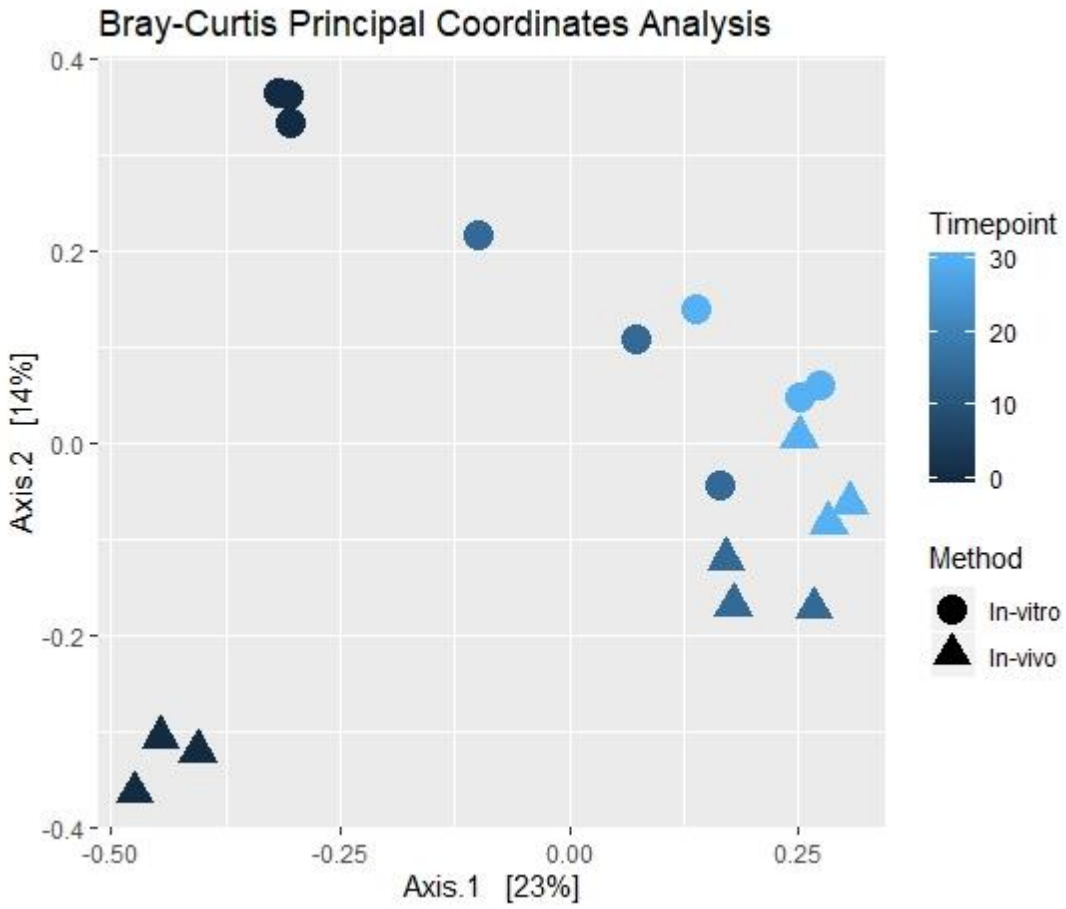

Figure 3 - Bray-Curtis Principle Coordinates Analysis of bacterial diversity indices plotting in-vivo and in-vitro propagated juveniles 0, 15 and 30 days after transformation

Discussion

In this study we tested the effects of the *in-vitro* propagation process on the microbiome of captively propagated juveniles. By sampling the juveniles at 0, 15 and 30 days after transformation, we were able to track the progression of the microbiome for both *in-vitro* and *in-vivo* propagated juveniles as well.

Culture-independent amplicon sequencing is a useful method for studying and understanding bacteria in aquatic environments. Aquatic invertebrates in particular have been extensively studied including Crustacea, Amphipoda and Echinodermata (Harris 1993). In malacology, the majority of bacterial community studies have focused on marine species with economic importance such as abalone (Huang et al. 2010, Tanaka et al. 2003), Sydney rock oysters (Green and Barnes 2010), Pacific oyster (Hernandez-Zarate and Olmos-Soto 2005, Lokmer and Wegner 2015), Eastern oysters (King et al. 2012, Pierce et al. 2016). These studies of commercially important species have been largely prompted by the potential for human interaction with harmful bacterial pathogens through consumption of these harvested and cultivated animals. Investigations of bacterial diversity can also be useful as aids to conservation efforts for species across many taxa (Bahrndorff et al. 2016).

Investigations of bacterial communities associated with Unionid bivalves have been limited. Starliper and coworkers (2008) researched freshwater mussel bacteria as a means to understand mussel die offs in the Clinch and Holston rivers. Alves and coworkers (2016) identified phytoplankton species present in *Diplodon enno* digestive tissue to optimize captive feeding protocols. Other studies have related freshwater mussel gut microbiome composition to host species (Weingarten et al. 2019) and collection site (Chittick et al. 2001). Aceves and coworkers (2018) used metagenomic methodologies to compare the gut microbiome composition

of wild and captively reared adult *Villosa nebulosa* and found significant differences in species composition but not in bacterial diversity. In reviewing the available literature, it is apparent that there is a gap in our knowledge of freshwater mussel microbiomes as they relate to varying captive propagation methods and the resulting juveniles.

Propagation

Long-term grow out of these broods of *L. cariosa* resulted more survival and higher growth rates of *in-vitro* propagated juveniles than those propagated *in-vivo*. The noted higher levels of survival and faster growth of *in-vitro* propagated juveniles was contradictory to previous findings that compared the success of *in-vitro* and *in-vivo* propagated juveniles (Fox 2014; Lima et al. 2012). Propagation methods used here are consistent with prior studies in our laboratory. It is possible that variability within the downwelling grow out systems could account for the variability however this is unlikely given that faster growth and for *in-vitro* propagated juveniles were consistent across all brood replicates grown in three different mucket buckets. Understanding that different species may react to the propagation process differently, we are hesitant to make generalized comparisons regarding these study animals. However, these counter intuitive results highlight the necessity of analyzing juvenile mussels using a variety of methods, such as metabolomic profiling, to more completely understand juvenile mussel health.

Microbiome Composition

Proteobacteria was first described by Stackebrandt and colleagues in 1988 and was the most abundant Phylum detected in both *in-vitro* and *in-vivo* propagated samples. It is a group of gram-negative bacteria and is the largest Phylum in the bacterial domain (Rizzatti et al. 2017).

Interestingly, these results differ from previous studies of adult mussels that found *Proteobacteria* to comprise only 5% of the bacterial Phyla detected (Aceves et al. 2018). It is possible that the microbiome composition shifts as the mussel ages as well.

At the point of transformation (day 0), the *Proteobacteria* Family *Moraxellaceae* was relatively more prominent in the *in-vivo* propagated juvenile's microbiome than in the microbiome of *in-vitro* propagated juveniles (Figure 2). In particular, bacteria from the Genus *Acinetobacter* were the most predominant and when the sequence was searched using NBCI Nucleotide BLAST, the highest ranking match (Max score – 780; Total score – 780) was *Acinetobacter beijerinckii*, a hemolytic, aerobic species that has been isolated from human, equine, soil and water samples (Nemec et al. 2009). The likely presence of this species in newly transformed *in-vivo* propagated juveniles suggests its role of encysted juvenile's ability to derive nutrients from the blood of host fish through the haemolytic action of the bacteria.

In *in-vitro* propagated juveniles, one of the most abundant Genera was *Delftia* from the *Burkholderaceae* Family. The NCBI Nucleotide blast of the most abundant sequence in this Genus returned *Delftia tsuruhatensis* and *Delftia lacustris* as the highest matching species (Max score – 789; Total score – 789). Both of these bacteria are capable of digesting peptidoglycan, a common component of bacterial cell walls (Jorgensen et al. 2009). Also, *Delftia tsuruhatensis* has been identified as having anti-fungal properties in testing with common plant root pathogens (Han et al. 2005). Their appearance in the newly transformed *in-vitro* juveniles is intriguing and may have contributed to the increased rates of survival and growth observed. For this reason, we have identified these species as candidates for future probiotic feeding experiments. In addition to their functional capabilities, they are readily cultured in commercially available media (e.g.

LB Broth or TSB) at 22°C which is critical to their practical use as a probiotic.

Microbiome Diversity

The gut microbiome has been found to be involved in metabolic pathways, immune response mechanisms and overall physiological condition in a wide variety of animals (Nicholson et al. 2012). These results confirm our hypothesis that *in-vitro* methodologies alter microbiome composition and diversity. Bacterial diversity was reduced in *in-vitro* propagated juvenile's microbiomes. However, analysis of diversity over time using a PCoA plot (Figure 3) shows that the diversity of microbiomes converges within 30 days of post-transformation grow out. These findings highlight the need for further refinement of our *in-vitro* methods to increase initial bacterial diversity. The observed increase of microbiota diversity by *in-vitro* propagated juveniles after 30 days is encouraging and suggests that probiotic feeding may be effective. Future experiments using probiotic feed supplements comprised of species identified as missing or in low abundance in *in-vitro* propagated juveniles (compared to *in-vivo* propagated juveniles) in this study are advised. By increasing the diversity of the *in-vitro* propagated juvenile's microbiome immediately after transformation, we may be able to "jump start" their microbiome to match the diversity levels that are acquired after 30 days in the grow out system.

Future Studies

The antibiotics used in this study, Rifampin and Gentamicin Sulfate, are broad spectrum antibiotics. Rifampin is a bactericidal antibiotic that kills a wide variety of gram-negative bacteria. Previous studies have investigated the effectiveness of various antimycotic treatments in controlling the fungal contamination of *in-vitro* mussel cultures (Owen et al. 2010). A similar

study testing a variety of antibiotics, particularly a variety of bacteriostatic antibiotics, would provide valuable insight into practical role of antibiotics in the culture medium. Bacteriostatic antibiotics inhibit the proliferation of bacteria rather than destroy them. The presence of potentially beneficial bacteria (*Delftia, Bacillis* and *Acinetobacter*) in the *in-vitro* propagated juveniles suggests that there is transmission of some bacteria from the gravid female to the glochidia, possibly preserved by the closure of the glochidia as they enter the MEM media. The use of this type of bacteriostatic antibiotic might allow for the preservation of the inherited microbiome while inhibiting the growth of additional culture contaminating microbes. Also, removal of all antibiotics from the culture media in the days immediately before transformation might aid the proliferation of these inherited beneficial bacteria.

These results offer our first glimpse into the microbiome of newly transformed juvenile mussels. The reduction of bacterial diversity and abundance as a result of the *in-vitro* propagation process highlights the need for more focus on this aspect of *in-vitro* methodology. We believe the insights gained from this study will effectively aid in the refinement of future *in-vitro* propagation efforts, making *in-vitro* methodologies increasingly effective tools for freshwater mussel conservation.

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Conclusions

This work further demonstrates that *in-vitro* methods are valuable tools for propagating rare freshwater mussel species such as the Dwarf Wedgemussel. This work also shows that modifications to common protocols are beneficial in optimizing these promising techniques. Serum component testing proved to be a vital task for successful *in-vitro* transformation of the Dwarf Wedgemussel and we believe that further trials exploring the use of different antibiotics and serum-free media will continue to advance the usefulness of *in-vitro* methodologies.

In-vitro techniques often result in higher transformation percentages than host fish based (*in-vivo*) propagation methods. The efficient use of larval broods using *in-vitro* methodologies reinforces their potential value for propagating rare species of freshwater mussels. However, long term grow out success of *in-vitro* propagated juveniles is often mixed.

To better understand the lagging survival and growth rates of *in-vitro* propagated juveniles, we looked to emerging technologies to assess previously unseen aspects of juvenile mussel health. The contributions of an animal's microbiome to its overall health has become the focus of many studies in recent years. Here, we used Next Generation Sequencing (NGS) technology to assess the microbiome of newly transformed *in-vitro* and *in-vivo* freshwater mussels by targeting the V3-V4 region of the 16S ribosomal RNA subunit.

The results of this investigation revealed that newly transformed *in-vitro* propagated juveniles have a reduced microbiome compared to juveniles raised on host fish. Despite lower initial microbiome diversity, *in-vitro* propagated juveniles acquire a microbiome with diversity measures similar to those of mussels propagated using *in-vivo* methods within 30 days of post-transformational growth. This finding highlights the need for future studies using probiotics in

the feed mixture to increase bacterial diversity earlier in the juvenile mussel's post transformational life.

We identified two species of bacteria, *Delftia tsuruhatensis and Delftia lacustris,* as good candidates for probiotic feeding trials. These bacteria were found to be present in newly transformed *in-vitro* propagated juveniles and are known to have desirable metabolic capabilities. Both species are capable of peptidoglycan digestion which may aid in the mussel's ability to process food items. Also, *Delftia tsuruhatensis* has been found to suppress pathogenic fungus growth when associated with plant root systems. Furthermore, both bacteria are readily cultured in a laboratory setting using commercially available media making them ideal candidates for probiotic trials.

The presence of bacterial species in newly transformed *in-vitro* propagated juveniles also suggests conference of the microbiome from the brooding female mussel to the larval brood. We believe that the antibiotics used in our *in-vitro* methods suppress this inherited microbiome. Therefore, trials testing the use of bacteriostatic antibiotics that suppress bacterial growth rather than destroy bacteria are recommended. The use of bacteriostatic antibiotics may allow the inherited bacterial species to persist through out the *in-vitro* propagation process and allow the bacteria to resume their potentially beneficial role in the newly transformed juveniles health upon leaving the *in-vitro* culture environment.

The insight gained from this work directs future studies in several ways. Exploration of the eukaryotic components of the juvenile mussel's microbiome using ITS sequencing will help to complete our picture of early juvenile mussel's gastrointestinal flora. Using this data, we can explore the role of fungal components of the mussel's microbiome that may reveal additional probiotic feeding candidates. Also, analysis of the newly transformed *in-vitro* and *in-vivo*

juvenile mussel's metabolomic profiles will give us an in depth view into the physiological health of juvenile freshwater mussels. The combination of microbiome analysis and metabolomic profiling would offer the most complete insight into juvenile mussel health available.

We hope that the insight gained from this research will aid in the refinement of *in-vitro* methodologies. *In-vitro* techniques are a valuable tool for the propagation of rare species of freshwater mussels and through continued improvement, we believe they will become even more vital to freshwater mussel conservation efforts.

APPENDICES

Appendix A: *In-vitro* Propagation Protocols

Laboratory –

1. Supplies
    - CO_2 infused, temperature controlled cell culture incubator
    - Deionized water source
    - Stir plate/stirbar
    - Glassware (Media storage bottles, beakers, graduated cylinders)
    - Autoclave
    - Vacuum pump or access
    - Class 2 Biological Safety Cabinet
    - Micropipettes, pipette aid, disposables (pipette tips, serological pipettes, wipes)
    - pH meter
    - 150 µm, 250 µm, 335 µm nitex mesh sieves
    - Microscopes – Inverted (10x-40x) and dissecting (1x-4x)

Media Formulation –

1. Sterile MEM "wash" media – 1 Liter
    a. Ingredients
        i. 1 - packet MEM media powder
        ii. 1L – 0.2 µm sterile filtered, deionized water
        iii. Sodium bicarbinate ($NaHCO_3$) adder per manufacturer's direction
        iv. HCL and/or $NaHCO_3$
    b. Materials
        i. 1L Sterile beaker
        ii. Stir plate/stirbar
        iii. pH meter
        iv. 0.2µm vacuum driven sterile filter
        v. 1L sterile media storage bottle
    c. Directions
        i. Combine MEM powder, sterile DI water and required sodium bicarbinate ($NaHCO_3$) in sterile 1L beaker. Mix thoroughly.
        ii. Measure pH
            1. Adjust pH to 7.3 using HCL and/or $NaHCO_3$
        iii. Inside a biological safety cabinet, filter sterilize the buffered media into 1L media storage bottle. Refrigerate at 10°C until use
2. Serum containing complete media – 1 Liter
    a. Ingredients
        i. 743mL - M199 media
        ii. 250 mL - serum
        iii. 2 mL - Gentamicin (50 mg/mL stock concentration)
        iv. 4 mL - Amphotericin B (250 µg/mL stock concentration)
        v. 1 mL - 0.1g Rifampin powder dissolved in 1 mL DMSO
        vi. HCL and/or $NaHCO_3$
    b. Materials
        i. 1L Sterile beaker

ii. Stir plate/stirbar
iii. pH meter
iv. 0.2µm vacuum driven sterile filter
v. 1L media storage bottles

c. Directions
i. Combine all ingredients (excluding HCL/$NaHCO_3$) into sterile 1L beaker. Mix thoroughly.
ii. Measure pH
1. Adjust pH to 7.3 using HCL and/or $NaHCO_3$
iii. Inside a biological safety cabinet, filter sterilize the buffered media into 1L media storage bottle. Refrigerate at 10°C until use. *Aliquot into 75 cm^2 0.2 µm filter cap cell culture flask as needed during mussel culture. Store aliquoted media in CO_2 infused, temperature controlled incubator. (See pg. 22 for further explanation)

Glochidia Culturing and Rearing -

1. Glochidia collection
a. Short term brooding (tachytictic) species
i. Evaluate mussel and estimate brood release date. Alternatively, induce release by increasing temperature or serotonin exposure. When brood release will occur within 24-36 hours complete the following steps.
ii. Clean outer shell of brooding mussel. Rinse inside of mussel with stream of sterile filtered deionized water.
iii. Transition gravid mussel to 1.5L sterile filtered dechlorinated and buffered holding water in a 2L sterile beaker. Gently aerate the water and do not feed the mussel.
iv. Upon completion of brood release, remove mussel and transfer glochidia to sterile dish. Separate glochidia from mucus and/or conglutinate material. Ensure no mucus remains with glochidia.
v. Transfer only the glochidia to the wash MEM media (see "Glochidia processing" below). Take care to transfer as little of the holding water to the MEM as possible.
b. Long-term brooding (bradytictic) species
i. Wipe outer surface of mussel with clean cloth and water until shell is clean. Rinse inside of mussel with 500 mL sterile filtered deionized water.
ii. Position mussel over sterile 500 mL beaker containing 200 mL sterile wash MEM. Hold the mussel with the posterior of the mussel pointing down. Using 18 gauge sterile needle and 10 mL syringe, flush the gills with sterile filtered deionized water. Flush glochidia directly into wash MEM media.
2. Glochidia processing
a. Once glochidia are in the wash MEM media (see "Glochidia collection" above), transfer glochidia to a 250 mL Erlenmeyer flask filled with 10 mL sterile wash MEM. Minimize the amount of media that is transferred with the glochidia between containers.
b. Agitate the glochidia inside the Erlenmeyer flask with a steady stream of sterile filtered wash MEM media.

c. Observe the flask. When the glochidia settle in the flask, pour off 80% of MEM media and contaminants into a clean waste media collection beaker. Take care to pour only the contaminants and nonviable glochidia (dead/open, underdeveloped, closed on neighboring glochidia) out of the flask. Repeat until all visible contaminants and nonviable glochidia have been decanted into the waste collection beaker. The remaining glochidia should settle in the beaker after agitation at a uniform rate and have a generally clean appearance.
d. Select nitex mesh sieve of appropriate mesh size for the species of mussel being propagated. Appropriately sized mesh allows individual closed glochidia to pass through while capturing nonviable glochidia. Rinse the glochidia through the mesh sieve into a clean dish using sterile MEM media. Under the dissecting microscope, observe the glochidia that passed through the filter. If nonviable glochidia pass through the sieve, reduce mesh size until all nonviable glochidia are captured by the sieve. The goal is to only have closed, singular and viable glochidia enter the *in-vitro* mussel culture.
e. Waste MEM collected in the waste media collection beaker is to be re-filtered and reused during the agitation and sieving steps.

3. Flask inoculation
    a. Inside of a Class 2 Biological Safety Cabinet, fill 25 cm^3 vented cap cell culture flasks with 5 mL complete serum containing media using sterile techniques. The number of flasks used will be determined by brood size. Target glochidia load ~1500 glochidia per cell culture flask.
    b. Transfer ~1500 washed and sieved glochidia (see "Glochidia processing") into cell culture flasks containing complete serum containing media using sterile techniques. Take care to minimize the amount of MEM media transferred to the cell culture flask when moving glochidia. Label flasks with mussel species, flask number, inoculation date and your initials.
4. Incubation and media changes
    a. Incubate flasks inside a thoroughly cleaned 3% ambient CO_2, temperature controlled incubator.
    b. Exchange 60% of culture media every 2^{nd} day during incubation using sterile techniques
        i. Use stock media that has been stored inside the 3% CO_2 incubator in 75cm^3 vented cap cell culture flask for media changes.
        ii. Inside of a Class 2 Biological Safety Cabinet, remove 3 mL media from the flask using a serological pipette and discard.
        iii. Refill flask with 3 mL fresh serum containing media from stock media contained.
    c. Observe glochidia using inverted microscope to monitor development and culture contaminant levels.
    d. Maintain cultures until transformation is observed. Transformation is indicated by the clear presence of 2 adductor mussels, clear movement of the foot, observation of vigorously beating cilia on the gills and/or foot of the mussel and foot movement outside of the shell. If unsure whether glochidia have transformed, dilute a sample of 10-20 glochidia from the culture to freshwater and monitor for

24 hours. Viable juveniles will begin moving and feeding in freshwater within 24 hours.

5. Dilution
    a. Upon transformation, dilute the serum containing culture media containing the glochidia at a rate of 10% per hour using sterile filtered, dechlorinated and buffered water. Water used for dilution should be sourced from the grow out system the juvenile mussels will be transitioned to. Water pH should match media pH, check and adjust if necessary prior to dilution.
    b. Store cell culture flasks containing glochidia in CO_2 infused incubator between dilution steps.
    c. Once diluted to 100% freshwater, transition juveniles to long term grow out system.

Appendix B: Juvenile Mussel DNA Sampling Protocol

Sample collection -

1. Remove juveniles from grow out system. Count and gather desired number of juveniles.
2. Using a sterile Pasteur pipette, transfer the sample and .5 mL of holding water or media to a sterile 2 mL snap cap conical centrifuge tube.
3. For each animal sample taken, collect a 4 mL holding water sample in 2 sterile 2 mL snap cap conical centrifuge tubes.
4. Freeze samples immediately at -20°C. Samples should remain frozen until DNA extraction.

DNA extraction -

1. Thaw and centrifuge collected animal samples for 1 min at 15,000 rpm. Using a sterile pipette tip, remove the supernatant water from the centrifuged animal sample leaving the animals in as little water as possible before continuing with the DNeasy Powersoil Pro Kit (Qiagen; Venlo, NE) protocol.
2. For water/media samples, centrifuge samples for 4 min at 15,000 rpm. Remove supernatant while avoiding the pellet in the tube. Proceed with DNeasy Powersoil Pro Kit (Qiagen; Venlo, NE) protocol.
3. Follow DNA extraction protocol outlined in the DNeasy Powersoil Pro kit (Qiagen; Venlo, NE).
    a. Add 20 µL of Protease K to bead beating tube with CD1 buffer
        i. Heat at 65°C for 10 min in dry bath, vortexing briefly 2-3 times during the heating period
    b. *Modified protocol* Recommended bead beating time – 5 min.
4. Quantify extracted DNA for each sample.
5. Label DNA samples and store at -20°C until PCR amplification.