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Modification of culture medium and identification of microbial contaminants for improved in vitro propagation of freshwater mussels

Raquel M. Wetzell
Virginia Commonwealth University

Modification of culture medium and identification of microbial contaminants for improved *in vitro* propagation of freshwater mussels

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By Raquel Wetzell, Center for Environmental Studies, Virginia Commonwealth University, wetzellrm@vcu.edu

Thesis Advisor: James Vonesh Ph.D. Professor, VCU Center for Environmental Studies

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Lastly, I would also like to thank my family and friends for always supporting me no matter where the wind takes me.

Vita

Raquel Maria Wetzell was born in Fairfax, Virginia in 1997 and graduated from Hayfield Secondary School, Alexandria, Virginia in 2015. She received her Bachelor of Science in Biology and Bachelor of Science in Anthropology from Virginia Commonwealth University, Richmond, Virginia in 2019. Before attending Graduate School at Virginia Commonwealth University in 2020, she was an intern and fellow at the Smithsonian Marine Station.

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CHAPTER 1

MODIFYING SERUM COMPONENT OF M199 MEDIUM FOR IMPROVED TRANSFORMATION OF *ATLANTACONCHA OCHRACEA* AND *LAMPSILIS CARIOSA*

Abstract

In vitro propagation efforts play an essential role in conserving and restoring threatened freshwater mussel populations by circumventing the need for a fish host. Across a broad range of taxa, transformation is induced with an artificial M199 medium and rabbit serum. However, such formulation may not be sufficient in culturing critical species with more specific physiological requirements. In this study, multiple serum mixtures were tested to improve *in vitro* transformation of two freshwater mussel species: yellow lampmussel (*Lampsilis cariosa*) and tidewater mucket (*Atlantaconcha ochracea*). These species were selected because they parasitize similar fish host species but have different rates of transformation in previous propagation trials. Juvenile transformation on rabbit serum only treatments was tested against juvenile transformation from treatments using rabbit serum supplemented with fish extract, blue catfish (*Ictalurus furcatus*) serum, or grass carp (*Ctenopharyngodon idella*) serum. *L. cariosa* showed an aptitude for a wide variety of serum types except for blue catfish, which showed signs of toxicity during early glochidia development. *A. ochracea* increased in transformation when cultured in full or partial carp serum compared to treatments utilizing only rabbit serum or rabbit serum with gill extract. Given the availability of local grass carp and the ability to mix with rabbit serum, it may be a preferred sera alternative for species like *A. ochracea* which exhibit poor transformation with serum.

Introduction

Freshwater mussels are among the most imperiled taxa in North America, with over 70% of species listed as endangered or threatened. Half of all mussel species found in the state of Virginia are listed at the federal or state level (Jones 2015). Filter feeding bivalves can provide important direct and indirect ecosystem services, most notably water filtration and habitat engineering as they work to remove suspended sediments, agricultural runoff, harmful fecal bacteria, and potentially manmade pollutants (Rott, 2019; Vaughn et al., 2008). As a result, there is a vested interest by the United States Fish and Wildlife Service (USFWS), Virginia Department of Wildlife Resources (VDWR), universities, and non-profit organizations to maintain and restore current mussel populations across the state of Virginia.

The unique lifecycle of a freshwater mussel begins with an introductory stage in which larvae (referred to as glochidia) require parasitic attachment to a host fish. However, even under pristine conditions, 99.9% of all glochidia released into the water column may never attach to a host (Lima et al., 2012). Glochidia attachment and juvenile mussel recruitment are further impacted by issues related to declines in fish host populations, stream contaminant presence, and habitat fragmentation (DWR, 2020). This low success in native recruitment serves as an opportunity for federal and state hatcheries to step in and form captive breeding programs for various freshwater mussel species. Hatchery operations mimic the freshwater mussel lifecycle by directly inoculating captive fish with glochidia taken from wild gravid females and netting the resulting juvenile drop-off. However, the process of maintaining fish hosts in captivity can become complex if the host is not identified, not abundant in the wild, or does not survive well in captivity (Lima et al., 2012). The need to remove the fish component and improve juvenile yields has led to the development of *in vitro* propagation.

In vitro propagation of freshwater mussels was first introduced in 1926 by Max Mapes Ellis, who removed encapsulated glochidia from the cells of a fish host and transformed them in an unknown artificial medium. More thoroughly documented experiments were conducted by Isom & Hudson (1984), whose culture medium successfully metamorphosed glochidia without the use of a fish host (Lima et al., 2012). Compared to natural reproduction, nearly all glochidia may transform with *in vitro* methods, which maximizes and increases juvenile yields for mussel species with low transformation rates or a limited number of gravid females (Owen, 2009; Uthaiwan et al., 2001, 2002, 2003). Recent *in vitro* research has simplified Isom and Hudson's process to determine the essential components of the culture medium that induce glochidia transformation (Escobar-Calderon et al., 2020; Lima et al., 2012; Uthaiwan et al., 2001, 2002, 2003).

To successfully transform glochidia, *in vitro* culture media must recreate the fish host physiological environment by providing necessary nutrients essential to metamorphosis and post-metamorphic survival. This can include a variety of essential and non-essential amino acids (obtained either from blood plasma/serum), salts, and lipids (Lima et al., 2012). Perhaps one of the most important components of the culture medium is serum – blood removed of all clotting components – which serves as source of protein for transforming glochidia (Owen, 2009). Fish serum is considered the most suitable sera for providing necessary nutrients for glochidia transformation given fish are the natural hosts of most freshwater mussel species, however, fish serum is not commercially available and cannot be purchased for *in vitro* propagation (Lima et al., 2012). Individual efforts to source fish sera require the repeated capture and euthanasia of multiple fish for blood draws and can limit *in vitro* production of mussels if organisms are not large in body size and widely available (Owen, 2009). Alternative sera that can be purchased

from commercial suppliers, including rabbit, equine, and fetal calf serum, have been used for successful propagation, but is limited to specific taxa (Lima et al., 2012). To fill this gap, studies have incorporated non-host fish plasma and serum for successful glochidia metamorphosis on taxa that do not respond to mammalian serum (Bagaria Osuna, 2016; Owen, 2009; Uthaiwan et al., 2001, 2002, 2003). While host fish can be unreliable sources of sera *in vitro*, access to common or invasive fish species could be preferred alternatives. Glochidia transformation has been documented using serum or plasma from common carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*), and buffalo (genus *Ictiobus*) (Bagaria Osuna, 2019; Owen, 2009; Mair, personal communication). However, differences in specific amino acid profiles and concentration composition between fish species may not make all non-host fish serum or plasma suitable for *in vitro* culture (Uthaiwan et al., 2001, 2002, 2003). In one instance, yellow catfish (*Pylodictis olivaris*) serum was suitable for transforming *Hyriopsis cumingii* and *Cristaria plicata* but failed to transform *Potamilus alatus* (Wen et al., 2018). If certain fish sera are incompatible with freshwater mussel glochidia, easier and alternative methods for introducing fish components to the *in vitro* medium should be explored, such as tissue extract derived from fish hosts.

Fish extract has been tested in culture medium to attempt to improve mussel propagation with limited success. Fish extract is derived from fish organ or body samples and, when compared to sourcing fish serum, takes less time for obtaining and processing subjects along with easier incorporation into the medium (Lima & Avelar, 2010). If combined with commercially available sera, it could provide the culture medium with certain developmental triggers found in fish host growth hormones (Collodi & Barnes, 1990; Henley & Neves, 2001; Joyce & Vogeler, 2018) that would otherwise be absent. However, little experimentation has

been published on the use of fish extract for mussel *in vitro* propagation. In one study, propagation of South American mussels *Diplodon rotundus* and *Diplodon greeffeanus* were improved with the addition of a concentrated freeze-dried fish extract made from macerated samples of a native fish species *Astyanax altiparanae.* Results showed higher metamorphic survival than *in vivo* experiments, with an improvement of 4-7.5 times more metamorphosized juveniles. After 50 days, post-metamorphic survival held at 75% (Lima & Avelar, 2010). However, lyophilization equipment for freeze-drying is not readily available in most laboratory setups. Fish cell culture – many techniques which have been taken and modified for the development of *in vitro* freshwater mussel propagation – features more extensive use of liquid fish extract for the development and maintenance of multiple cell lines and could be modified appropriately for *in vitro* propagation of mussels. However, these experiments have incorporated fish embryo extract (Chen et al., 2003; Collodi & Barnes, 1990) and it is unknown if an extract derived from other fish components will have the same impact or lead to redundancy in the regular *in vitro* medium.

In this study, we aimed to improve juvenile mussel transformation by 1) adding an experimental fish gill extract to provide additional nutrients to rabbit serum and 2) attempt to transform glochidia with fish sera from widespread non-host species that have been the subject of invasive species removal. Two mussel species with similar life histories were chosen for this study: *Atlantaconcha ochracea* and *Lampsilis cariosa*. Both species have a wide distribution across the Atlantic Slope region but are listed as vulnerable, imperiled, or critically imperiled throughout their range (Cummings & Cordeiro, 2012; NatureServe, 2020). In both species, gravid females brood long-term, wintering with glochidia until release in the spring. White perch is the only identified host of *A. ochracea* and it may be host-specific, while *L. cariosa* is a host-

generalist with a wide range of identified hosts including yellow perch (*Perca flavescens*), white perch (*Micropterus salmoides*), and largemouth bass (*Morone americana*) (NatureServe, 2020; Mair & Watson, personal communication). By comparing these two species, we hope to compare sera preferences among host-specific and host-generalist mussels and apply this information to other species with similar difficulties transforming *in vitro*.

Methods

Experimental design overview

Two experiments were conducted with each species (*A. ochracea* and *L. cariosa*) at separate time intervals based on availability of broodstock. In the first *in vitro* trial with *L. cariosa*, juvenile transformation in rabbit serum only treatments were compared against rabbit serum treatments supplemented with fish gill extract. Rabbit serum treatments with fish extract consisted of concentrations of 1, 2, 4 mL of fish extract (per liter of media) sourced from two fish species, white perch and largemouth bass (Table 1.1). In this trial, *L. cariosa* juvenile transformation was also compared between rabbit serum only treatments and blue catfish serum only treatments. All resulting treatments were replicated 5 times (n = 5). This trial was conducted at the Virginia Fisheries and Aquatic Wildlife Center (VFAWC; Charles City, Virginia) *in vitro* mussel propagation lab based at the VCU Rice River Center from August 16 to August 30, 2021.

The second *in vitro trial* also compared juvenile transformation of *A. ochracea* on rabbit only serum and rabbit serum supplemented with fish extract treatment. Rabbit serum treatments with fish extract consisted of concentrations of 1, 2, 4 mL of fish extract (per liter of media) sourced from white perch (*Micropterus salmoides*) or largemouth bass (*Morone americana*), as seen in Table 1.1. This experiment was conducted at the VFAWC *in vitro* mussel propagation

lab from December 10 to December 27, 2021. In a final trial, *A. ochracea* juvenile transformation was also compared between rabbit serum only, grass carp serum only, and 50:50 rabbit-carp sera mixture treatments. This experiment was conducted at the VFAWC *in vitro* mussel propagation lab from January 21 to February 9, 2021. All resulting treatments were replicated 5 times (n = 5).

Preparation of fish gill extract

Fish collected by the VFAWC to use as host fish for freshwater mussel propagation were not released and were instead euthanized by the request of Virginia Department of Wildlife Resources (VDWR). For this experiment, individuals intended for euthanasia were used to create fish gill extract. Excised gill tissues of largemouth bass (*Micropterus salmoides*) and white perch (*Morone americana*) were obtained from fish sacrificed by VFAWC staff or that were recently deceased (of natural causes within a 6-hr period). A 10% concentrate of fish extract was created by macerating tissue in basal M199 media (without serum) and centrifuging at 15,600 x *g* for 30 minutes. Resulting supernatant was preserved in freezer (0^{o}C) for later addition into *in vitro* culture medium. This procedure was modified from cell culture techniques found in Chen et al. (2003) and Collodi & Barnes (1990). Treatment levels were devised for a dosing regimen as illustrated in Table 1.1.

Preparation of fish serum

Blood was obtained from grass carp (*Ctenopharyngodon idella*) and blue catfish (*Ictalurus furcatus*) caught during invasive fish removal efforts on the James River. Fish were sacrificed by VFAWC staff and VDWR/VCU biologists via pithing/decapitation. Blood from the fish was obtained via cardiac or caudal vein puncture. Blood samples were aliquoted into 50-mL

conical tubes and centrifuged separately in the laboratory to remove clotting component. All sera was combined and filter sterilized using 0.45 and then 0.2 micron filters (Owen, 2009; Rosa et al., 2010).

Media preparation and incubator parameters

Basal media was created using a modified recipe from Owen (2009) and Ryan (2020). Serum (33%) was added to basal medium (67%) in a 1:3 ratio. Incubator was kept at 23-25^{o}C with a CO_2 level of 1.5% to maintain pH of 7.65. See Appendix A and B for ingredient list and more extensive media preparation protocol.

Glochidia extraction, viability, and plate inoculation

Gravid *L. cariosa* and *A. ochracea* females were collected by the VFAWC (from Nottoway River, Franklin, Virginia) and housed in a 6-8^{o}C flow through system with weekly 10% water changes until day 0 of the experiment. Glochidia were obtained by rupturing a gravid mussel's gills with a 22G needle and flushing out contents with autoclaved pondwater. Three 25-uL subsamples from each female were tested for viability (>80%) with emersion in a saline solution (Neves et al., 1985). Prior to inoculation in media, glochidia were once again rinsed in autoclaved water and basal media to remove debris and potential contaminants. Glochidia were loaded into 60 x 15 mm petri dishes containing 5mL of full medium using a 25-uL drop (estimated to contain 50-200 glochidia). After glochidia extraction, all females were tagged and returned to site of collection.

Assessing transformation and dilution protocol

Glochidia received media changes every 3-4 days and were monitored for media contamination and developmental growth. Transformation was assessed by the appearance of

adductor muscles and juvenile foot movement outside of the shell of at least one individual. All dishes were removed at the same time. To remove glochidia from media, 1 mL of chlorine-free water (at pH of 7.65) was added to each dish. After 15 minutes, an additional 2 mL was added. After another 15 minutes, an additional 3 mL was added. 3 mL of the diluted media mixture was removed before each dish was returned to the incubator and left to sit overnight. The next day, live juveniles were observed for foot movement and counted. Juveniles were converted to freshwater by being gently spun to the center of the dish and slowly diluted using a wash bottle with chlorine-free water until mixture was no longer tinged red from remaining media in mixture. Juveniles were then transferred to culture tanks.

Data analysis

Pictures of each replicate were taken on day 0 of the experiment and on dilution day. Glochidia/live juveniles were counted using the count function in ImageJ image processing program. A transformation rate was calculated for each replicate and defined as the difference between the number of glochidia in medium on day 0 and the number of transformed juveniles at the end of the *in vitro* trial. Any replicates that developed microbial contamination at any point during the study were removed from data analysis due to the likelihood of a decreased transformed rate compared to non-contaminated replicates.

To compare the effect of different serum treatment groups and combinations, one-way Analysis of Variance (ANOVA) models were run for each experiment using the ‘aov’ function in base R. Proportional data did not follow a normal distribution, so all data were arcsine transformed. Diagnostic plots using resulting ANOVA residuals were used to assess model fit and that ANOVA assumptions had not been violated. If ANOVA diagnostics were poor, non-parametric tests were conducted instead. Any significant differences identified from experiment

results ($p < 0.05$), were assessed with a Tukey's post-hoc test to examine significant differences between treatment groups.

Results

L. cariosa on blue catfish serum

On day 3 of *in vitro* trial, all replicates on blue catfish serum (n = 4) contained glochidia that were open in media and showed arrested tissue development; all were discarded. On day 7, two control replicates became contaminated and were removed from data analysis. All remaining control replicates (n = 3) had transformed juveniles by day 14 *in vitro* (Figure 1.1). For initial glochidia counts and final juvenile counts of uncontaminated replicates, refer to Table 1 in Appendix C.

A one-way ANOVA could not be performed between control and blue catfish treatments as diagnostic plots revealed a non-normal data distribution of arcsine transformed data points. A Mann-Whitney U Test was performed instead and there was a significant difference between the two treatments groups ($W= 0$, $p = 0.03$).

Comparison of fish extract treatments in L. cariosa *and* A. ochracea

L. cariosa replicates were transformed on Day 14 and transformation was high overall, ranging from 78-100% (Figure 1.2). Two control replicates developed contamination on Day 7 and were discarded (n = 3). Minor contamination appeared across all treatment groups and, while these replicates still resulted in juveniles, these replicates were removed from the ANOVA. Treatment groups were not significantly different from control group ($F_{6,16} = 2.74$, $p = 0.05$). Although marginally significant, we proceeded with a post-hoc Tukey test. L2 was the only treatment group different from the control group (p adjusted = 0.033). As a result, there appeared

to be no effect on the amount of fish extract in the medium and treatment groups were combined by host species (WHP, n = 10; LMB, n = 10). For initial glochidia counts and final juvenile counts of uncontaminated replicates, refer to Table 2 in Appendix C.

A. ochracea replicates were transformed *in vitro* by day 17 and transformation was low, ranging from 3-40% (Figure 1.3). Contamination did not appear in any replicate (n = 5 for all). Treatment groups were not significantly different from control group ($F_{2,32} = 2.04$, $p = 0.156$). There also appeared to be no effect on the amount of fish extract in the medium or difference between host and non-host fish extract. For initial glochidia counts and final juvenile counts of uncontaminated replicates, refer to Table 3 in Appendix C.

A. ochracea *full and mixed grass carp serum treatments*

Transformation for *A. ochracea* in this *in vitro* trial was much higher on average than the previous as replicates were given an extra day *in vitro* and diluted by day 18. As seen in Figure 1.4, control group transformation rate ranged from 55-70% (n = 5) and treatment groups (grass carp, n = 4; rabbit-carp, n = 5) had transformation rates upwards of 75%. Results of the ANOVA revealed a significant difference between groups ($F_{2,11} = 11.87$, $p = 0.002$). Post-hoc analysis showed no significant difference between carp serum treatments and rabbit-carp treatments (p adjusted = 0.441) but both treatment groups were significantly different from control replicates (p adjusted = 0.02, 0.001). For initial glochidia counts and final juvenile counts of uncontaminated replicates, refer to Table 4 in Appendix C.

Discussion

Blue catfish serum

All *L. cariosa* replicates in blue catfish treatments were dead by Day 3. In these replicates, all glochidia were open or showed signs of abnormal and arrested tissue development. Other species, including *Atlantaconcha ochracea* and *Strophitus undulatus* were also tested with blue catfish serum and showed similar results (Wetzell, unpublished data), suggesting a component in blue catfish sera was toxic to glochidia development. Similar effects were observed with yellow catfish (*Pylodictis olivaris*) plasma tested on *Potamilus alatus* and was also thought to be the result of "fatal factors" (Wen et al., 2018). Wen et al. (2018) proposed a pyramid model to understand the defensive barriers in place that could lead to success or failure of *in vitro* transformation, with fatal factors acting as the primary barrier to transformation before nonspecific immune factors and nutrient limiting factors (Wen et al., 2018). Organic and inorganic compounds in fish sera vary widely between fish species (Uthaiwan et al., 2003) and it may be important to trace what compounds are not only needed to induce transformation, but also compounds that are toxic to glochidia development. Additionally, future studies should continue to explore different fish sera sources, particularly from fish species that are readily available in the environment and perhaps the subject of invasive species removal.

Addition of fish extract

Given *L. cariosa*'s affinity for multiple fish hosts and previous high transformation *in vitro*, it was not surprising to find that fish extract had little to no effect on juvenile transformation in this study. Though there may have been a positive trend seen with largemouth bass extract, power of the ANOVA was limited by the small sample size. This result was also independent of fish tissue concentration and likely inconclusive. Fish extract also had no effect in the *A. ochracea* trial, which was unexpected as *A. ochracea* was a host-specific mussel with low metamorphosis on rabbit serum. These results are reminiscent of Fox (2014), where fish cell

lines incorporated in the *in vitro* culture medium had no effect on transformation despite being absorbed by glochidia. This was hypothesized to be the result of a lack in host-specific nutrients but, given that host fish were used in this study, it is possible that the concentration of fish tissue was too dilute or insufficient to make a significant change in nutrition of the media. An increased concentration in future experiments could prove to affect transformation. Additionally, a freeze-dried extract may remain a more appropriate form for incorporation into the M199 medium (Lima & Avelar, 2010).

Grass carp serum

The results of this study support grass carp serum as a beneficial addition to media for *in vitro* propagation of *Atlantaconcha ochracea*. First, however, it is important to note that transformation of the control group was much higher in this experiment than the control group used during the fish extract experiment, where transformation ranged from 3-40%, likely due to the extra day in media allotted to juveniles. It is possible that *in vitro* juveniles may have slightly different timelines for reaching full development, much like *in vivo* counterparts that drop off from host fish over a series of days or weeks (Lima et al., 2012), and were pulled too early from the experiment. Rather than wait for the appearance of adductor muscles to signify the end of development, movement or gaping from a few individuals in media was considered a better indicator. Nevertheless, range of average transformation in the control group (55-70%) was still lower than average transformation of grass carp or rabbit-carp treatments (75-100%). Unlike catfish, carp taxa have been used for successful transformation in other *in vitro* laboratory setups (Owen, 2009) and may be preferred for non-host serum usage if these taxa lack fatal factors (Wen et al., 2018).

Transformation rates of grass carp and rabbit-grass carp treatments were not significantly different from each other. Grass carp supply is limited due to difficulties associated with sourcing and processing blood from test organisms. However, if only half the amount of grass carp sera is needed per *in vitro* trial, this could extend the shelf life of grass carp sera while also cutting down costs and time related to fish blood extraction. Future experiments should attempt to further reduce ratio of grass carp serum to rabbit serum and identify a threshold of inducing transformation. Due to the wide availability of grass carp in hatcheries or caught during invasive species removal, this could be a preferred alternative for not only *A. ochracea* propagation but also other mussel species that do not transform with mammalian sera. Later experiments were attempted on *Dromus dromas* and *Cyprogenia stegaria* using grass carp serum, but results were inconclusive due to the limited supply (Wetzell, unpublished).

Conclusion

Through this study, we observed that different mussel species require different nutritional components in the *in vitro* culture media that may not be incorporated into widely used M199 media formulations utilizing commercially available sera such as rabbit. This has made *in vitro* propagation difficult for species such as *Atlantaconcha ochracea,* a mussel with only one identified host that does not do well in captive settings. However, the incorporation of grass carp sera – a non-host fish of *A. ochracea* – was able to significantly improve juvenile yields. In future investigations, we plan to use a rabbit-carp sera mixture for other freshwater mussel species that have struggled to transform *in vitro* in the hope that we can continue to refine our propagation toolbox towards conservation efforts.

Tables

Table 1.1. Treatment groups for testing concentration of fish extract added to rabbit serum. Treatments were organized by amount of gill extract per liter of media (1, 2, or 4 mL) and fish source – white perch (*Morone americana*) or largemouth bass (*Micropterus salmoides*). Fish species chosen were based on host/non-host species of *Atlantaconcha ochracea.* Each treatment was replicated five times (n = 5).

	Treatment groups		
Amount of liquid gill extract per liter of media	1 mL	2 mL	4 mL
White Perch (Host) replicates	W1	W2	W3
Largemouth Bass (Non-Host) replicates	L1	L2	L3

Figures

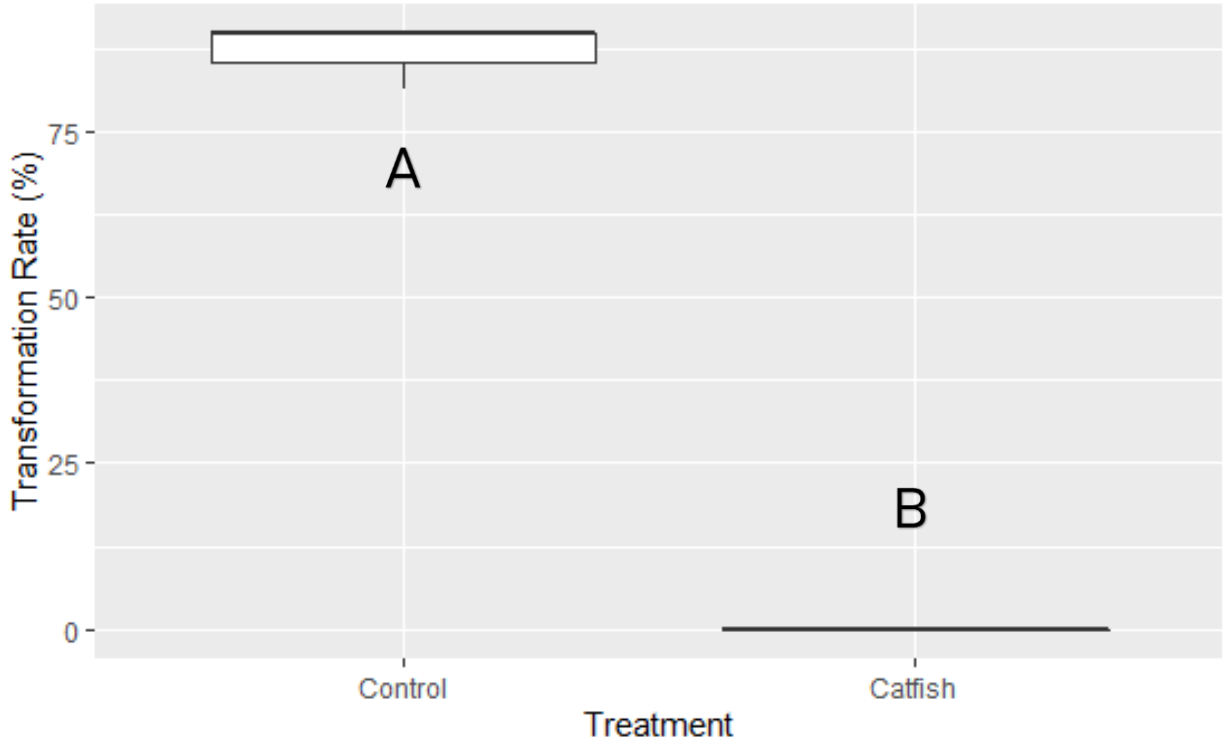

Figure 1.1. Transformation rate of *Lampsilis cariosa* cultured *in vitro* using different serum treatments: rabbit control (n = 3) or blue catfish (n = 4). Transformation ranged from 0-100%. Significant differences between A and B (W= 0, p = 0.03).

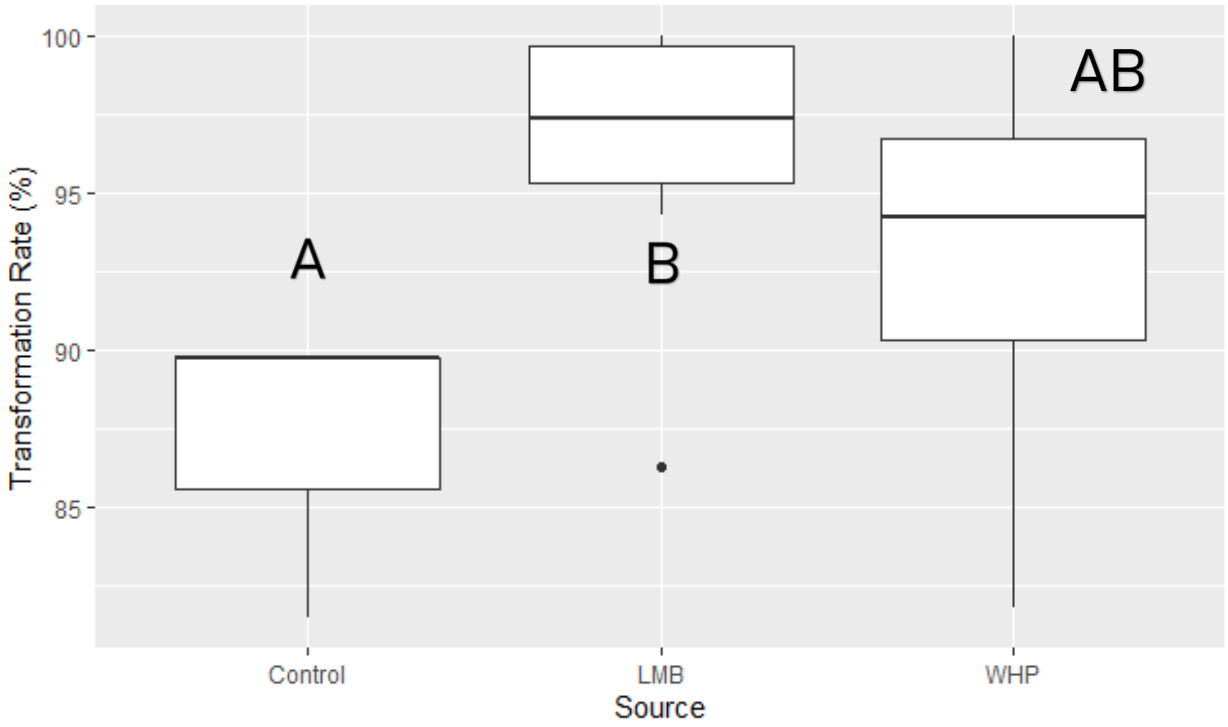

Figure 1.2. Transformation rate of *Lampsilis cariosa* cultured *in vitro* on rabbit serum with different liquid fish extract treatments: control (n = 5), largemouth bass (LMB; n = 10) or white perch (WHP; n = 10). Transformation ranged from 78-100%. ANOVA results were close to significance ($F_{6,16}$ = 2.74, p = 0.0501) and post-hoc suggested difference between A and B (p-adjusted = 0.033).

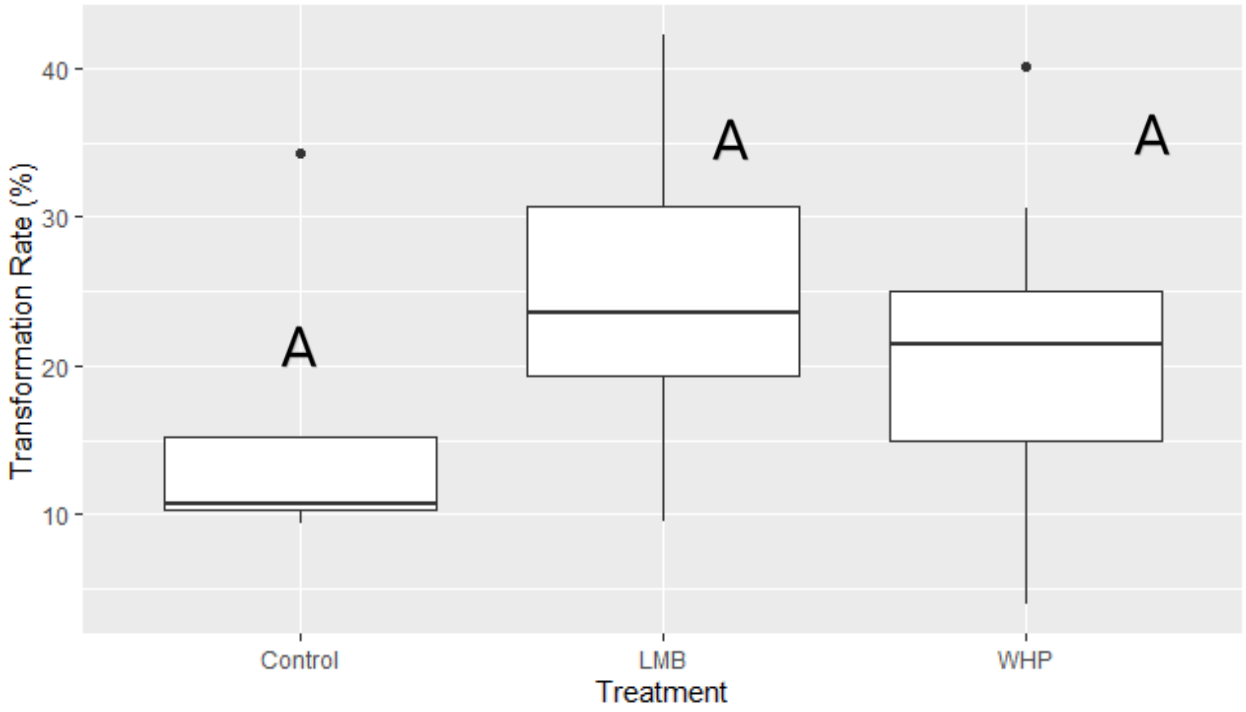

Figure 1.3. Transformation rate of *Atlantaconcha ochracea* cultured *in vitro* on rabbit serum with different liquid fish extract treatments: control (n = 5), largemouth bass (LMB; n = 15) or white perch (WHP; n = 15; $F_{2,32} = 2.04$, $p = 0.156$). Transformation ranged from 3-45%.

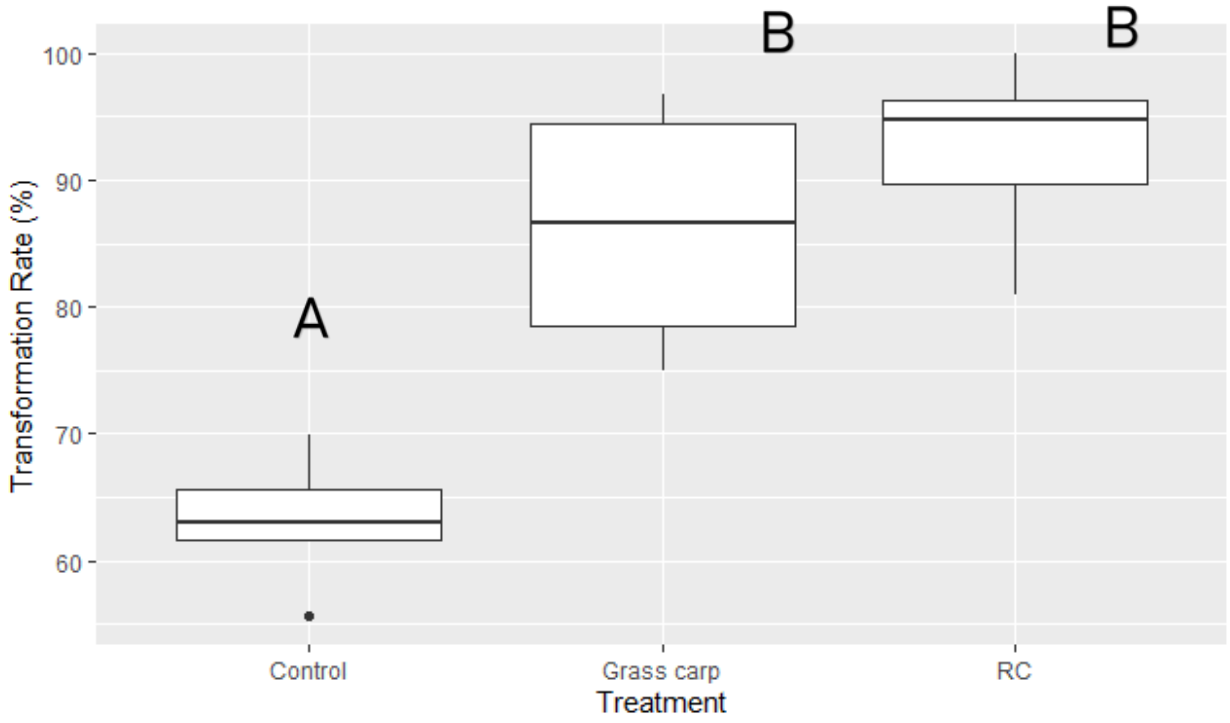

Figure 1.4. Transformation rate of *Atlantaconcha ochracea* cultured *in vitro* using different serum treatments: rabbit control (n = 5), grass carp (n = 4), and 50:50 rabbit-grass carp mix (n = 5). Transformation ranged from 55-100%. $F_{2,11} = 11.87$, p = 0.002. Significant difference between A and B (p adjusted = 0.02, 0.001).

Citations

Bagaria Osuna, M. (2016). Cultiu in vitro de nàiade allargada (*Unio mancus* Lamarck 1819) en condicions de laboratori. Universitat de Girona. Thesis.

Chen, S.-L., Sha, Z.-X., & Ye, H.-Q. (2003). Establishment of a pluripotent embryonic cell line from sea perch (Lateolabrax japonicus) embryos. *Aquaculture*, *218*(1–4), 141–151.

Collodi, P., & Barnes, D. W. (1990). Mitogenic activity from trout embryos. *Proceedings of the National Academy of Sciences*, *87*(9), 3498–3502.

Cummings, K., & Cordeiro, J., 2012. *Leptodea ochracea*. The IUCN Red List of Threatened Species. https://dx.doi.org/10.2305/IUCN.UK.2012.RLTS.T11686A503405.en.

DWR. (2020). *Freshwater Mussels.* Virginia Department of Wildlife Resources. https://dwr.virginia.gov/wildlife/freshwater-mussels/

Escobar-Calderón, F., Vodáková, B., & Douda, K. (2020). Early responses of in vitro cultured fish-growing glochidia: The effects of taurine, lipids and sera on *Margaritifera margaritifera*. *Aquaculture Research, 51*(3), 1069-1076.

Fox, T. R. (2014). *Studies on the in vitro Propagation of Freshwater Mussels with Implications for Improving Juvenile Health.* North Carolina State University. Thesis.

Henley, W. F., & Neves, R. J. (2001). Behavioral responses of glochidia of freshwater mussels (Bivalvia: Unionidae) to chemical cues of fish. *American Malacological Bulletin*, *16*(1/2), 131-136.

Isom, B.G. & R.G. Hudson. (1984). Freshwater mussels and their fish hosts; physiological aspects. *The Journal of Parasitology*, 70:318-319.

Jones, J. W. (2015). Freshwater mussels of Virginia (Bivalvia: Unionidae): An introduction to their life history, status and conservation. *Virginia Journal of Science*, *66*(3), 6.

Joyce, A., & Vogeler, S. (2018). Molluscan bivalve settlement and metamorphosis: Neuroendocrine inducers and morphogenetic responses. *Aquaculture, 487*, 64–82.

Kern, M. A. (2017). *Simplifying methods for in vitro metamorphosis of glochidia*. Missouri State University. Thesis.

Lima, P., Lima, M. L., Kovitvadhi, U., Kovitvadhi, S., Owen, C., & Machado, J. (2012). A review on the *in vitro* culture of freshwater mussels (Unionoida). *Hydrobiologia*, *691*(1), 21–33.

Lima, R. C., & Avelar, W. E. (2010). A new additive to the artificial culture medium for freshwater bivalve culture *in vitro*. *Invertebrate Reproduction & Development*, *54*(2), 89–94.

Ma, X.-Y., Wen, H.-B., Zou, J., Jin, W., Hua, D., Gu, R.-B., & Xu, P. (2018). An improved method for *in vitro* culture of glochidia in freshwater mussel *Cristaria plicata* (Mollusca, Bivalvia). *Hydrobiologia*, *810*(1), 133–144.

NatureServe. (2020). *NatureServe Explorer*. https://explorer.natureserve.org/

Neves, R.J., L.R. Wever, and A.V. Zale. 1985. An evaluation of host fish suitability for glochidia of *Villosa Vanuemi* and *V. nebulosi* (Pelecypoda: Unionidae). The American Midland Naturalist 113:13-19.

Owen, C. T. (2009). *Investigations for the conservation and propagation of freshwater mussels*. University of Louisville. Dissertation.

Rosa, J., Laizé, V., Dias, J., & Leonor Cancela, M. (2010). *Preparation of fish serum for the culture of marine fish cell lines*. Association of European Marine Biological Laboratories. https://www.assemblemarine.org/assets/Uploads/Documents/tool-box/Preparation-of-fish-serum-for-the-culture-of-marine-fish-cell-lines.pdf

Rott, N. (2019, December 6). Nature's "Brita Filter" Is Dying And No One Knows Why. *National Public Radio*. https://www.npr.org/2019/12/06/784422726/natures-brita-filter-is-dying-and-nobody-knows-why

Ryan, J. (2020). *In-vitro Propagation and Fish Assessments to Inform Restoration of Dwarf Wedgemussel (Alasmidonta Heterodon)*.University of Massachusetts Amherst. Thesis.

Uthaiwan, K., Noparatnaraporn, N., & Machado, J. (2001). Culture of glochidia of the freshwater pearl mussel *Hyriopsis myersiana* (Lea, 1856) in artificial media. *Aquaculture*, *195*(1–2), 61–69.

Uthaiwan, K., Pakkong, P., Noparatnaraporn, N., Vilarinho, L., & Machado, J. (2002). Study of a suitable fish plasma for in vitro culture of glochidia *Hyriopsis myersiana*. *Aquaculture*, *209*(1–4), 197–208.

Uthaiwan, K., Pakkong, P., Noparatnaraporn, N. V., Vilarinho, L., & Machado, J. (2003). Studies on the plasma composition offish hosts of the freshwater mussel, *Hyriopsis myersiana*, with implications for improvement of the medium for culture of glochidia. *Invertebrate Reproduction & Development*, *44*(1), 53–61.

Vaughn, C. C., Nichols, S. J., & Spooner, D. E. (2008). Community and foodweb ecology of freshwater mussels. *Journal of the North American Benthological Society*, *27*(2), 409–423.

Walter, M. J. (2020). *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.* North Carolina State University. Thesis.

Wen, H. B., Jin, W., Ma, X. Y., Zheng, B. Q., Xu, P., Xu, L., Hua, D., Yuan, X. H., & Gu, R. B. (2018). Vitro culture of axe-head glochidia in pink heelsplitter *Potamilus alatus* and

mechanism of its high host specialists. *PloS one, 13*(2), e0192292.

https://doi.org/10.1371/journal.pone.0192292

CHAPTER 2

COMBATTING MICROBIAL CONTAMINATION DURING *IN VITRO* PROPOGATION OF *ELLIPTIO COMPLANATA*

Abstract

In vitro propagation allows for transformation of freshwater mussel juveniles without a fish host using modified cell culture techniques. However, microbial contamination can greatly decrease the likelihood of successful transformation. A broad-spectrum antimicrobial mixture of rifampicin, carbenicillin, gentamycin, and amphotericin b (RCGA) is used to curb the proliferation of microbes, but this may not be suitable for all types of contamination. Additionally, some antimicrobial compounds such as amphotericin b can negatively impact juvenile transformation at higher concentrations. In this study, an alternative antimicrobial mixture, Primocin™ (InvivoGen, San Diego, California, Cat. #ant-pm-2), was considered for *in vitro* propagation of *Elliptio complanata*. Primocin™ was assessed against the original RCGA mixture to determine its efficacy and test for toxicity to transforming juveniles. Antimycotic components were also tested at lower concentrations to determine if microbial contamination can still be controlled without impact on glochidia development. Contaminated replicates underwent DNA extraction and analysis to identify bacterial and fungal pathogens. While Primocin™ successfully curbed microbial proliferation, *Elliptio complanata* transformers showed no signs of tissue development. In RCGA treatments, there was no significant difference between replicates with or without amphotericin b. Results of DNA analysis identified unique contamination for each replicate without antimicrobials. Contamination could be attributed to known pathogens that were ubiquitous across a range of environments or common in shellfish and aquaculture production.

Introduction

Freshwater mussel propagation operations have expanded in recent years as an important part of conservation and stream restoration initiatives. Captive breeding programs have taken the form of artificial inoculation of captive fish or modified cell culture techniques to induce transformation without a fish host (Lima et al., 2012). *In vitro* propagation can be the preferred method for mussel species with hosts that are unidentified or difficult to keep in captivity, as well as mussels with low population numbers lacking adequate numbers of gravid females or low glochidia production (Owen, 2009; Lima et al., 2012). However, to promote successful propagation, the *in vitro* medium must provide adequate nutrients for glochidia development while incorporating antibacterial and antimycotic compounds to curb microbial proliferation in such a nutrient rich environment (Owen, 2009; Owen et al., 2010).

Microbes enter the *in vitro* culture medium with glochidia, likely originating from the gravid female. As freshwater mussels are filter-feeding bivalves, they accumulate both harmless and pathogenic microorganisms from the surrounding aquatic environment. Problems associated with this behavior become more pronounced as bivalves begin to exist in more human-impacted watersheds (Potasman et al., 2002). Microorganisms, like other particles, are processed through the gills which is also the residing place of glochidia within the female mussel (Kern, 2017; Potasman et al., 2002). Once entering the *in vitro* culture medium, any bacteria and fungi carried by glochidia – particularly those with pathogenic traits associated with quick growth and toxin production – may become more prevalent to take advantage of the shift in nutrient availability (Brown et al., 2012). Poor sterile techniques can also result in the introduction of additional microbes (Barile, 1973). This contamination, if left unchecked, will eventually starve or poison

glochidia before proper transformation can occur. Likelihood of *in vitro* contamination also increases with mussel species that require longer incubation periods (Kern, 2017; Owen, 2009).

Accepted antibiotics for *in vitro* freshwater mussel propagation (at concentrations of 100 ug/mL) include rifampicin, gentamycin, and carbenicillin together with antimycotic amphotericin b (concentration of 5 ug/mL). Low concentrations are essential for avoiding potential toxicity to glochidia development, particularly in the case of amphotericin b, which binds to sterols and disrupts multicellular tissue development (Ryan, 2020). Cytotoxicity may be species-dependent, as development was altered in species in concentrations as low as 5ug/mL (Owen 2009; Ryan, 2020) whereas *Cristaria plicata* tolerated a concentration as high as 50 ug/mL (Ma et al. 2018; Ryan, 2020). Given amphotericin b is also a broad-spectrum antimycotic, it may be worth seeking out other antimicrobials targeting specific pathogens and with less impact on glochidia development.

This study aims to better control microbial contamination in *Elliptio complanata* by 1) comparing an alternative antimicrobial mixture, Primocin™ (InvivoGen, San Diego, California, Cat. #ant-pm-2), to the original rifampicin, carbenicillin, gentamycin, and amphotericin b (RCGA) mixture and assessing toxicity of Primocin™ on transforming juveniles, 2) determining the efficacy of low amphotericin b concentrations on reducing fungal contamination, and 3) identifying potential pathogenic microbes that may appear in the *in vitro* culture medium to target future control efforts.

Methods

Experimental design overview

This *in vitro* trial was conducted at the Virginia Fisheries and Aquatic Wildlife Center (VFAWC; Charles City, Virginia) *in vitro* mussel propagation lab based at the VCU Rice River Center from June 15 to June 23, 2021. Basal media was created according to Ryan (2020), modified from Owen (2009) and serum (33%) was added to both basal media groups (67%) in a 1:3 ratio.

RCGA basal media was created with rifampicin, carbenicillin, and gentamycin at concentrations of 100 mg per liter of media. Primocin™ basal media was created with at a concentration of 2mL per L (100 mg/L), which was deemed comparable to RCGA antibiotic concentrations according to the manufacturer's instructions. Dishes were directly inoculated with antifungal prior to glochidia inoculation. RCGA dishes received 0, 0.25, or 1 ug/mL of amphotericin b. Primocin™ dishes received 0.25 or 1 ug/mL of Fungin™; because the antimycotic compound Fungin™ is already incorporated into Primocin™, the creation of a Primocin™ treatment with no antimycotic was not possible. Refer to Table 2.1 for description of treatment groups.

Broodstock collection and housing

This study was conducted with *Elliptio complanata* due to their wide availability and presence of conglutinates. *Elliptio complanata* are short-term brooders, releasing glochidia in the summer months in a mucus conglutinate which easily adheres to contaminated surfaces in holding troughs. Stringent protocols including glochidia rinses and appropriate antimicrobial doses are essential for avoiding contamination while the species is *in vitro.* Gravid females were collected by the VFAWC from Broad Run (Manassas, Virginia) and housed in short-term brooding troughs at 18 degrees Celsius with weekly 10% water changes until day 0 of the experiment. After glochidia extraction, all females were tagged and returned to site of capture.

Glochidia extraction, viability, and plate inoculation

Glochidia were extracted from gravid females by piercing gills with a 22G needle and flushing out contents with autoclaved pondwater (Ryan, 2020). Three 25 uL subsamples from each female were tested for viability (>80%) with emersion in a saline solution (Neves et al., 1985). Prior to inoculation, glochidia were once again rinsed in autoclaved water and basal media. See Appendix A and B for ingredient list and more extensive media preparation protocol.

Glochidia were loaded into 60 x 15 mm petri dishes containing 5mL of full medium using a 25-uL drop (estimated to contain 50-200 glochidia). Incubator parameters were kept at 23-25^{o}C with a CO_2 level of 1.5%. Glochidia received media changes every 3-4 days and were monitored for media contamination and developmental growth.

Assessing transformation and dilution protocol

Transformation was observed with the appearance of adductor muscles and juvenile foot movement outside of the shell of at least one individual. To remove from media, 1 mL of chlorine-free water (at pH of 7.65) was added to each dish. After 15 minutes, an additional 2 mL was added. After another 15 minutes, an additional 3 mL was added. 3 mL of the diluted media mixture was removed before each dish was returned to the incubator and left to sit overnight. The next day, juveniles were once again observed for foot movement and counted. Juveniles were converted to freshwater by being gently spun to the center of the dish and slowly diluted using a wash bottle with chlorine-free water until mixture was no longer tinged red from remaining media in mixture. Juveniles were then transferred to culture tanks at the VFAWC.

DNA extraction and analysis of contaminated samples and Elliptio complanata *juveniles*

2 mL of media were sampled from all control replicates that developed full media contamination – resulting in glochidia death – and stored on ice at -20 degrees C for later DNA analysis to identify primary pathogens. Any transformed juveniles from *in vitro* trial were left to grow out in culture tanks for six months before being sacrificed for background analysis of freshwater mussel microbiome diversity. Five *Elliptio complanata* juveniles were removed from grow-out systems and frozen at -20 degrees Celsius in 2 mL of holding water.

All samples were processed and analyzed with the ZymoBIOMICS® Targeted Sequencing Service (Zymo Research, Irvine, CA). Any *Elliptio complanata* tissue was macerated with 2mm and 0.5mm bashing beads to prevent DNA lysing. All samples were run through an automized 96-well mag bead kit ran through automation (Zymo Research, Irvine, CA). Bacterial 16S ribosomal RNA gene targeted sequencing was performed using the Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine, CA). Fungal ITS gene targeted sequencing was performed using the Quick-16S™ NGS Library Prep Kit with custom ITS2 primers substituted for 16S primers (Zymo Research, Irvine, CA). The sequencing library was prepared using qPCR fluorescence readings and sequenced on Illumina® MiSeq™ with a v3 reagent kit (600 cycles). Resulting taxonomic assignment was performed using Uclust from Qiime v.1.9.1 and referenced through the Zymo Research Database, which is internally designed and curated (Callahan et al., 2016; Caporaso et al., 2010; Segata et al., 2011).

Data analysis

Pictures of each replicate were taken on day 0 of the experiment and on dilution day. Glochidia/live juveniles were counted using the count function in ImageJ image processing program. A transformation rate was calculated for each replicate and defined as the difference

between the number of glochidia in medium on day 0 and the number of metamorphosed juveniles at the end of the *in vitro* trial.

To compare the effect of antimicrobial mixture type and antimycotic concentrations, a one-way Analysis of Variance (ANOVA) model was run on different treatment groups using the 'aov' function in base R. Proportional data did not follow a normal distribution, so all data were arcsine transformed. Diagnostic plots using resulting ANOVA residuals were used to assess model fit and that ANOVA assumptions had not been violated. If ANOVA diagnostics were poor, non-parametric tests were conducted instead. Any significant differences identified from experiment results ($p < 0.05$), were assessed with a Tukey's post-hoc test to examine significant differences between treatment groups.

Results

Elliptio complanata and antimicrobials

By day 3 of the experiment, all controls showed visible signs of microbial contamination (i.e., pH color change from red to orange, cloudy media, appearance of biofilm). If possible, 2 mL of contaminated media were sampled and frozen at -20 degrees Celsius for further analysis. By day 6 of the experiment all Primocin™ treatments showed individuals with little to no organ development and were discarded, even though no microbial contamination appeared (Figure 2.2).

Fungal contamination appeared in one RCGA replicate without amphotericin b and any viable glochidia remaining were transferred to a new petri dish with clean media. Although contamination did not reappear, glochidia transformation was reduced compared to other replicates with no amphotericin b. Transformed individuals in all RCGA treatments showed

signs of adductor muscles and were transferred into grow out tank systems on Day 12. For initial glochidia counts and final juvenile counts, refer to Table 5 in Appendix C.

A significant difference was found between Primocin™ and RCGA treatment groups ($W = 0$, $p < 0.001$) but no significant in-group differences were found between RCGA treatments ($F_{2,11} = 1.445$, $p = 0.28$). However, replicates with no antifungal qualitatively had a wider variation in average transformation (Figure 2.1). For microbial contamination, both RCGA and Primocin™ treatments inhibited microbial growth (Figure 2.2).

Microbial composition of contaminated samples

Media samples from the contaminated control groups were observed to be less diverse than *Elliptio complanata* microbiome samples and no bacterial taxa found in juveniles were found in contaminated control samples. However, *Elliptio complanata* juveniles and contaminated control samples qualitatively had similar in-group diversity according to the Bradley-Curtis plot of bacterial beta diversity (Figure 2.3).

As seen with observations noted during sample collection, results of DNA analysis also revealed Control 4 contained some fungal contamination with an absolute abundance of 353 fungi genes and 2 fungi genome copies per uL. However, the fungi remained unidentified following DNA analysis. DNA sequences were then uploaded to the Basic Local Alignment Search Tool (BLAST) by the National Institutes of Health (Altschul et al., 1990). Though an exact identification was not produced, this sequence was similar to other sequences within the genus *Penicillium.*

Controls 1-3 and 5 only had bacterial contamination, with zero fungal genome copies per uL. Unique primary pathogens were found in controls 1 and 3. *Psedomanas* spp. accounted for

85% of control 1 species composition. *Aeromonas veronii* accounted for 68% and 87% of control 2 and control 5 species composition respectively. *Vibrio cholerae* and *Vibrio cholerae-mimicus* accounted for 99% of control 3 species composition. *Acinetobacter bouvetti-johnsonii* accounted for 86% of control 4 species composition. All bacterial taxa present (>0.01) in control samples are listed in Table 2.2.

Discussion

RCGA verses Primocin™

Transformation of *Elliptio complanata* juveniles was improved using the original RCGA antimicrobial mixture with water soluble derivatives of rifampicin and amphotericin b. Both antibiotics were originally used in their insoluble forms and their efficacy in the *in vitro* M199 media may have decreased as insoluble components are easily removed during filter sterilization. Primocin™, an alternative to the original RCGA mixture, was also proposed for controlling contamination if repeated outbreaks occurred (Henley, personal communication). However, all resulting replicates showed signs of toxicity early in development (Figure 2.1). While microbial contamination was controlled (Figure 2.2), *Elliptio complanata* glochidia in Primocin™ replicates showed little to no signs of tissue development when compared to RCGA replicates and were removed from the trial. Fungin™, the antimycotic component in Primocin™, was tested on *Strophitus undulatus* as an alternative to amphotericin b. This compound showed no signs of toxicity at low concentrations and transformation remained high (Wetzell, unpublished). Either a different antibacterial compound in Primocin™ may have been responsible for toxicity or lack of development was the result of a species-specific sensitivity (Ma et al. 2018).

Antimycotic concentration

All RCGA replicates resulted in successful transformation of *Elliptio complanata* juveniles despite varying levels of antimycotic. This is in contrast to other research highlighting the potential toxicity of amphotericin b (Ryan, 2020), though our study used lower concentrations of the antimycotic than described in Owen (2009). Additionally, while there was no significant difference in treatment groups with 0 ug/mL, 0.25 ug/mL, or 1 ug/mL of amphotericin b, range of average transformation across replicates with no antimycotic were qualitatively more variable than RCGA treatment groups that did contain amphotericin b. Only one replicate with antimycotic developed fungal contamination and, while the contamination did not reappear throughout the trial, this replicate had the lowest rate of transformation in this treatment group. It is possible that other replicates without antimycotic did not develop fungal contamination by chance due to stringent aseptic techniques than perceived antimycotic effect (Ryan et al. 2022). To prevent all possibility of contamination, amphotericin b should be added to M199 medium even in low concentrations (0.25-1 ug/mL).

Microbial Composition

Microbial identification of potential contaminants in the *in vitro* culture is essential for combatting and mitigating potential outbreaks, particularly when broad-spectrum antimicrobials used in the RCGA mixture are not effective. While contamination symptoms appeared similar across most controls, different pathogens appeared in each sample and at different abundances. In control 1 replicate, primary pathogens belonged to the genus *Pseudomonas*, which contains multiple known pathogens responsible for human infection and can be found in a wide variety of environments including soil, water, and vegetation. Some *Pseudomonas* species, while a few can develop antibiotic resistant strains, are susceptible to carbenicillin and gentamycin (Igleweski, 1996). The primary pathogen of control replicates 2 and 5 was *Aeromonas veronii*, a common

opportunistic pathogen in aquaculture with a wide variety of hosts, though it is moderately sensitive to rifampicin (Wang et al. 2021). Control 4 replicate featured the primary pathogen *Acinetobacter bouvetti-johnsonii,* a genus with known opportunistic pathogens able to persist in the environment (Kämpfer, 2014). Control 3 replicate contained the lowest bacterial diversity of all control samples, with primary pathogens *Vibrio cholerae* and *Vibrio cholerae-mimicus* making up the vast majority. *Vibrio cholerae* is another pathogen associated with shellfish aquaculture as bivalves filter feed in areas contaminated with human sewage. This bacterium is likely to persist in the gills of bivalves, the same location in which glochidia are held within a freshwater mussel prior to release (Potasman et al., 2002). It is predicated *Vibrio* species may become more common as water temperatures rise because of climate change (Baker-Austin et al. 2016).

Only one control developed fungal contamination, but this fungal species could not be identified using the Zymo Research database. BLAST results revealed the fungal species as potentially belonging to the genus *Penicillium* (Altschul et al., 1990), which lacks a robust reference database compared other fungi species (Visagie et al., 2014). Most *Penicillium* species are ubiquitous in the environment and present wherever there is organic material. The ability to produce mycotoxins, that is characteristic of most species within this genus, can make *Penicillium* sp. a significant threat to glochidia development (Visagie et al., 2014). Previous identification of fungal contamination *in vitro* was attributed to other potential pathogens, such as *Candida parapsilosis*, a species also common in aquatic and human environments (Ryan, 2020).

Of the primary bacterial species identified from control replicates, two are known bacterial pathogens in aquaculture not likely to be found in a laboratory setting and may have

originated from the gravid female mussel (Potasman et al., 2002; Wang et al. 2021). The other two bacterial pathogens, as well as the *Penicillium* fungus from Control 4 replicate, are ubiquitous across multiple environments and it is unknown if contamination originated from the gravid female mussel or mishandling in the laboratory setup. Nevertheless, *Pseudomonas* sp. and *Aeromonas veronii* are both sufficiently inhibited by the current RCGA antimicrobial mixture (Igleweski, 1996; Wang et al. 2021).

Identifying the bacterial and fungal pathogens responsible for contamination enabled us to compare to microbial contamination found in other *in vitro* laboratory setups for freshwater mussel propagation. For researchers at the Freshwater Mussel Conservation Center (FMCC, Virginia Tech, Blacksburg, VA) primary pathogens were identified as *Stenotrophomonas maltophilia, Delftia acidovarans,* and *Chryseobacterium* sp. The bacterial pathogens, unlike those identified at VFAWC, can develop high levels of antibiotic resistance and were not susceptible to the original RCGA mixture proposed by Isom and Hudson (Jones & Watson, personal communication). Combined with the fungal identification of *Candida parapsilosis* as a contaminant during other *in vitro* trials (Ryan, 2020), it is likely that microbial contamination varies between laboratory setups due to geographic location and broodstock source. Therefore, individual identification of primary pathogens as contamination arises and choosing appropriate, non-toxic antimicrobials is essential for *in vitro* propagation success.

Conclusion

Microbial contamination remains one of the greatest hindrances to successful *in vitro* propagation of freshwater mussels (Owen et al. 2010). Despite the use of antimicrobials within the *in vitro* culture medium, microbial pathogens can persist if these compounds are not in appropriate forms (ie. water solubility) or are not suitable for controlling specific bacterium. This

research suggests that pathogens entering the *in vitro* medium are likely unique in different laboratory setups. Future researchers should identify their own microbial contamination and reconsider whether the general antibiotic formulation used for *in vitro* – rifampicin, carbenicillin, gentamycin, and amphotericin b (RCGA) – are appropriate for tackling these primary pathogens. However, some antimicrobial compounds, while successful at controlling contamination like Primocin™, can be toxic to glochidia development and require testing before incorporation into the *in vitro* protocol. By effectively managing microbial contamination without causing harm to glochidia, this could allow for more successful *in vitro* transformation, particularly for imperiled species where all glochidia must be maximized to their fullest potential and used towards freshwater mussel conservation efforts.

Tables

Table 2.1. Treatment groups for testing antibiotic mixture and antimycotic concentration on transformation of *Elliptio complanata*. Treatments were organized by antibiotic mixture (RCGA or Primocin™) and antimycotic concentration (none, 0.25 ug/mL, 1.0 ug/mL). As antimycotic compound Fungin™ is already present in Primocin™ formulation, creation of a Primocin™ treatment with no antimycotic was not possible. Each treatment was replicated 5 times (n = 5).

Treatment Group	Type of antibiotic mixture	Antimycotic concentration
Control	No antibiotics	No antimycotics
RCGA	RCG	No amphotericin B
RCGA	RCG	0.25 ug amphotericin B per mL
RCGA	RCG	1.0 ug amphotericin B per mL
Primocin™	Primocin™	0.25 ug Fungin™ per mL
Primocin™	Primocin™	1.0 ug Fungin™ per mL

Table 2.2. Microbiome analysis of potential bacterial pathogens found in control replicates 1-5. Only bacterial taxa present in the community above 1% are listed.

Contaminated Media Samples	**Primary Bacterial Pathogens (Present > 0.01)**	**Proportion of Community Makeup**
Control 1	*Pseudomonas* sp.	0.85
	Rhizobium rosettiformans-selenitireducens-vitis	0.12
	Unidentified, Family Rhizobiaceae	0.02
Control 2	*Aeromonas veronii*	0.68
	Acidovorax defluvii	0.12
	Acinetobacter tjernbergiae	0.11
	Rhizobium rosettiformans-selenitireducens-vitis	0.07
Control 3	*Vibrio cholerae*	0.88
	Vibrio cholerae-mimicus	0.11
Control 4	*Acinetobacter bouvetii-johnsonii*	0.86
	Rhizobium rosettiformans-selenitireducens-vitis	0.13
Control 5	*Aeromonas veronii*	0.87
	Aeromonasallo saccharophila	0.10

Figures

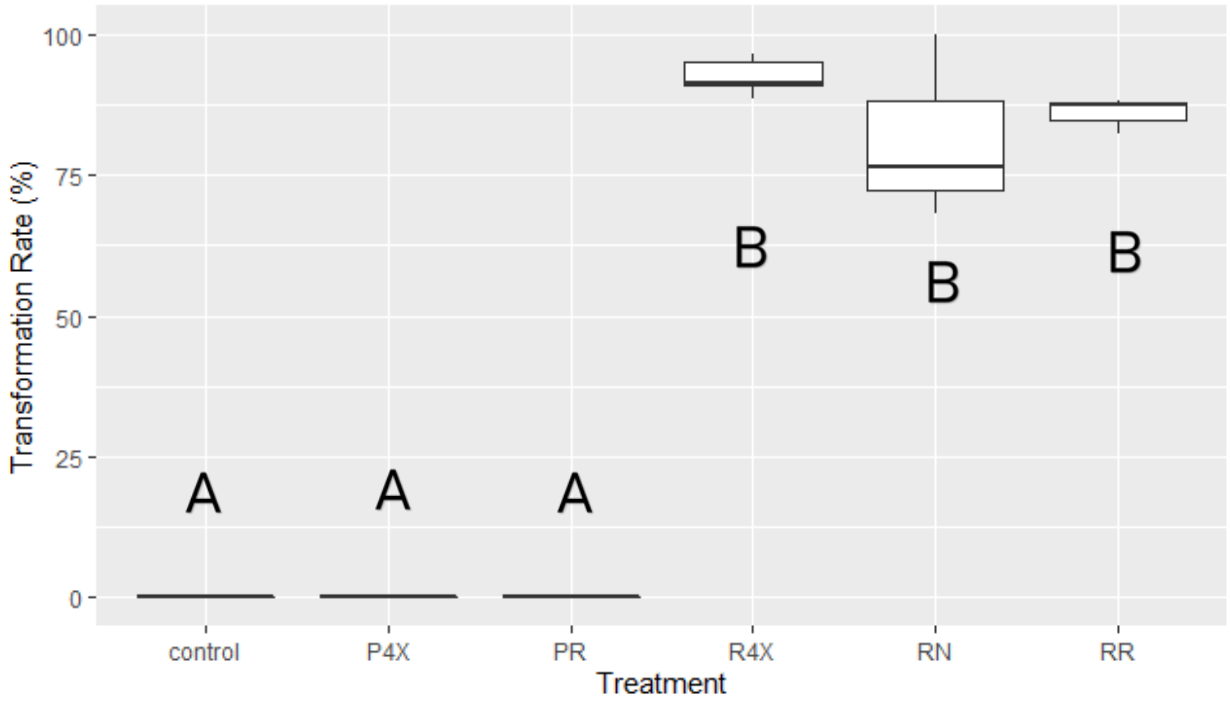

Figure 2.1. Transformation of *Elliptio complanata* in RCGA antimicrobial treatments. Control and Primocin™ antimicrobial treatments died prematurely and resulted in zero juvenile transformation. RCGA treatments were the only groups that survived until the end of the *in vitro* trial. Transformation rate ranged from 0-100%. A significant difference was found between Primocin and RCGA treatment groups ($W = 0$, $p < 0.001$) but no significant difference was found between RCGA treatment groups ($F_{2,11} = 1.45$, $p = 0.28$).

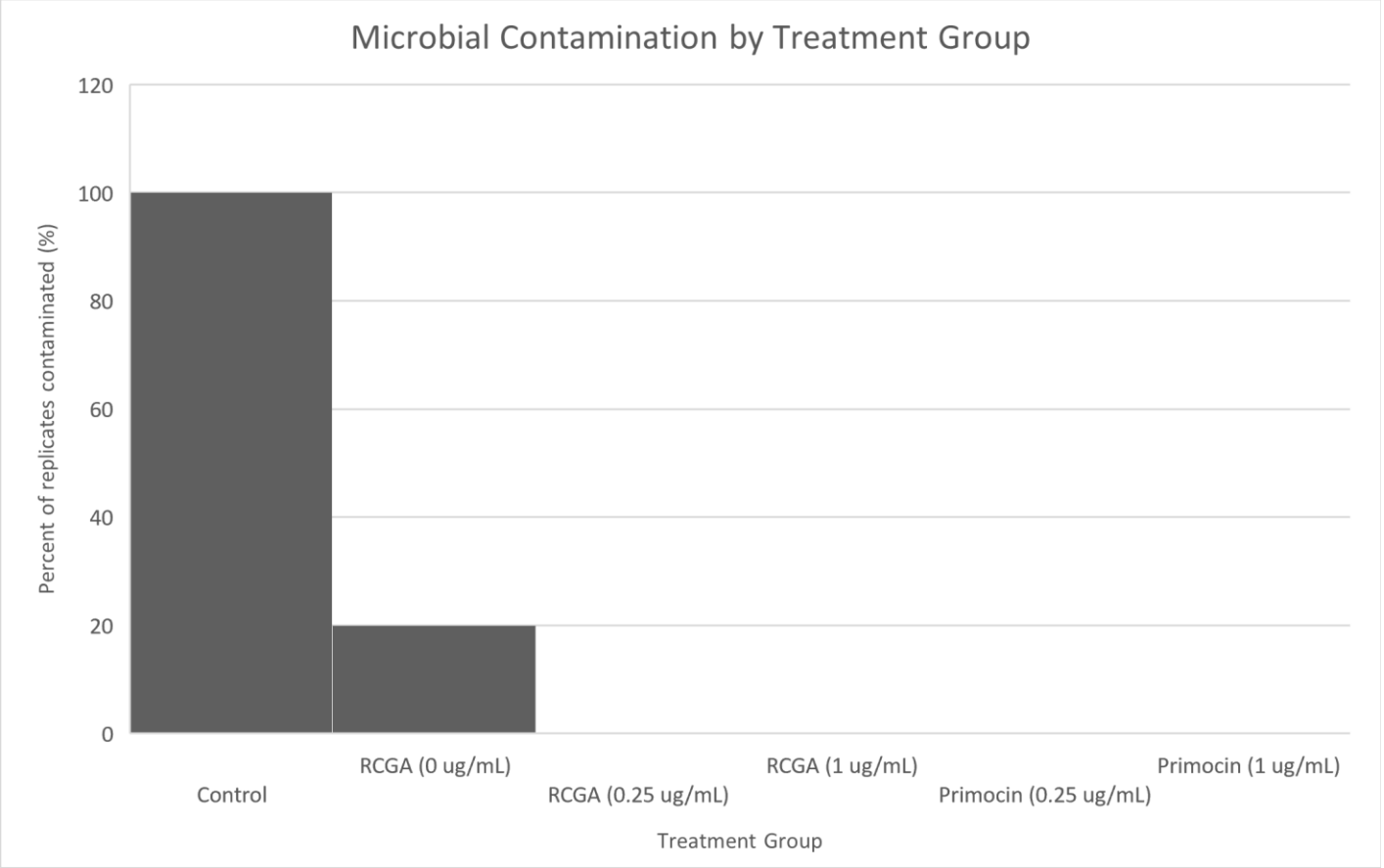

Figure 2.2. Percentage of replicates with microbial contamination per treatment group. Microbial contamination appeared in all control replicates (100%) and one RCGA replicate with no amphotericin b (20%). All other treatment groups had no microbial contamination.

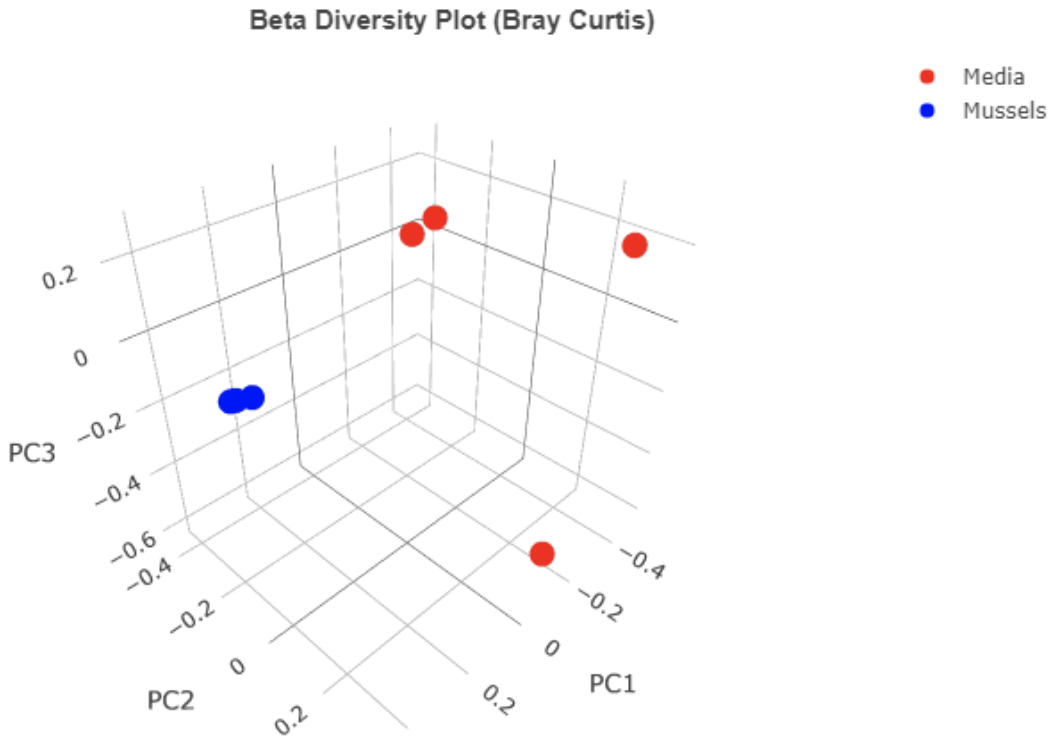

Figure 2.3. Bradley-Curtis plot of bacterial beta diversity between contaminated control replicates and *Elliptio complanata* juveniles performed with Qiime v.1.9.1 (Caporaso et al., 2010). Contaminated control replicates were less diverse than and grouped separately in the matrix from *Elliptio complanata* juveniles. Some bacterial taxa were also unique between control replicates and points are grouped much farther apart than *Elliptio complanata* juveniles.

Citations

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*. 215:403-410.

Baker-Austin, C., Trinanes, J. A., Salmenlinna, S., Löfdahl, M., Siitonen, A., Taylor, N. G., & Martinez-Urtaza, J. (2016). Heat wave–associated vibriosis, Sweden and Finland, 2014. *Emerging infectious diseases*, 22(7), 1216.

Barile, M. F. (1973). Mycoplasma contamination of cell cultures: Incidence, source, prevention, and problems of elimination. In *Tissue Culture* (pp. 729–735). Elsevier.

Brown, S. P., Cornforth, D. M., & Mideo, N. (2012). Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. *Trends in microbiology*, *20*(7), 336–342. https://doi.org/10.1016/j.tim.2012.04.005

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., Holmes, S.P. (2016). DADA2: High resolution sample inference from Illumina amplicon data. Nat Methods 13(7):581-3. doi: 10.1038/nmeth.3869

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K. ... Knight R. (2010). QIIME allows analysis of high-throughput community sequencing data. Nat Methods (7): 335-336. doi: 10.1038/nmeth.f.303

DWR. (2020). *Freshwater Mussels*. Virginia Department of Wildlife Resources. https://dwr.virginia.gov/wildlife/freshwater-mussels/

Iglewski BH. (1996). *Pseudomonas*. S. Baron (Ed.), Medical Microbiology (Fourth edition), University of Texas Medical Branch, Galveston TX (1996), Chapter 27.

Kämpfer, P. (2014). Acinetobacter. C.A. Batt, M.L. Tortorello (Eds.), Encyclopedia of Food Microbiology (Second edition), Academic Press, Oxford (2014), pp. 11-17

Kern, M. A. (2017). *Simplifying methods for in vitro metamorphosis of glochidia*. Missouri State University. Thesis.

Lima, P., Lima, M. L., Kovitvadhi, U., Kovitvadhi, S., Owen, C., & Machado, J. (2012). A review on the "in vitro" culture of freshwater mussels (Unionoida). *Hydrobiologia*, *691*(1), 21–33.

Ma, X.-Y., Wen, H.-B., Zou, J., Jin, W., Hua, D., Gu, R.-B., & Xu, P. (2018). An improved method for in vitro culture of glochidia in freshwater mussel Cristaria plicata (Mollusca, Bivalvia). *Hydrobiologia*, *810*(1), 133–144.

NatureServe. (2020). *NatureServe Explorer*. https://explorer.natureserve.org/

Owen, C. T. (2009). *Investigations for the conservation and propagation of freshwater mussels*. University of Louisville. Dissertation.

Owen, C. T., Alexander Jr, J. E., & McGregor, M. (2010). Control of microbial contamination during in vitro culture of larval unionid mussels. *Invertebrate Reproduction & Development*, *54*(4), 187-193.

Potasman, I., Paz, A., & Odeh, M. (2002). Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. *Clinical Infectious Diseases*, *35*(8), 921-928.

Rott, N. (2019, December 6). Nature's "Brita Filter" Is Dying And No One Knows Why. *National Public Radio*. https://www.npr.org/2019/12/06/784422726/natures-brita-filter-is-dying-and-nobody-knows-why

Ryan, J. (2020). *In-vitro Propagation and Fish Assessments to Inform Restoration of Dwarf Wedgemussel (Alasmidonta Heterodon)*. University of Massachusetts Amherst. Thesis.

Ryan, J. E., Roy, A. H., Hazelton, P. D., Perkins, D. L., Warren, T., Walsh, C., & Wick, R. (2022). Assessing methods for mitigating fungal contamination in freshwater mussel in vitro propagation. *Hydrobiologia*, 849(11), 2487-2501.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., & Huttenhower, C. (2011) Metagenomic biomarker discovery and explanation. Genome Biology (12): R60. doi: 10.1186/gb-2011-12-6-r60

Visagie, C. M., Houbraken, J., Frisvad, J. C., Hong, S. B., Klaassen, C. H., Perrone, G., Seifert, K. A., Varga, J., Yaguchi, T., & Samson, R. A. (2014). Identification and nomenclature of the genus Penicillium. *Studies in mycology, 78*, 343–371. https://doi.org/10.1016/j.simyco.2014.09.001

Walter, M. J. (2020). *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.* North Carolina State University. Thesis.

Wang, B., Mao, C., Feng, J., Li, Y., Hu, J., Jiang, B., ... & Su, Y. (2021). A First report of *Aeromonas veronii* infection of the sea bass, *Lateolabrax maculatus* in China. *Frontiers in Veterinary Science*, 7, 600587.

Appendix A: Basal media ingredients

Ingredient list modified from Ryan, 2020.

- 1500 mL Chlorine-free water
- 10 g M199 powder
- 2.6 g D-(+)- Galactose
- 2.0 g D- (+) - Glucose
- 25 mg 99% L-Ornithine monohydrochloride
- 40 mg L-Taurine
- 200 mg Carbenicillin disodium salt solution
- 200 mg Gentamicin sulfate salt solution
- 200 mg Rifampicin (in DMSO solution)
- 1mg Amphotericin B (in DMSO solution)
- 1.5 mL Lipid Mixture
- 1.5 mL MEM Vitamins
- 1.5 mL Menhaden oil
- 0.75 mL MEM Nonessential Amino Acid solution
- 1.5 mL MEM Amino Acid solution

Appendix B: Media preparation

Steps for media preparation were based on techniques described in Ryan 2020.

1. Serum should take up a third of complete media solution (33%); 500 mL of rabbit serum were added to 1500 mL of basal media.
2. Defrosted rabbit serum was heat-treated by warming it in a 56 degrees Celsius hot water bath for 30 minutes.
3. Basal media ingredients (Appendix A) were combined in 2L Erlenmeyer flask. Stirring rod was used to agitate media until all solid compounds were dissolved.
4. Heat-treated rabbit serum and basal medium were combined.
5. Using sodium hydroxide (NaOH), complete medium was buffered to a pH of 7.65. If medium went above desired pH, hydrochloric acid (HCL) was used to bring pH back down.
6. Complete medium was sterilized through a 0.45-micron filter (using vaccum filter setup).
7. Medium was sterilized again through a 0.2- or 0.1-micron filter.
8. Under sterile laminar flow hood, media into 50 mL aliquots. Aliquots were then stored in freezer (-20 degrees Celsius).

Appendix C: Supplementary tables

Table C. 1. *L. cariosa* glochidia and juvenile counts from control and blue catfish treatment groups. Each treatment was started with five replicates (n = 5), but any replicates that developed contamination were removed from analysis. Each replicate was inoculated with an estimated 50-200 glochidia but it is possible pipetting differences resulted in replicates with more than 200.

Treatment Group	Replicate	Initial # of glochidia	# of juveniles
Catfish	1	107	0
Catfish	2	151	0
Catfish	3	102	0
Catfish	4	92	0
Control	2	265	238
Control	3	146	131
Control	4	275	224

Table C.2. *L. cariosa* glochidia and juvenile counts from control, rabbit serum with white perch gill extract (WHP), and rabbit serum with largemouth bass gill extract (LMB). Each treatment group was started with five replicates (n = 5), but any replicates that developed contamination were removed from analysis. Each replicate was inoculated with an estimated 50-200 glochidia but it is possible pipetting differences resulted in replicates with more than 200.

Treatment Group	Concentration Level	Replicate	Initial # of glochidia	# of juveniles
Control	0	2	265	238
Control	0	3	146	131
Control	0	4	275	224
WHP	1	3	182	176
WHP	1	4	165	135
WHP	2	1	219	197
WHP	2	2	175	171
WHP	2	3	73	70
WHP	2	4	162	150
WHP	3	1	272	272
WHP	3	2	419	405
WHP	3	3	127	116
WHP	3	4	157	138
LMB	1	1	158	156
LMB	1	2	112	112
LMB	1	3	184	177
LMB	1	4	193	182
LMB	2	1	120	120
LMB	2	2	302	302
LMB	3	1	51	44
LMB	3	2	148	141
LMB	3	3	133	127
LMB	3	4	195	192

Table C.3. *A. ochracea* glochidia and juvenile counts from control, rabbit serum with white perch gill extract (WHP), and rabbit serum with largemouth bass gill extract (LMB). Each treatment group was started with five replicates (n = 5), but any replicates that developed contamination were removed from analysis. Each replicate was inoculated with an estimated 50-200 glochidia but it is possible pipetting differences resulted in replicates with more than 200.

Treatment Group	Concentration Level	Replicate	Initial # of glochidia	# of juveniles
Control	0	1	219	75
Control	0	2	409	42
Control	0	3	417	39
Control	0	4	190	29
Control	0	5	242	26
WHP	1	1	212	85
WHP	1	2	283	56
WHP	1	3	205	44
WHP	1	4	609	150
WHP	1	5	268	68
WHP	2	1	297	31
WHP	2	2	282	11
WHP	2	3	224	28
WHP	2	4	265	57
WHP	2	5	292	42
WHP	3	1	367	57
WHP	3	2	228	56
WHP	3	3	350	107
WHP	3	4	590	175
WHP	3	5	305	66
LMB	1	1	321	108
LMB	1	2	325	31
LMB	1	3	279	118
LMB	1	4	266	53
LMB	1	5	255	60
LMB	2	1	278	69
LMB	2	2	202	49
LMB	2	3	201	63
LMB	2	4	265	102
LMB	2	5	305	65
LMB	3	1	326	46
LMB	3	2	194	35
LMB	3	3	322	60
LMB	3	4	305	92
LMB	3	5	331	73

Table C.4. *A. ochracea* glochidia and juvenile counts from control, grass carp, and rabbit-carp treatment groups. Each treatment group was started with five replicates (n = 5), but any replicates that developed contamination were removed from analysis. Each replicate was inoculated with an estimated 50-200 glochidia but it is possible pipetting differences resulted in replicates with more than 200.

Treatment Group	Replicate	Initial # of glochidia	# of juveniles
Control	1	259	144
Control	2	192	121
Control	3	249	174
Control	4	163	107
Control	5	206	127
Grass carp	1	502	400
Grass carp	2	203	190
Grass carp	3	259	194
Grass carp	4	371	359
Rabbit-carp	1	405	363
Rabbit-carp	2	173	164
Rabbit-carp	3	239	239
Rabbit-carp	4	237	228
Rabbit-carp	5	273	221

Table C.5. *Elliptio complanata* glochidia and juvenile counts from control (no antibiotics), Primocin (0.25 or 1 ug/mL), and RCGA (0, 0.25, 1 ug/mL) treatments. Each treatment group was started with five replicates (n = 5). Each replicate was inoculated with an estimated 50-200 glochidia but it is possible pipetting differences resulted in replicates with more than 200.

Treatment Group	Replicate	Initial # of glochidia	# of juveniles
Control	1	106	0
Control	2	96	0
Control	3	119	0
Control	4	109	0
Control	5	65	0
Primocin (1 ug)	1	50	0
Primocin (1 ug)	2	102	0
Primocin (1 ug)	3	72	0
Primocin (1 ug)	4	111	0
Primocin (1 ug)	5	53	0
Primocin (0.25 ug)	1	199	0
Primocin (0.25 ug)	2	61	0
Primocin (0.25 ug)	3	58	0
Primocin (0.25 ug)	4	79	0
Primocin (0.25 ug)	5	79	0
RCGA (1 ug)	1	112	102
RCGA (1 ug)	2	162	156
RCGA (1 ug)	3	96	85
RCGA (1 ug)	4	103	98
RCGA (1 ug)	5	88	80
RCGA (none)	1	121	35
RCGA (none)	2	150	122
RCGA (none)	3	72	55
RCGA (none)	4	100	100
RCGA (none)	5	50	34
RCGA (0.25 ug)	1	110	96
RCGA (0.25 ug)	2	92	81
RCGA (0.25 ug)	3	REMOVED	MISSING
RCGA (0.25 ug)	4	82	68
RCGA (0.25 ug)	5	181	149