Propagation and monitoring of freshwater mussels released into the Clinch and Powell rivers, Virginia and Tennessee

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ABSTRACT

Freshwater mussels (Unionidae) in the United States have experienced dramatic declines, and 25% species are listed as federally endangered. Hence, recovery plans for endangered species proposed a strategy of propagation of young mussels for release to natal rivers to augment declining populations. In this study, I conducted laboratory experiments, assessed site suitability for mussel restoration, and evaluated survival and growth rates of released mussels to meet the requirements of recovery plan.

I conducted multiple experiments to develop an improved protocol for juvenile mussel propagation and culture. Significantly greater survival and growth rates were found in newly metamorphosed juveniles of the rainbow mussel (*Villosa iris*) reared in a substrate of fine sediment and one-month-old juveniles of wavy-rayed lampmussel (*Lampsilis fasciola*) fed on natural food in pond water. Bio-filter media greatly increased water quality by reducing the concentration of ammonia and nitrite. The negative impacts of flatworm predation and filamentous algae in juvenile culture were controlled, and juvenile escapement was prevented. Juvenile mussels were successfully produced and cultured to stockable size (>15 mm) for release.

I released laboratory-propagated mussels at three historically important sites in Clinch and Powell rivers for the assessment of site suitability. Use of cages was the most effective method to determine site suitability because the free-released mussels (untagged, tagged) had low catchability. Mussels released at Horton Ford, Clinch River, exhibited significantly faster growth. Horton Ford is the most suitable site, while environmental conditions at Fugate Ford, Powell River, are deemed unsuitable for mussel restoration and recovery.

To facilitate the detection of released mussels, I applied Passive Integrated Transponder tags to laboratory-produced juveniles of the endangered Cumberlandian

combshell (*Epioblasma brevidens*) and released them near Brooks Bridge, Powell River. The detection probability increased above 98%. I developed a set of hierarchical Bayesian models incorporating individual variations, seasonal variations, periodic growth stages and growth cessation to estimate survival, detection probability and growth of released mussels in a changing environment. Mussels of *E. brevidens* exhibited great survival (> 99% per month) and growth, indicating suitable conditions for recovery of this endangered species at this site.

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ATTRIBUTION

Four co-authors contributed significantly to this dissertation, which is a compilation of four publications and manuscripts. This section specifies the contributions of each co- author to this work. Yan Jiao and Richard J. Neves are my academic advisors and primary project supervisors. They contributed heavily to the project design and analyses, and edited each chapter and manuscript.

Jess Jones co-authored Chapter 3-1 and Chapter 3-2. He co-supervised the project and provided funding for my study. He revised manuscripts critically for important technical content. Matthew S. Johnson co-authored Chapter 1-2. He involved in the conception, data collection, analyses, and writing of the manuscript.

Chapter 1-1 was published in *Aquaculture* in 2013, and Chapter 3-1 is in press in *Ecology and Evolution*. Chapter 1-2 has been submitted to *Aquaculture* and Chapter 3-2 has been submitted to *Methods in Ecology and Evolution*.

CHAPTER 1: General Introduction

"*We consider species to be like a brick in the foundation of a building. You can probably lose one or two or a dozen bricks and still have a standing house. But by the time you've lost 20 per cent of species, you're going to destabilize the entire structure. That's the way ecosystems work*."

- Donald Falk, Christian Science Monitor, 26 May 1989

Overview of freshwater mussels in North America and research goals

The greatest diversity of freshwater mussels (Unionidae) is concentrated in North America (Neves 2008) with roughly 297 recognized species, 281 full species and 16 subspecies (William et al. 1993). Although the recorded existence of freshwater bivalves dates back to the late Devonian period (Gray 1988), this fauna has become the most imperiled group of freshwater animals in North America in recent times (Neves 2008, Haag 2012). More than 29 species are considered to have gone extinct over the last 100 years (Haag and Williams 2014), and 75 species are listed as federally endangered, 13 species are threatened (Williams et al. In press), and that number is expected to increase (Shannon et al. 1993). The U.S. Fish and Wildlife Service listed 4 additional mussel species in 2013 as endangered under the Endangered Species Act (Fish and Wildlife Service (USFWS) 50 CFR Part 17. 2012, USFWS 2013). Five Pennsylvania freshwater mussel species have been proposed for threatened or endangered status (Austen 2008). Similarly in Kansas, five of the 46 species of resident mussel species are now state- listed as endangered, four as threatened, and 12 species in need of conservation. Additionally, four species are thought to be extirpated (Simmons 2004). The decline of this fauna is mostly caused by habitat degradation and destruction due to sedimentation, dam, pollution, coal mining, exotic species invasion, over-exploitation, and other anthropogenic disturbances (Williams et al. 1993, Neves et al. 1997, Parmalee and Bogan 1998, Neves 1999, Neves 2008). Without immediate efforts to recover these federally protected species in watersheds throughout the country, extinction of additional species is imminent. The goal of my reserch was to release juvenile mussels at sites within two rivers to test the feasibility of expanding population ranges

with laboratory-produced mussels, to monitor the survival and growth of released cohorts, and to evaluate the suitability of mussel release sites and restoration efforts.

Freshwater mussels are ecologically and economically important. They purify rivers by removing suspended inorganic particulates, detritus, and plankton due to their suspension - feeding mechanisms. One mussel can filter 1 to 2 L of water per hour (World Bank project 2008, Allen 1914). The impacts of filtering in a high-density mussel assemblage can be extremely significant. An experiment of mussel filtration demonstrated their ecological significance (Figure 1). This advantage has been employed in successfully cleaning the polluted Dianchi Lake in China (World Bank project 2008). Freshwater mussels are also recognized as 'indicators' of the health of aquatic ecosystems because they are sensitive to water quality and have little capacity to move long distances. As long-lived suspension-feeders, they accumulate contaminants in their bodies and shells. Therefore, they are considered as biomonitors to indicate exposure risk in the ecosystem. Freshwater mussels are an important food source for fish and some furbearers such as raccoon, muskrat, and otter. They also provide nutrition and micro-habitats for other aquatic organisms by depositing feces on the stream bottom and promoting aeration of riverbeds by burrowing in the substrate. The calcium in mussel shells is often preserved over thousands of years with the annual growth lines remaining intact, providing valuable archaeological and geological information (Peacock and Seltzer 2008). Additionally, some freshwater mussels provide economic value by producing great numbers of pearls in the pearl culture industry (Hua 2001). Mussel shells have been used as the raw material for buttons and beads used to produce pearl nuclei (Williams et al. 1993), jewelry and crafts (shell carving), and as an animal food additive (Hua 2001, Hua et al. 2003).

Life cycle and reproduction

Most freshwater mussels are dioecious with separate sexes (Howells et al. 1996), and have a unique life history, which requires a parasitic stage to complete the life cycle. Mature males release sperm into the water, which are filtered from the water by the females via the inhalant aperture. Fertilization occurs internally in the female mussel (Haggerty 2005). Fertilized

eggs are brooded within the female's gills (also called marsupia) and develop into larvae, called glochidia. Mature glochidia then are dispersed to the water where they must parasitize and encyst on the gills, fins or epidermis of a suitable host fish to start the metamorphosis stage; otherwise, they will perish (Neves and Widlak 1987). Once glochidia attach to a suitable fish host, they will develop further, and metamorphose into juveniles. Juveniles will then excyst and drop off the fish host to start their benthic, free-living stage on the bottom of a river or lake, and grow into adults.

One gravid female mussel can release many glochidia, ranging from 75,000 to 3,000,000 individuals, depending on the species (Surber 1912, Coker et al. 1921). However, only those glochidia that successfully attach to the appropriate fish hosts can develop into juveniles. The percentage of glochidia that reach this stage is extremely low. For instance, the transformation rate of the glochidia of *Margaritifera margaritifera* is estimated to be only about 0.001% (Young and Williams 1984). In addition to above, only those juveniles that reach suitable habitat are likely to survive (Howard 1922), and young juveniles are considered to be the most vulnerable stage in the life cycle (Dimock and Wright 1993). Therefore, mortality is always much higher at this stage when compared to those later in development.

Conservation status and efforts

Research on freshwater mussel propagation was initiated by the U.S. Bureau of Fisheries in 1914, in response to the over-harvest of mussel shells (Neves 2008). However, conservation efforts didn't achieve success until after passage of the Endangered Species Act in 1972, when recovery plans for those recognized threatened and endangered species of freshwater mussels were prepared. Under the Act, recovery plans for listed threatened and endangered species recommended a strategy of propagation and release of young mussels to their natal rivers in order to augment existing populations and to reintroduce populations into historic habitats (Jones et al. 2005, Neves 2008). That requirement ignited studies on the life cycle and environmental requirements of freshwater mussels, principles and methodology of propagation, laboratory culture of juveniles, and restoration and translocation in the early 1990s at the Freshwater

Mollusk Conservation Center (FMCC) at Virginia Polytechnic Institute and State University in Blacksburg, Virginia. Dr. Richard Neves succeeded in resolving the difficulty of propagation methods and established a propagation protocol. Since then, the techniques of propagation and culture of freshwater mussels have been studied, involving biology, ecology, physiology, toxicology, hydrochemistry, and nutrition. The propagation sequence includes obtaining gravid female mussels from the river; collecting appropriate host fish from either the wild or commercial sources; maintaining mussels and host fish in captivity; obtaining glochidia; infesting host fish with glochidia; obtaining juvenile mussels after transformation from infested host fish; culturing juvenile mussels; testing protocols to improve culture technology; and growing juvenile mussels to a desired size for release to natal rivers.

This protocol has been used to guide national conservation efforts in propagating various endangered species. Following establishment of FMCC, other propagation facilities have been developed in 12 other states; in eastern and mid-western federal and state hatcheries, university laboratories, and NGO agencies to conserve and restore endangered mussels. The programs have expanded to include mussel surveys, propagation, juvenile releases, adult translocations, contaminant and physiological studies, genetic conservation, and habitat monitoring and management. A comprehensive understanding of environmental influences on mussel population dynamics will significantly accelerate the national conservation program.

Since 1996, research scientists at FMCC have released approximately 50,000-100,000 juveniles per year into rivers of mostly Virginia and Tennessee. By the end of 2007, nearly 7 million young mussels, of which 570,000 juveniles representing 13 endangered species, have been released to streams and rivers in Tennessee and Virginia (Neves et al. 2007). However, these stocking programs have had limited success due to unavailable culture methods to grow juveniles to larger sizes before release. Propagated juveniles experience high mortality after 1 or 2 mo of culture, and this has become a bottleneck in the propagation process. The specific ecological, physical, and chemical requirements of juvenile mussels at each stage were unknown for most species. Therefore, one of my research goals was to resolve this bottleneck, and propagate and grow mussel juveniles to a size for tagging before release into natal rivers.

Modeling in freshwater mussels

Population dynamic models in fisheries have been developed for more than 100 years (Ricker 1975, Quinn and Deriso 1999). However, monitoring freshwater mussel populations has just begun in very recent years, even though their life histories have been studied since the early 1900s. Because freshwater mussels have very complicated life cycles, and the populations are largely influenced by environmental factors, evaluations of their population dynamics have been very limited. During this decade, monitoring the mortality and recruitment of mussels has initiated age- and stage-structure models, with or without density-dependence (Bauer 1992, Hruska 1992, Hastie et al. 2001, Howard 2006) and invasive species effects (Stoeckel 2004, Casagrandi et al. 2007).

Tag and recapture of many species or population monitoring have been used to estimate key life history parameters (Williams et al. 2002, Pledger et al. 2003). Exponential mortality has been widely used in modeling aquatic species, such as mussels (Quinn and Deriso 1999; King 2007). Being conscious of the errors in age estimation in mussels by using inappropriate models (Anthony et al. 2008), mark-recapture models were introduced to estimate survival, recruitment and rate of population growth in native mussels such as *Elliptio complanata*, *E. fisheriana*, and *Lampsilis cariosa* (Villella et al. 2004), *Lampsilis siliquoidea*, and *Pyganodon grandis* (Anthony et al. 2008), as well as *Elliptio dilatata* (Hart et al. 2001). The advantage of mark-recapture methods typically allows repeated sampling of a targeted population through marked individuals to decrease uncertainty. However, the traditional tagging methods in monitoring mussel populations using numbered glue-on tags (Villella et al. 2004, Peterson et al. 2011) or direct curved labels on mussel shells (Hua 2005) are inefficient due to low recapture rate (Rogers 1999, Villella et al. 2004, Peterson et al. 2011). Natural mortality is an important parameter used in such models to indicate the decline in population size. However, it is one of the most difficult parameters to estimate (Hewitt et al. 2007), and can significantly affects model results, biological conclusions, and management recommendations. Hence, a reliable and efficient tagging methodology is desirable to effectively recapture released mussels.

Effective conservation and restoration strategies of endangered mussel species require knowledge of population dynamics and predictions of population growth. Applying inadequate models can create various noises, resulting in biased predictions (Jiao et al. 2008). The traditional mark-recapture models often assume homogeneity in animal survival, capture probabilities and individual variability, which likely introduces bias into model selection and parameter estimation (Pledger et al. 2003), resulting in misunderstanding of life-history traits (Cam et al. 2002). To estimate variability among individuals, models have been developed and constructed to allow individual heterogeneity using computer programs, and thus have enabled significant applications for understanding population biology (Pledger et al. 2003). Markov Chain Monte Carlo (MCMC) algorithms used in Bayesian statistical inference provide a mathematical framework to circumvent the problem of high-dimensional integrals and allow the likelihood function to be conditional on the unobserved variables in models, simplifying and expediting Bayesian parameter estimation (Wade 2000, Gimenez 2008).

Growth of aquatic animals often reveals strong seasonal oscillations, mainly due to fluctuations of temperature, food supply, body reserves and even social behavior (Shul'man 1974, Alanara et al. 2001, Bacon et al 2005). Hence, the traditional von Bertalanffy growth model has been discussed and modified to allow for seasonal oscillations during specific growth periods. Currently, the estimates of growth parameters (K and L_∞) have not incorporated individual variations, seasonal variations, and growth cessations for freshwater mussels, primarily due to absence of reliable data and advanced demographic modeling techniques.

Historical and current mussel and fish assemblages in Clinch and Powell rivers

The Clinch River is about 480 km long, formed by the junction of two forks in southwest Virginia near Tazewell, VA and flows generally southwest through the Great Appalachian Valley, to the Tennessee River at Kingston, TN. An important tributary of the Clinch River is the Powell River, which arises in southwest Virginia and flows approximately 185 km through Virginia and Tennessee. The Clinch and Powell drainage basins are separated by Powell Mountain.

The Clinch and Powell rivers support a unique assemblage of aquatic life and collectively foster the greatest number of endangered mussel species in the United States. The Clinch River historically contained 55 mussel species, which is more than any other river of comparable size, and over 100 species of fish (Ortmann 1918, Ahlstedt et al. 2005). However, 5 mussel species are now extinct and 18 out of 41 extant mussel species are listed as federally endangered species. Additionally, four species of fish in the Clinch River are federally endangered (Ahlstedt et al. 2005). In the Powell River, 46 mussel species were documented historically (Ortmann 1918, Johnson et al. 2012), of which three species are extinct. Although at least 32 mussel species are still extant, several species are nearing extirpation, and 7 species are federally listed as endangered (Ahlstedt et al. 2005). The causes of decline or extinction of aquatic animals most likely are attributed to agriculture, forestry, coal mining, and urbanization. In addition to the above, two major toxic spills have occurred in the Clinch River in the last 30 yr and killed mussels and fish at the spill sites and downstream. For example, 16 species of mussels (7,036 individuals) were eliminated by the 27 August 1998 chemical tanker spill in the Clinch River at Cedar Bluff, Virginia. Dead mussels in the affected zone occurred from CRM 322.5 downstream to CRM 317.0 at Richlands (Jones et al. 2001).

Trends in population size have differed in the Clinch and Powell rivers in Virginia, compared to the overall population trends in Tennessee. From 1979 to 2004, Ahlstedt et al. (2005) have monitored mussel populations at 12 fixed-station sites in the Clinch and Powell rivers and indicated that mussel populations in the Clinch River have declined by 70 % at investigated sites in Virginia, while population abundance of mussels at sites in Tennessee increased 2.5-fold. Mussel population abundance at sites in the Powell River, in Tennessee and Virginia, decreased by 63%. The investigated data apparently revealed variability in mussel population changes at sites in the Clinch and Powell rivers. For instance, mussel density at Pendleton Island in Scott County, VA in Clinch River declined from 25 / m^2 in 1979 to 4.6 / m^2 in 2004. Mussel populations have dramatically declined in the river reach from near St. Paul downstream to Clinchport, VA, while mussel aggregation upstream from Nash Ford to Carbo, VA show signs of recovery. In the river reach from Woodway upstream to Appalachia, VA in

the Powell River, mussel occurrences were extremely low or absent, while adult mussel abundance remains high in the remote section of this river along the Virginia-Tennessee border, but with low population recruitment (Johnson et al. 2012). The practical assessment of site suitability by release of juveniles, measurement of water quality, and evaluation of physical habitat factors are essential for the application of conservation and restoration strategies for endangered mussel species.

Research rationale

Most agencies have realized the need to investigate the impacts of biological, water quality and physical parameters to mussel populations. Mussels are sensitive to environmental changes such as water quality, habitat, and fish communities (Smith and Jepsen 2008). Mussels will be stressed under conditions of low dissolved oxygen and sedimentation. Loss of host fish reduces mussel reproduction and can eventually result in the decline of mussel populations.

Research objectives

My specific objectives during this study were as follows:

Objective 1: To optimize laboratory conditions for culturing juveniles of *Villosa iris*, *Lampsilis fasciola* and the endangered *Epioblasma capsaeformis,* and develop an improved protocol for juvenile mussel culture.

Specifically, 1) to test the influence of algal density, water flow and substrate type on growth and survival of juveniles of *V. iris*; 2) to test the effects of algae and pond water on growth and survival of juveniles of *L. fasciola*; 3) to compare the growth and survival of juveniles of *E. capsaeformis* in various culture systems; and 4) to test the effectiveness of bio-filter media in juvenile mussel culture.

Objective 2: To assess site suitability for restoring laboratory-reared juvenile mussels to historic river reaches in Virginia and Tennessee.

Specifically, 1) to investigate survival and individual growth of juveniles of *V. iris* and *L. fasciola* in 3 locations; 2) to record heavy metal concentrations in released mussels; 3) to evaluate site suitability for establishing laboratory-reared juvenile mussels.

Objective 3: To model the survival, catchability, and growth oscillations in released federally endangered Cumberlandian combshell (*Epioblasma brevidens*) using a hierarchical Bayesian approach in mark-recapture studies incorporating individual variations.

Specifically, 1) to develop a recapture method to increase mussel recapture rates; 2) to develop empirical models to investigate heterogeneity of individual variations and seasonal changes in the estimation of survival and catchability of released mussels of *Epioblasma brevidens*; 3) to model the growth oscillations incorporating individual variations of released mussels.

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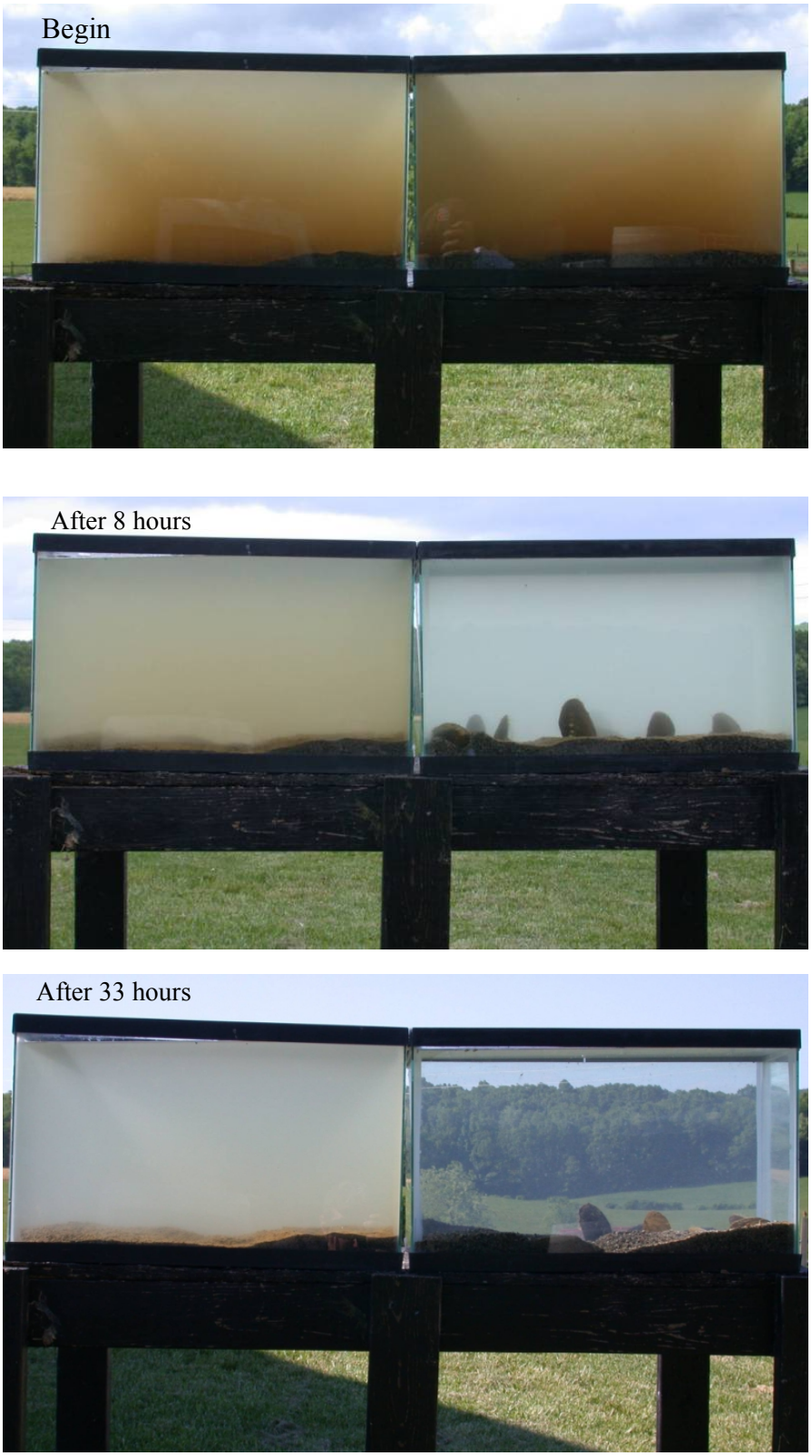

Figure 1. Experiment of mussel filtration conducted in Freshwater Mollusk Conservation Center by Jacob Rash and Aaron Liberty (Left tank without mussel, right tank with mussels).

CHAPTER 2-1: Effects of algal density, water flow and substrate type on culturing juveniles of the rainbow mussel (*Villosa iris*) (Bivalvia: Unionidae) in a laboratory recirculating system

Abstract

The effects of algal density, water flow and substrate type on the survival and growth of newly metamorphosed juveniles of the rainbow mussel (*Villosa iris*) were investigated in 40 L recirculating culture systems. A split-plot design with a whole plot factor combining algal density and water flow, and a split-plot factor of substrate was applied in this study to test the three factors. Juveniles reared in 4 juvenile culture units (550 ml) with fine sediment (< 200 μm), sand (300-500 μm), limestone sand (800-1000 μm) and without substrate in each recirculating system received water flow at levels of 3.75 ml s^{-1} or 7.5 ml s^{-1}; algae concentrations of 35,000 cells ml^{-1}, 87,500 cells ml^{-1}, or 175,000 cells ml^{-1}; and were sampled every 10 days during a 30-day experiment. Substrate type significantly affected survival and growth of juveniles ($p < 0.0001$), while algal density and water flow had no significant influence on their survival or growth ($p > 0.05$). Juveniles had significantly higher survival and faster growth in a substrate of fine sediment when compared to other substrate types ($p < 0.05$). Escapement rate of juveniles exceeded 39% overall in culture units. Water quality was consistent throughout the experimental duration and within a suitable range for juvenile mussels. Results confirmed that fine sediment is the best substrate for juvenile culture in a laboratory recirculating system. The split-plot design was effective for conducting this multi- factor experiment and saved significant time and resources.[·]

Keyword: freshwater mussel, rainbow mussel (*V. iris*), algal density, water flow, substrate, split-plot design, recirculating system.

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1. Introduction

Propagation and grow-out of sufficient numbers of juvenile mussels for reintroduction to natal rivers has become a primary strategy to recover and restore populations of endangered species (National Native Mussel Conservation Committee, 1998). However, this strategy has been limited by the difficulty of rearing large numbers of juveniles under controlled laboratory conditions. The requirements of habitat and food supplies for juvenile mussels have not been adequately quantified, and testing of a combination of nutritional and environmental factors is needed, using appropriate experimental design and analysis.

Although the test of multiple factors on rearing juvenile mussels has not been conducted, some single-factor experimental studies or survey results have provided useful information. Newly metamorphosed mussels feed in the sediment with foot ciliation until their gills are completely developed for suspension-feeding (Yeager et al., 1994). Silverman et al. (1997) also stated that juveniles fed on suitable particles of algae, bacteria, and organic detritus through their ciliated feeding mechanisms. Further studies indicated that substrate type influences the growth and survival rate of juveniles. For example, Hua (2005) studied the effects of substrate and algal diets on newly metamorphosed mussels of pink heelsplitter, *Potamilus alatus,* and found that higher survival and growth rates occurred in fine sediment. Similarly, several studies showed that moderate deposition of silt in a mussel bed could increase the growth and survival of newly metamorphosed juveniles (Marking and Bills, 1980; Zimmerman, 2003). However, juveniles of *Villosa iris* reared in fine sand (500-800 μm) had a higher survival than those in fine sediment (< 120 μm) (Rogers, 1999). Liberty (2004) found that juveniles of *V. iris* had a higher survival rate in coarse sand, but exhibited greater growth in fine sediment. Beaty and Neves (2004) concluded that growth and survival of juveniles of *V. iris* cultured in fine sediment (< 120 μm) or coarse sediment (120-600 μm) were not significantly different.

Algae serve as an important food source for juvenile and adult mussels, and have been widely used as a major dietary component for rearing juvenile mussels (Hudson and Isom, 1984; Gatenby et al., 1996). The importance of using various algal species to rear juvenile mussels is due to their physical characteristics and nutritional properties. Juvenile mussels selectively feed

by algal size rather than by species due to their feeding mechanisms as suspension-feeders. Juveniles can shift to select algal species based on nutritional value when they are fed algae of similar size (Beck and Neves, 2003). Besides the quality of diet for juvenile mussels, the quantity of algae is important in juvenile culture (Winter, 1978). Rogers (1999) fed juvenile mussels with algae (*Scenedesmus spp*.) at a level of 20,000 cells ml^{-1}. Henley et al. (2001) suggested that a concentration of algae of *Scenedesmus spp.* at approximately 30,000 cells ml^{-1} was sufficient to meet the requirement of juveniles in indoor recirculating water systems. Barnhart (2006) found that a cell concentration of green algae *Neochloris oleoabundans* at about 10,000 –15,000 cells ml^{-1} was suitable for rearing juvenile mussels in a bucket system. Bush (2008) suggested that optimum rations of the alga *Neochloris oleoabundans* for adult *Epioblasma* spp. could range from 40,000-120,000 cells ml^{-1}.

An inadequate feeding rate can cause nutritive stress in mussels. Mussel fed with too low a ration will continuously ingest food, resulting in stress or mortality due to high-energy cost and low caloric ingestion (Widdows, 1991). Conversely, Foster-Smith (1975) reported that too high a food ration could result in a low filtration rate and high pseudofeces production in marine mussel species. In free-flowing rivers, algal density and species dominance fluctuate based on changes in nutrient levels and environmental conditions on a seasonal basis. The minimum algal density in rivers occurs in winter or under oligotrophic conditions, while the highest density typically occurs in summer or with eutrophic conditions. Algal density in rivers varies from single digit to 100,000 cells ml^{-1} (Swale 1969; Aykulu, 1978; Jones and Barrington, 1985). Therefore, the determination of suitable algal feeding rates is essential to successfully produce healthy juveniles and adult mussels under controlled propagation.

Water flow is the other critical factor to freshwater mussels. High water velocity can cause passive transport of mussels downstream. Conversely, too low a water velocity may be incapable of providing sufficient food and oxygen to sustain mussels. Unfortunately, flow requirements for freshwater mussels are not fully characterized and vary widely among species (Mummert, 2001). Water velocity in a flow-through pond environment for mussel culture ranged from almost 0 to 4.8 cm s^{-1} (Mummert, 2001). High water flow was adopted in bucket

culture systems, with a flow rate of roughly 400 L h^{-1} (Barnhart, 2006). Effect of water flow on native mussel mortality in the lower Flint River Basin was inversely related, resulting in high mortality of unionids when flow velocity was less than1.0 cm s^{-1} (Paula et al., 2001).

Coker et al. (1921) reported that newly metamorphosed juvenile mussels are highly mobile, such that locomotion of juveniles can result in escapement during juvenile mussel culture. The escapement of newly transformed juveniles of *V. iris* has been observed, resulting in an average escapement rates from 5% to 70 % among substrate types (Liberty et al., 2007). Therefore, the capture of escaping mussels is essential in quantitative culture experiments and in efficient juvenile mussel production.

Split-plot designs were proposed by Fisher (1925) for use in agricultural experiments. Their use has been extended to industrial experimentation (Box et al., 2005), in split-plot fractional factorial designs (Bingham et al., 2004), split-plot designs for response surface and mixture designs (Vining and Kowalski, 2008), and for optimal split-plot designs (Jones and Goos, 2009). Those designs have been extensively used by statisticians in many scientific disciplines due to their low cost and efficiency (Box et al., 2005). In our study, the split-plot design was used to test the influence of the multiple factors of substrate, water flow and algae density on growth and survival of juvenile mussels. The experimental species, *V. iris,* is a common species widely distributed in eastern North America; however, populations of this species have been declining in its western range (Cummings and Mayer, 1992), and it is listed as an endangered species in Illinois. This species has commonly been selected for toxicity testing and as a surrogate for reintroduction of rare species (Mummert et al. 2003, Wang et al. 2007).

The goal of this study was to improve propagation and culture methods for juvenile mussels by conducting a multiple-factor experiment, to compare growth and survival among varying conditions of algal density, water flow and substrate.

2. Materials and Methods

2.1 System design and components

This experiment was conducted in recirculating culture systems (RCS) at the Freshwater Mollusk Conservation Center (FMCC), Virginia Polytechnic Institute and State University. Major components of this system included juvenile culture units (JCUs), water conditioning and distribution tanks, air distribution lines, pump, and chiller and cooling system (Fig. 1). Each recirculating system consisted of 4 JCUs with a capacity of 550 ml for each container; a water deliver tube, drain pipe and a nylon sieve bag per JCU; and one water conditioning and distribution tank to store 40 L of water. Four containers in each JCU held 3 types of substrate with a layer of 1-2 mm of fine sediment (particle size < 200 μm), sand (particle size 300-500 μm), limestone sand (800-1000 μm), and without substrate. Water flow rate was controlled by valves on the water delivery tubes. Water drained to the conditioning and distribution tank through a central drainpipe, and a sieve bag of mesh size 150 μm was used to catch juvenile mussels escaping from the container to ensure the accuracy of survival rates of juvenile mussels at the sampling occasions. Air was delivered to the conditioning and distribution tanks from a 1.86-kw Sweetwater air blower via PVC pipes (ϕ = 3.8 cm). Aerated water supplied to JCUs was a mix of dechlorinated municipal water and well water. Water depth in each JCU was 10 cm. A magnetic drive pump (Little Giant, Magnetic Drive Pump, 3-MDX) was installed on each JCU to deliver algal diets and create a recirculating flow through PVC pipes (ϕ = 1.3 cm) and silicon rubber tubing (ϕ = 0.3 cm). Water temperature was controlled by a chiller (Aqua Logic Trimline, Delta Star ½ HP TLD-5) and held at 20^{o}C throughout the experimental period. Each recirculating culture system was independent for application of statistical analyses.

2.2. Experimental design

Juvenile mussels used for this experiment were one-day old species of *V. iris* from the peak period of transformation to minimize variation among treatments. They were fed with a commercially prepared mixed algal diet (50% Shellfish diet 1800 and 50% *N. oculata* 3600 purchased from Reed Mariculture Inc) at mean concentration levels of (1) 35,000 cells ml^{-1}, (2)

87,500 cells ml^{-1}, and (3) 175,000 cells ml^{-1}, respectively. The Shellfish Diet 1800 consists of four species of marine microalgae: *Isochrysis sp, Pavlova sp*, *Thalossiosira weissflogii*, and *Tetraselmis sp*. The Nanno 3600 is a concentration of *Nannochloropsis*. These species have demonstrated success in culturing bivalve species due to their high nutritional content. The mixed diet of Shellfish Diet 1800 and Nanno 3600 has been used at FMCC since 2006. The feeding rates for juvenile mussels were determined from previous studies; a ration of approximately 30, 000 cells ml^{-1} was shown to be sufficient for juvenile mussels (Henley et al., 2001). Substrate sizes used in this experiment were categorized as (A) fine sediment, (B) sand, (C) limestone, and (D) a container without substrate served as a reference trial. Fine sediment was collected from the New River, McCoy, Blacksburg, VA and sieved through a 200 μm mesh. It was then aerated for 2-3 days to facilitate oxidation. Sand and limestone sand were used as the other substrates. All substrates were autoclaved for disinfection before use. Water flows were set at 3.75 ml s^{-1} (L) and 7.5 ml s^{-1} (H). The higher level of water flow (7.5 ml s^{-1}) was determined from tests of maximum velocity without disturbing juvenile mussels and their substrate (especially fine sediment). All equipment was disinfected using an acid solution, followed by a rinse in clean water.

A Split-Plot Design (SPD) was applied to test the multiple factors in this study. The whole plot design was a combination of two factors of algae density (3 levels) and water flow (2 levels), randomly assigned to 12 recirculating systems in two sets of 6 (2 blocks). The split-plot factor was substrate (4 types), randomly assigned into JCUs in each whole plot (recirculating system). Four 550 ml containers associated with treatments of substrate, and no substrate, were placed in one JCU above the conditioning and distribution tank to receive the treatment combinations of water flow and algal diet (Table 1). One hundred juvenile mussels of *V. iris* were randomly assigned to each JCU and fed twice per day. A total of 4,800 juvenile mussels were used in this study.

2.3. Sampling

This experiment lasted 30 days, starting with newly metamorphosed *V. iris* at 1day old. Juvenile mussels were sampled initially and at 10-day intervals (4 sample periods) to determine survival and growth. Ten juveniles per experimental unit were randomly selected to measure shell length using a dissecting microscope equipped with reticule to assess growth rate (N=240). Substrates in each unit were rinsed through a 300 um sieve on top and a 150 um sieve on the bottom respectively to collect juveniles. Fine sediment went through the bottom sieve, while sand and limestone sand collected in the top sieve. Live juveniles in each JCU, including escaped juveniles collected in the sieve bag, were counted to calculate survival rate. Juvenile shells were counted as dead mussels. Water in each RCS was exchanged at every sampling occasion. Live juveniles were then returned to their original containers filled with fresh substrate for continued testing.

2.4. Water quality

Temperature, dissolved oxygen (DO), pH, hardness, and total ammonia nitrogen were tested during each sample period. Temperature and DO were determined using a YSI DO meter (Model: 55/ 12 FT, Yellow Springs, Ohio). Hardness was measured using a Hach TM test kit (HACH Company, Loveland, CO), and pH was measured with a pH meter (Orion, Model: 290A). Total ammonia nitrogen was determined by the Nessler method using a HACH DR/ 2400 spectrophotometer.

2.5. Data analyses

Survival rate was calculated as the percentage of juveniles initially placed in each experimental unit that were still alive in the culture unit and sieve bag at time of sampling. Escaped juvenile mussels were included into the calculation of survival and growth rates even though they were temporarily absent from substrates. However, they remained in the same treatments of feed and water flow as those in the JCU. Additionally, the majority of escaped mussels came from those JCUs without sediment, representing the same conditions as in the JCU. Escapement rate was calculated as live juveniles caught within the sieve bag, as a

proportion of total live individuals from the sieve bag and culture unit combined. Survival rates of juvenile mussels during the 30-day period, and growth rates and escapement rates during each 10-day period were tested among treatments of algae density, water flow, and substrate using SAS Version 9.2. A logistic regression model and GLIMMIX procedure for SPD (1) was used to test survival of juveniles for significant differences at α = 0.05. Juvenile growth were compared using the SPD GLM model (2) (full model and reduced model), and HSD (Tukey's Honest Significant Difference) to test for significant differences among treatments at α = 0.05. Juvenile escapement was tested using the Logistic GLM model.

y_{tijkl} ~ Binomial (100, $\{p_{tijkl}\}$)

$$\log\left(\frac{p_{tijkl}}{1-p_{tijkl}}\right) = \mathrm{f}\,(p_{tijkl}) = \mu + \delta_t + \theta_i + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \epsilon_{tijk} + \gamma_l + (\alpha\gamma)_{jl} + (\beta\gamma)_{kl} + (\alpha\beta\gamma)_{jkl} \quad (1)$$

Where y_{tijkl} is a response variable of count of survived mussels ; p_{tijkl} was the probability of the live mussels and 1- p_{tijkl} is the probability of dead mussels; μ was the overall (constant) population mean; δ was the fixed effect of time (t =1,2,3); θ_i was the fixed effect of block (i =1, 2); α_j was the fixed effect of 'water flow' (j =1, 2); β_k was the fixed effect of 'algal density' (k =1, 2, 3); γ_l was the fixed effect of 'substrate'(l = 1, 2, 3, 4); and $(\alpha\beta)_{jk}$, $(\alpha\gamma)_{jl}$, $(\beta\gamma)_{kl}$, $(\alpha\beta\gamma)_{jkl}$ were interactions between 'water flow' and 'algal density', 'water flow' and 'substrate', 'algal density' and 'substrate'; and 'water flow', 'algal density' and 'substrate', respectively.

$$y'_{ijkl} = \mu + \theta_i + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \epsilon_{ijk} + \gamma_l + (\alpha\gamma)_{jl} + (\beta\gamma)_{kl} + (\alpha\beta\gamma)_{jkl} + \epsilon_{ijkl} \quad (2)$$

y'_{ijkl} was the response variable of shell length (um), and μ, θ_i, α_j, β_k, γ_l, $(\alpha\beta)_{jk}$, $(\alpha\gamma)_{jl}$, $(\beta\gamma)_{kl}$, $(\alpha\beta\gamma)_{jkl}$ denoted the same factors or interactions as those in equation (1).

3. Results

Mortality of juveniles was observed after 10 days, with highest mortality in containers without substrate, followed by those in containers with sand and limestone sand. The juveniles reared in containers with fine sediment were the only group that survived after 30 days (Figure 2). Results of juvenile survival from the two blocks were identical ($p = 0.242$). Substrate significantly affected juvenile survival ($p < 0.0001$), while factors of algal density and water flow had no significant influence on juvenile survival ($p > 0.05$). A time series analysis was conducted, showing significant changes in mussel survival over time ($p < 0.0001$) (Table 2). Among substrate types, juveniles achieved their highest survival in fine sediment, followed by sand, gravel, and no substrate, respectively, in all time series sampled (Fig. 2).

Results of juvenile growth were similar to those for survival through the first10-day period of sampling data. Substrate type significantly influenced juvenile growth ($p < 0.0001$), while algal density ($p > 0.05$) and water flow ($p > 0.05$) did not. No significant difference was found between blocks ($p = 0.39$) (Table 3). Interactions among factors of substrate, algae, and water flow were included in full model analyses. Of those, the interactions between flow and algal density, flow and substrate, algal density and substrate, and flow and algal density with flow did not significantly affect juvenile growth ($p> 0.05$). A follow-up reduced model was tested after excluding the above interactions. Results from the reduced model were similar to those from the full model (Table 3). The effect of levels of substrate on juvenile growth were tested using Least Square Difference (LSD), Tukey's Honest Significant Difference (HSD), Bonferroni (Dunn), and Scheffle's test (Table 4). Juvenile mussels reared in fine sediment grew significantly faster than those in other substrates ($p < 0.05$). Juveniles reared in containers without substrate exhibited significantly slower growth than those in substrates ($p < 0.05$). Effects of sand and limestone sand on juvenile growth were not significantly different ($p > 0.05$). Results from the full model and reduced model were the same. Effects of algae and water flow on juvenile growth also were tested; no significant difference was shown (Fig. 3). The normality test was applied using QQ-plot of residuals, resulting in a normal distribution pattern.

During each sampling event, juveniles of *V. iris* exhibited active escapement in each

experimental unit, resulting in varying escapement rates among trials. Escapement rate was significantly different among substrate types ($p<0.0001$), while not significantly different among levels of algal density ($p > 0.05$) and water flow ($p > 0.05$). The levels of escapement were highest without substrate, followed by limestone sand, sand and fine sediment. The escapement rate in fine sediment was significantly less than the other three-substrate types. There was no significant difference between sand and limestone, but a significant difference between sand and no substrate (Fig. 4).

The water quality characteristics of temperature, DO, pH, hardness, and total ammonia nitrogen (TAN) provided suitable conditions for rearing juvenile mussels. Un-ionized ammonia nitrogen was calculated based on the TAN and associated pH and temperature. There were no significant differences ($p>0.05$) among water quality values during the experimental period (Table 5).

4. Discussion

The success of propagation and production of mussels mostly depends on techniques to improve survival rate of newly transformed juveniles, since high mortality is often observed in the culture of newly metamorphosed mussels due to poor environmental conditions and inadequate food supply (Buddensiek, 1995; Gatenby et al., 1996; Gatenby et al., 1997; Beck and Neves, 2003). The early life of juvenile mussels aged from day 1 to 1 or 2 mo. is a crucial stage in the life cycle. In our study, survival rates of juvenile mussels significantly decreased over time, and averaged 0 – 40% after 30 days. However, juveniles in fine sediment exhibited significantly higher survival, around 80% at 10-days old; and 40% after 30 days, when compared to survival in other substrate types, indicated a more suitable condition. Other substrate trials for the 30-day period approached 100% mortality. Obviously, those substrates are not suitable for rearing newly metamorphosed mussels in indoor culture systems. Similarly, substrate had a significant influence on juvenile growth. Juveniles in fine sediment grew significantly faster (average length of 430.8 μm) than those in other containers after10 days. These results confirm those of previous studies, that juvenile mussels survive and grow better in fine sediment (Gatenby et al., 1996;

Henley et al., 2000; Mμmmert, 2001; Zimmerman, 2003; Hua, 2005). Gatenby et al. (1996, 1997) concluded that mussels reared in sediment grew significantly faster with higher survival rate than those reared without sediment. Similarly, Hudson and Isom (1984) reported that juveniles of *Anodonta imbecilis* reached a high survival rate when placed in silt as an additional supplement, at a concentration of 700 mg silt L^{-1}. Newly metamorphosed mussels are pedal-feeders through foot ciliation, and bacteria and organic matter in sediment provide additional food resources (Lopez and Holopainen, 1987; Gatenby et al.; 1997; Hua, 2005), while sand and gravel do not contain these food supplements. After sampling, juveniles were observed to burrow into sediment within a few seconds when placed back into the containers. Their guts always exhibited a green and brown color in fine sediment, while only green was observed in guts of juveniles reared in sand, gravel and without substrate. These results agree with observations in a previous feeding study of *P. alatus* (Hua, 2005); juveniles ingested suitable-sized particles from fine sediment, and digested and gained nutrition from these particles. Juveniles in other substrates (sand and limestone sand) or without substrate could feed only on algae retained in interstices among sand and gravel, or on the surface or bottom and wall of culture units. Although other studies have shown that juveniles exhibit better or equivalent growth and survival in coarse substrate (Rogers, 1999; Beaty and Neves, 2004; Liberty et al. 2007), these observations are likely due to differences in experimental set-up, or lack of consideration for juvenile escapement. River water used in those studies of Liberty et al. (2007) and Beaty and Neves (2004) consisted of detritus, bacteria, phytoplankton, and microscopic zooplankton. Perhaps, those components accumulated in interstitial spaces of sand or coarse substrate, and sustained juveniles that were pedal-feeding. Yeager et. al (1994) found that newly transformed juveniles of *V. iris* burrowed themselves quickly into the sediment and fed interstitially rather than to suspension-feed. Gut analysis showed that particles ranged from 2 to 5 μm along most of the digestive tract. The river water might increase survival rate of those juvenile mussels reared in sand and coarse substrate. Adult mussels of *V. iris* occur in sand and gravel, even along edges of emerging vegetation (Parmalee and Bogan, 1998). From our field experience, juveniles can be found mostly in silt or a mixture of sand and clay. Water used in our study was a mix of

dechlorinated municipal water and well water, and algal diet was the only food supplied to juveniles. It would seem that natural food including organic detritus, microscopic zooplankton, bacteria, and algae as along with fine sediment plays important roles in juvenile growth and survival. Similar results were found in juvenile production using pond water at FMCC; newly metamorphosed juveniles of *V. iris* grew faster with higher survival rates in those culture systems with fine sediment (< 200 μm) than bucket systems without sediment (Hua et al., 2011).

High mortality of juvenile mussels typically occurs from an improper diet and ration (Winter, 1978; Buddensiek, 1995; Henley et al., 2001). Previous studies have examined quality of juvenile diets (Gatenby, 1997; Zou et al., 2000), but studies with quantitative evaluations are limited. A mixed commercial algal diet (Shellfish 1800 and Nanno 3600) is commonly used for commercial bivalve production and was tested in our study. Lower algal density of 35,000 cells ml^{-1} was applied based on a suggested algal feeding rate of 30,000 cells ml^{-1} (Henley et al., 2001). Results showed that the algal density at 35,000 cells ml^{-1}, 87,500 cells ml^{-1} and 175,000 cells ml^{-1} had no significant effect on survival. There are two possible explanations for the non-significance of algal density. The testing range of algal density may have been too high or too low at all three levels to show significant differences; or the range within three levels of algal density may be suitable for rearing juvenile mussels. The potentially limited food supply could not meet the requirement of mussels, resulting in nutritive stress and constant ingestion. However, algal density is much lower in natural rivers. It varies from a few hundred to a few thousand cells per milliliter, but peak production can reach 100,000 cells ml^{-1} (Palmer, 1964; Aykulu, 1978; Jones and Barrington, 1985). The maximum cell density of algae in the River Derwent was 3,800 cells ml^{-1} (Jones and Barrington, 1985), 6,625 cells ml^{-1} in River Avon (Aykulu, 1978), and 40,000-50,000 cells ml^{-1} in the Stour and Severn rivers in England (Swale, 1969). Palmer (1964) studied algae production in fifteen North American rivers and listed the highest algal concentration in Arkansas, with a peak density at 101,200 cells ml^{-1}, followed by the Mississippi River with an algal concentration of 53,000 cells ml^{-1}. In our study, the algal density levels of 35,000 to 175,000 cells ml^{-1} were certainly comparable to those in natural

rivers. Therefore, algal density ranging from 35,000 cells ml^{-1} to 175,000 cells ml^{-1} are adequate for culturing young juveniles of *V. iris*.

Studies on the relationship between water flow and growth of juvenile mussels are limited. Barnhart (2006) cultured juvenile mussels in an enclosed bucket system with a flow rate at 400 l h^{-1} (equivalent to 111.1 ml s^{-1}). The flow rate in our study was set at 3.75 ml s^{-1} and 7.5 ml s^{-1}, with a maximized high flow that could retain fine sediment on the bottom of containers, and a low water flow comparable to the range of 3.16 to 4.2 ml s^{-1} used for juveniles of *V. iris* by Yeager et al (1994). Results showed that water flow had no statistically significant effect on juvenile survival. Seemingly, juvenile mussels can accommodate a wide range of flow conditions, and the flow from 3.75 ml s^{-1} to 7.5 ml s^{-1} in our recirculating system provided sufficient dissolved oxygen.

Water quality values in all culture units were stable. The importance of water quality for juvenile mussel culture has been recognized and widely studied (Mummert et al., 2003; Wang et al., 2007). Un-ionized ammonia is a risk factor, and can dramatically affect survival and growth of juvenile mussels, especially young mussels. Mummert et al. (2003) indicated that newly metamorphosed juveniles or even 2-mo-old juveniles (Wang et al., 2007) of *V. iris* were more sensitive to un-ionized ammonia when compared to other species of freshwater mussels. The LC 50 of un-ionized ammonia for newly metamorphosed *V. iris* has been tested, producing a mean value of 0.11 mg L^{-1} NH_3-N (96-h) (Mummert et al., 2001) and 1.0 mg L^{-1} NH_3-N (96-h) (Wang et al., 2007). In aquaculture, 5% of the LC 50 is a safe application concentration, which is suitable for most natural toxins such as NH_3-N (Boyd, 2005). According to these data, the un-ionized ammonia level in our study, 0.003 to 0.005 mg L^{-1}, was a safe concentration for newly metamorphosed *V. iris* based on the application concentration (0.0055 mg L^{-1}) calculated from the lowest LC 50 (96-h) (Mummert et al., 2001). Other water quality parameters were within the suitable ranges for juvenile mussels, when compared to results of previous studies (Henley et al., 2001, Wang et al., 2007).

Escapement of juvenile mussels has not been specifically studied during juvenile production except by Liberty et al (2007), who reported escapement of juveniles of *V. iris* in a flow-through

system. In our study, juvenile mussels exhibited high mobility in all culture units. Juveniles moved from culture units to sieve bags, resulting in escapement rates from 39 to 92.5% among substrate types after the initial 10-day culture period. Movement in this early life stage is believed to correspond with active feeding. The newly metamorphosed juveniles rely on pedal-feeding through foot ciliation (Gatenby et al., 1996; Beck and Neves, 2003), and pedal-feeding occurs in almost all juvenile bivalves (Reid et al., 1992). Statistical analyses of escapement rates among substrate types showed significant differences, with the highest value in culture units without substrate and the lowest value in those with fine sediment ($p < 0.0001$). Although juveniles in fine sediment had less active movement compared to those in other substrate types, escapement rate was still considerable at 39%. The movement of juvenile mussels has been observed in all species of juvenile mussels cultured at FMCC, and lasts for a few months or even years based on species. Obviously, prevention of escapement of juvenile mussels caused by their feeding movements is essential during the culture process. Henley et al (2001) first introduced a recirculating culture system at FMCC for juvenile mussel culture. Kovitvadhi et al (2008) adapted and modified Henley's recirculating system with a supplement of macrophytes and a biological filter to control water quality. However, the escapement of juvenile mussels was not noticed, hence, not prevented in their recirculating systems. In our experiment, a nylon sieve bag with a proper mesh size (150 μm) was installed and had been tested as a useful device to solve the problem of escapement of juvenile mussels. Recapture of those escaped juveniles is critical in any experiments to ensure an accurate assessment of survival rate or in culturing juvenile mussels.

Split-plot designs have been used commonly in industrial experiments, though developed for use in agricultural experiments (Fisher, 1925). The primary advantage of a split design is its efficiency. The cost of a split-plot design is generally less than the cost to run a completely randomized design (Jones and Nachtsheim, 2009), and can save time and equipment in experiments, when compared to a completely randomized design. In this study, four types of substrate were grouped by using the spilt-plot experimental design. Otherwise, four times sets of separate experimental units would be required to complete the study. Additionally, split-plot

designs are considered more efficient, with greater overall precision for experiments, compared to completely randomized designs (Goos and Vandebroek, 2004). Moreover, the split-plot design has even been used to run unreplicated factorial tests (Loeppky and Sitter, 2002). Hence, the application of a split-plot design to test multiple factors improved the temporal and statistical efficiency of our experiment, and can be manipulated in any experimental design to compare multiple factors at less cost.

5. Conclusion

A split-plot design was successfully used to test the effects of algal density, water flow, and substrate type on the culture of juveniles of *V. iris* in a recirculating system. Substrate was the most significant factor in culturing newly metamorphosed mussels. Juvenile mussels reared in fine sediment exhibited significantly greatest growth and survival, while those reared in culture units without substrate had the lowest growth and survival. No significant difference in growth and survival was recorded in those juvenile mussels reared in sand or gravel substrates. Algae at three concentrations and water flow at two levels did not result in significant differences in growth and survival. Based on these results, we recommend a substrate of fine sediment, an algae feeding density of 35,000 cells ml^{-1}, and a flow rate from 3.75 to 7.5 ml s^{-1} for rearing juvenile mussels of this and similar species. Ammonia is a critical parameter for juvenile mussel culture, and we recommend a maximum of NH_3-N 0.02 mg L^{-1} from this experiment and previous culture tests (Henley et al., 2001; Hua, 2005). We also suggest a source of natural food (such as pond water or stream water) to supplement the commercial diet for juvenile mussels or to directly feed mussels if those waters contain an abundance of natural food materials. An amplified recirculating culture system derived from this experiment, including 4 JCUs (37 L aquarium per each JCU) with 1-2 mm of fine sediment on the bottom of aquaria and one water conditioning and distribution tank (200 L), has been used to successfully produce a great number of juvenile mussels at FMCC (Hua, 2011).

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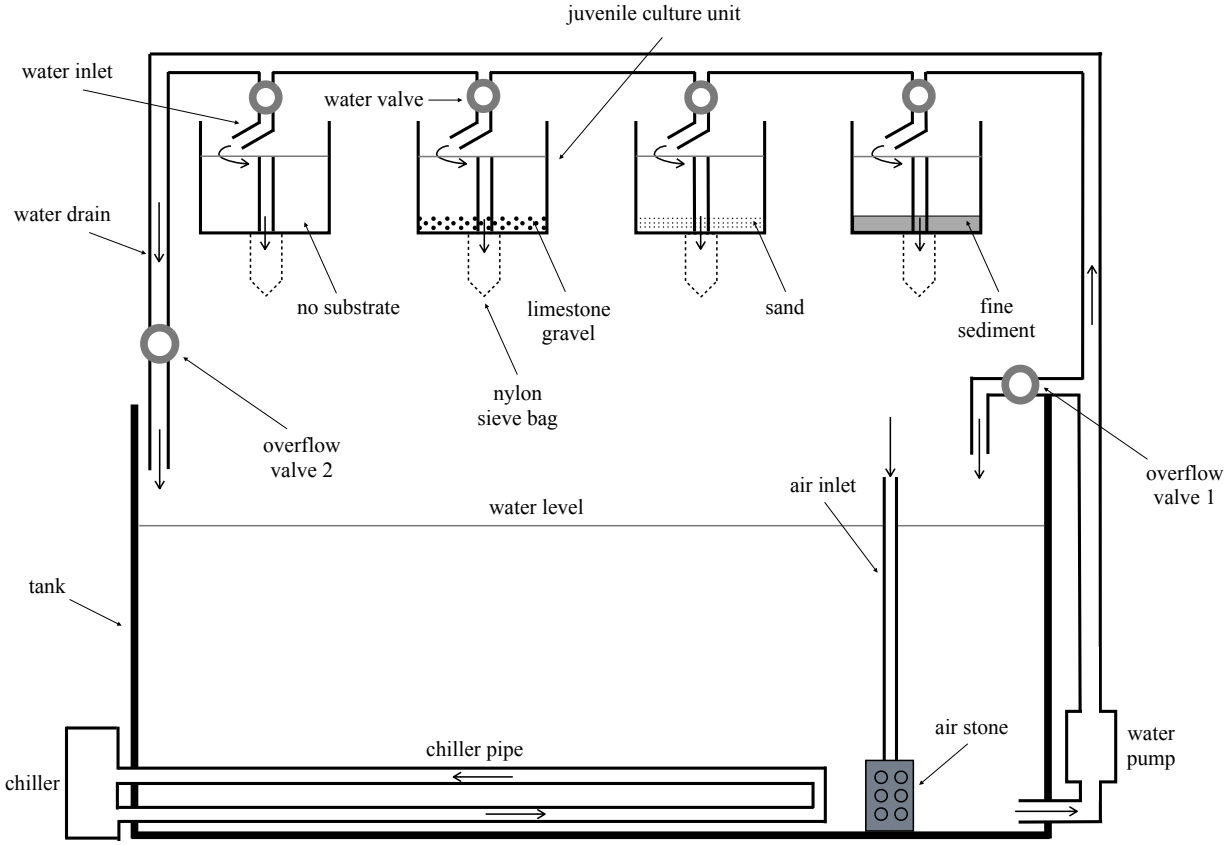

Fig. 1. Recirculating culture system used to rear juvenile mussels of *V. iris*.

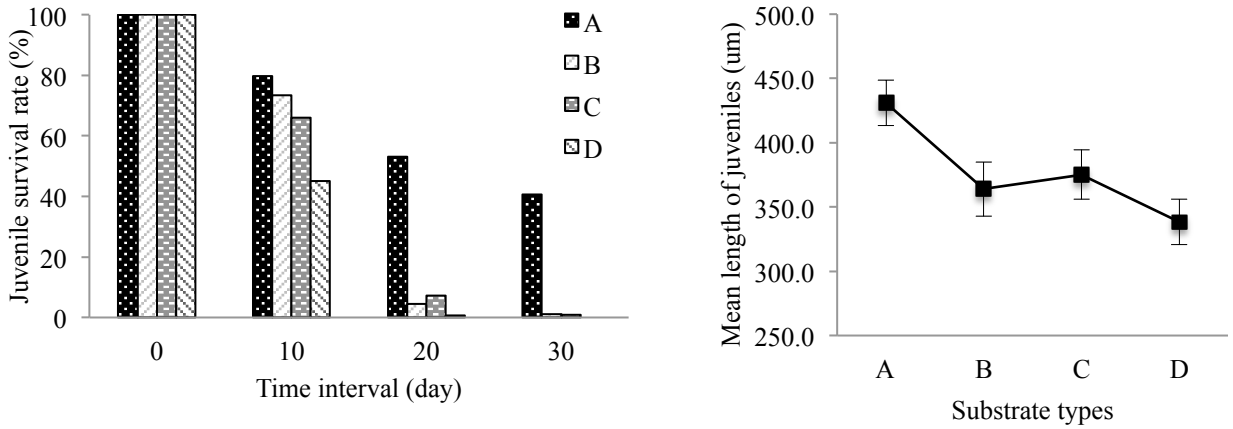

Fig. 2. Comparison of juvenile survival rates (left) and growth (right, Mean ± SD) among substrate types: A= fine sediment, B = sand, C = limestone sand, and D = no substrate.

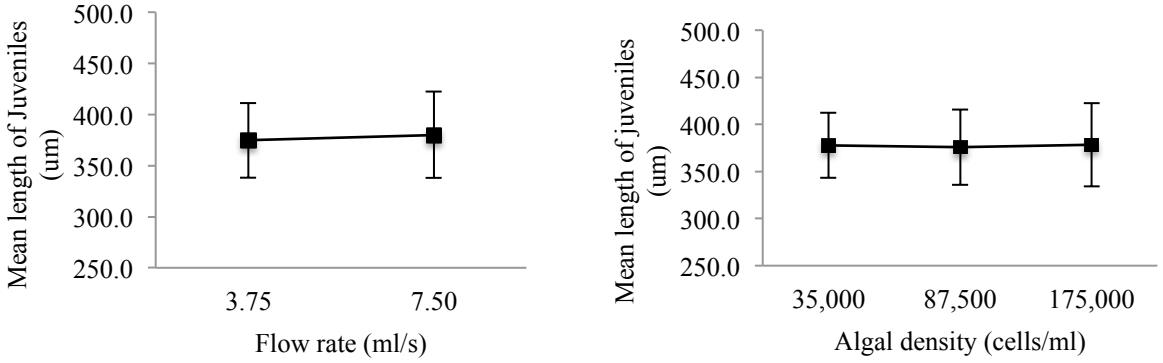

Fig. 3. Comparison of juvenile growth (mean length ± SD) at multiple levels of water flow (left) and algal density (right).

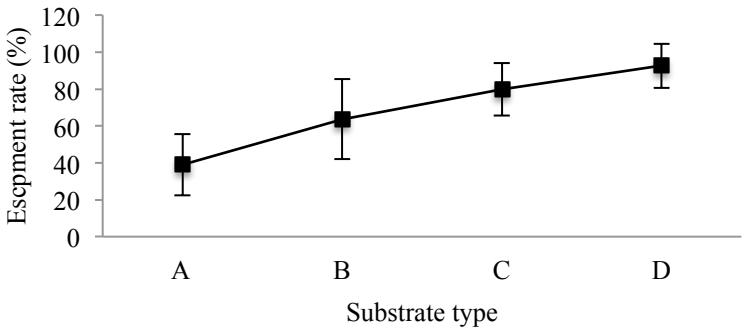

Fig. 4. Comparison of juvenile escapement rate (Mean ± SD) among substrate types: A= fine sediment, B = sand, C = limestone sand, and D = no substrate.

Table 1. Split-plot design for testing the effects of algal density (3 levels), water flow (2 levels) and substrate types (4 levels) on juvenile mussel growth and survival.

Whole plot		Split plot
Algae density	Water flow	Substrate type
1	H	A B C D
2	L	B C A D
3	L	D A B C
2	H	C D A B
3	H	D C A B
1	L	A D B C

Table 2. Effects of substrate, algal density and water flow on juvenile survival from logistic GLM model ($\alpha = 0.05$).

Source	DF	F value	Pr > F
Block	1	22.21	0.2423
Time	2	53.94	< 0.0001
Algal density	2	0.45	0.6418
Water flow	1	1.09	0.3084
Substrate	3	41.24	< 0.0001

Table 3. Effects of substrate, algal density and water flow on juvenile growth derived from full model and reduced model analyses ($\alpha = 0.05$).

		Full model		Reduced model	
Source	DF	F Value	Pr > F	F Value	Pr > F
Block	1	0.09	0.7729	0.14	0.7264
Flow	1	0.30	0.6056	0.21	0.6681
Algal density	2	0.02	0.9785	0.02	0.9809
Flow*Algal density	2	0.50	0.6328	0.39	0.6984
Substrate	3	74.31	<.0001	73.59	<.0001
Flow*Substrate	3	0.41	0.7464		
Algal density*Substrate	6	0.97	0.4760		
Flow* Algal density *Substrate	6	1.44	0.2567		

Table 4. Effect levels of substrate type on juvenile growth. Comparisons of significant at the 0.05 level are indicated by *** derived from full model and reduced model (HSD: Tukey's Honest Significant Difference).

	Full model				Reduced model			
Substrate Comparison	Difference Between Means	Simultaneous 95% Confidence Limits			Difference Between Means	Simultaneous 95% Confidence Limits		
A - C	0.28750	0.19423	0.38077	***	0.28750	0.19768	0.37732	***
A - B	0.34538	0.25001	0.44075	***	0.34538	0.25354	0.43722	***
A - D	0.47667	0.38339	0.56994	***	0.47667	0.38685	0.56649	***
C - A	-0.28750	-0.38077	-0.19423	***	-0.28750	-0.37732	-0.19768	***
C - B	0.05788	-0.03749	0.15325		0.05788	-0.03396	0.14972	
C - D	0.18917	0.09589	0.28244	***	0.18917	0.09935	0.27899	***
B - A	-0.34538	-0.44075	-0.25001	***	-0.34538	-0.43722	-0.25354	***
B - C	-0.05788	-0.15325	0.03749		-0.05788	-0.14972	0.03396	
B - D	0.13129	0.03592	0.22666	***	0.13129	0.03945	0.22313	***
D - A	-0.47667	-0.56994	-0.38339	***	-0.47667	-0.56649	-0.38685	***
D - C	-0.18917	-0.28244	-0.09589	***	-0.18917	-0.27899	-0.09935	***
D - B	-0.13129	-0.22666	-0.03592	***	-0.13129	-0.22313	-0.03945	***

A = fine sediment, B = sand, C = limestone sand and D = no substrate.

Table 5. Measurements of water quality (mean values) during the 30-day experimental period ($\alpha = 0.05$).

JCU	Hardness	NH3-N (mg L^{-1})	PH	DO(mg L^{-1})	Temperature (^{o}C)
1	239.25	0.005	8.53	8.21	21.73
2	217.00	0.005	8.53	8.21	21.90
3	225.75	0.003	8.54	8.34	22.05
4	245.75	0.005	8.53	8.09	21.63
5	224.00	0.003	8.56	8.19	21.63
6	245.25	0.003	8.55	8.50	21.70
p value	0.23	0.64	0.85	0.32	0.99

·CHAPTER 2-2: Optimization of laboratory conditions for culturing juvenile freshwater mussels

Abstract

Juvenile mussels fed on natural food in pond water and various algal diets were studied to compare the effects of diet on survival and growth. Juvenile ambient environmental variables including substrate types, water quality parameters (ammonia, nitrite, dissolved oxygen, pH, and temperature), predation, and interspecies competition in culture systems were also examined. Juveniles of wavy-rayed lampmussel (*Lampsilis fasciola*) fed on only food in pond water had significantly greater survival and growth rates than those fed diets of commercial algae. In the test of two algal diets, juveniles of rainbow mussel (*Villosa iris*) exhibited significant differences in survival rates; however their growth rates were not significantly different. Besides common species, the endangered oyster mussel (*Epioblasma capsaeformis*) was successfully produced and grew in systems with fine sediment (< 150) and pond water. To improve culture condition and water quality in juvenile culture system, bio-filter media was used and greatly reduced the concentration of ammonia (NH_3) and nitrite ($NO2^{-1}$) to a level below 0.06 mg N L^{-1} and 0.02 mg N L^{-1}, respectively. The negative impacts of flatworm predation and filamentous algae to the survival and growth of juveniles were controlled through host fish quarantine, pond water filtration, and appropriate manipulation to prevent filamentous algal growth. Juvenile escapement during the culture period was prevented by using sieve bags with a proper mesh size. An initial protocol for juvenile mussel propagation and culture was developed on the basis of studies during the past decade, and recommendations of further research on essential nutrient requirement of mussels was addressed in this study.

Keyword: wavy-rayed lampmussel (*Lampsilis fasciola*), rainbow mussel (*Villosa iris*), oyster mussel (*Epioblasma capsaeformis*), juvenile mussel culture, essential nutrient.

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1. Introduction

Freshwater mussels play a significant role in aquatic ecosystems. As suspension-feeders, they improve water quality by removing biotic and abiotic matter from water body through their feeding process. Because they remain relatively sedentary throughout their adult lives, they have been used as indicator species to evaluate ecosystem health (Van Hassel and Farris, 2007). Due to juvenile mussels' sensitivity to environmental contaminants, the United States Environmental Protection Agency (USEPA) (2013) recently added juvenile mussels to the list of standard aquatic organisms used for toxicity testing. This sensitivity, in addition to other confounding factors, has caused freshwater mussels to experience significant declines over the past 50 yr in North America (Williams et al., 1993). The propagation and release of hatchery-raised juveniles has been identified in species recovery plans as a primary strategy for restoring or augmenting imperiled populations (Neves, 2008). Consequently, efforts to improve propagation and culture techniques have been a focus of resource managers in recent decades. Great advancements have been made such as identification of suitable fish hosts, understanding the timing and duration of spawning seasons, feeding behaviors, and other primary life history characteristics of freshwater mussels. Despite the technological and procedural advances in the culture process however, standard protocols for juvenile mussel culture are not fully developed.

In recent years, a variety of juvenile mussel culture systems have been developed ranging in not only size, but also cost and complexity. These systems range from a simple, self-contained bucket system (Barnhart, 2006) to a trough-modified system, which utilizes recirculating pond water (Hua and Neves, 2007) as well as Pan and upweller systems (Mair, 2013). In addition to culture system design, ambient environmental conditions in culture systems have also been studied. Fine sediments have been shown to significantly increase survival and growth rates of juvenile mussels in a laboratory recirculating system (Zimmerman, 2003; Hua et al., 2013). Natural water sources; i.e., raw or coarsely filtered water from ponds, streams, or rivers, have also been shown to increase juvenile survival and growth (Barnhart, 2006; Hua et al., 2013) because they contain suitable foods including bacteria, organic matter, microscopic zooplankton, and algae.

Water quality is critical to juvenile mussel survival. The greatest risk to satisfactory water quality during mussel culture is un-ionized ammonia, which significantly affects survival and growth (Hua et al., 2013). Wang et al. (2007) conducted chronic toxicity tests of ammonia on multiple species of two-month-old juvenile mussels, and indicated that chronic toxicity values as low as 0.37 to 1.2 mg total ammonia N L^{-1} would influence the survival of juvenile mussels and 0.37 to 0.67 mg total ammonia N L^{-1} would affect growth of juveniles (at the ambient pH of 8.2-8.4 and temperature of 20^{o}C). Similar results were found in acute toxicity tests, that genus mean acute toxicity values to juvenile mussel survival ranged from 2.56 to 8.97 mg total ammonia N L^{-1} (at the pH of 8 and temperature of 12-25^{o}C) (Augspurger et al., 2003), which is the equivalent chronic toxicity value of 0.3 to 1.0 mg total ammonia N L^{-1}. Glochidia and young juveniles have been show to be more sensitive to unionized ammonia than adult mussels (Mummert et al., 2003; Augspurger et al., 2003). Nitrite (NO_2-N) is known to be toxic to a range of aquatic fauna, including fish and crustaceans (Lewis and Morris, 1986; Tahon et al., 1988; Chen and Chen, 1992). However, its chronic toxicity on juvenile mussels has not been tested. The acute toxicity tests of nitrite on juvenile mussels were conducted and showed dramatically different results among tests. USEPA (2010) documented that the LC_{50} value (96 hr, 20^{o}C) of nitrite to juvenile *Lampsilis siliquoidea* was 177 mg N L^{-1}, however Myers-Kinzie's (1998) reported the LC_{50} value of 0.45-0.63 mg N L^{-1} (48 hr, pH = 8.2-8.4, 24^{o}C) for the same species.

The other impacts that could jeopardize juvenile survival are predation and interspecific competition during mussel culture. Flatworm predation on newly metamorphosed juvenile mussels has been verified. Within 24 hr, flatworms were observed to consume 88.3% of juvenile mussels during an experiment (Zimmerman et al., 2003). Filamentous algae are common in natural water bodies and can be detrimental to juveniles by clinging to the periostracum and interfering with siphoning (Hua et al., 2001). Filamentous algae were observed to cause high mortality of juveniles in our previous culture systems. In addition, juvenile escapement during the culture period can affect juvenile production (Hua et al., 2013; Liberty et al., 2007). Without a suitable culture protocol to control these impacts, juvenile mussel culture often results in high mortality.

The purpose of this study was to optimize laboratory conditions by reducing mortality and increasing juvenile mussel production and grow-out of endangered mussel species. Specifically, our objectives were: 1) to test the effects of pond water and various algal diets on the survival and growth of juvenile mussels; 2) to compare the growth and survival of juveniles in different culture systems supplied with fine sediments; and 3) to develop an appropriate method to control water quality, flatworm predation, filamentous algae, and juvenile escapement. Our final goal was to establish a practical protocol to guide juvenile mussel culture for most species.

2. Materials and Methods

2.1 Juvenile mussels and culture systems

Juvenile mussels used for experiments were produced at the Freshwater Mollusk Conservation Center (FMCC), Department of Fish and Wildlife Conservation, Virginia Polytechnic Institute and State University. They were reared in three water re-circulating facilities, including Juvenile Culture Units (JUC), Barnhart Bucket Systems (BBS), and Trough System (TS) to be tested in experimental designs. The details of the three water circulation facilities are described below.

A. Juvenile Culture Unit (JCU): juveniles were placed in 4 JCU (70 L aquaria per each JCU) with 1–2 mm of fine sediment on the bottom of each aquarium. Fine sediment was collected from the New River in Montgomery County, VA and sieved through a 150 μm mesh size, so that sediment was of a smaller diameter than newly-metamorphosed juveniles. Sediments were autoclaved to kill any organisms within the sediment and then aerated for 2–3 days to facilitate oxidation, and then placed into each JCU at a depth of 1-2 mm on the bottom. JCUs were connected to a water conditioning and distribution tank (245 L) with aeration and water recirculation.

B. Barnhart Bucket Systems (BBS): juveniles were placed in enclosed chambers that were covered on each end with mesh sieve material (150 to 500 μm based on juvenile size). Each bucket (20 L) contained 7 chambers and a mini - jet pump built centrally in the bucket to create a

downweller of water flow through the partition between the two buckets that comprise the main structure of the system. A detailed diagram of the system design can be found in Barnhart (2006).

C. Trough Systems (TS): juveniles were placed in plastic containers (38 *cm* x 24 *cm* x 23 *cm*) with a fine layer (1-2 mm) of sediment covering the container bottom. The sediment used within this culture system was prepared in the same manner as the sediment used in the JCUs. Each trough (185 L) held up to 4 containers and was fed food in recirculated pond water through a conditioning and distribution tank (70 L).

Water quality parameters, including ammonia (NH_3), nitrite (NO_2^- - N), dissolved oxygen (DO), pH, and temperature were measured daily (unless specifically noted) throughout the duration of the experimental to monitor water quality.

2.2 Experimental design and data analyses

2.2.1 Effect of algae and pond water on juvenile mussel growth and survival

Two experiments were conducted in recirculating Barnhart bucket systems at the FMCC to determine the suitability of commercial algae and pond water to juvenile mussel growth and survival. The first experiment was to test the quality of commercial algae (50% Shellfish diet mixed with 50% Nanno 1800, Reed Mariculture Inc.) and pond water for juvenile survival and growth and implemented from June 8 to September 18, 2007. One-month-old laboratory-produced juveniles of *L. fasciola* 350 μm (300-400 μm) in mean length were used in this experiment. Juveniles were reared in conditioned water (dechlorinated municipal water mixed with well water and aerated), fed commercial algae twice a day at a density of 30,000 cells L^{-1} in each bucket system. Juveniles reared in pond water with natural food were not fed supplemental algae. Pond water was filtered through 200 μm and 100 μm sieves prior to use. Each bucket system was an independent unit with 3 chambers to hold juveniles. One hundred juveniles were randomly assigned to each chamber seated in the buckets to receive the commercial algal mixture or pond water. Ten individuals per experiment unit (chamber) were randomly selected and were measured for length. A completely randomized design (CRD) with subsampling was

applied; and each treatment was replicated 3 times using 3 buckets with a sample size of 90. Juvenile growth and survival were monitored monthly. Buckets were placed in a water bath to maintain 20^{o}C. The other facilities were the same as *2.1.B.*

The second experiment was to test the effect of two types of commercial algae on juvenile growth and survival and was conducted in BBS from February 13 to March 27, 2009. One-month-old laboratory-produced second generation juveniles of *V. iris* were used and fed commercial algae (shellfish diet 3600 and Nanno 1800 (Reed Mariculture Inc.) and an algae mixture produced by White Sulfur Springs Hatchery, WV. The feeding rate of algae was determined by a chlorophyll method (American Public Health Association, American Water Works Association, Water Environment Federation, 1998). Juveniles were reared in conditioned water (dechlorinated municipal water mixed with well water and aerated). Each bucket contained 7 chambers. Forty juveniles were randomly assigned into each chamber seated in the bucket (40 juveniles/chamber, 7 chambers/bucket) and received the same amount of algae (total chlorophyll 0.12-0.14 mg L^{-1}) fed twice a day. A CRD with subsampling was applied in this experiment with 3 replicates and sample size of 210 for each treatment. Juvenile growth and survival were monitored every 2 wk.

Live juveniles were counted to assess survival rate and then returned to their chamber after sampling. Ten individuals per experiment unit (chamber) were randomly selected and were measured for length using an ocular micrometer and dissecting microscope to monitor growth rate. A total of live juveniles was used for the measurement if the count of live juveniles in the chamber were less than 10 individuals. Hence, the experimental analysis was incorporated with unbalanced data. Survival and growth rates were analyzed using SAS 9.3. ANOVA was used to test for significant differences at $\alpha = 0.05$.

2.2.2 Effect of culture system on growth and survival rates of the endangered oyster mussel (Epioblasma capsaeformis)

This experiment was conducted in two recirculating systems of JUC and TS at the FMCC from June 5 to December 3, 2008. The newly metamorphosed juveniles of *E. capsaeformis* were from the same cohort and produced at the FMCC laboratory. Juveniles placed in JCU systems

received the combination diets of instant algae (50% Shellfish diet mixed with 50% Nanno 1800, Reed Mariculture Inc.) and pond water, while those reared in the TS continuously received pond water through a recirculating system connected to the pond. Water in the JCU was replaced with fresh pond water at the time of sampling; juveniles in the JCU were fed commercial algae twice daily. Fine sediment was sieved through a 150 μm mesh, prepared as *2.1.A.* and placed in both systems. A total number of 2346, 4505 and 4585 juveniles was randomly placed in the 3 containers (38 *cm* x 24 *cm* x 23 *cm*) seated in one TS, while 5510 juveniles were randomly placed in one JCU at the beginning of the experiment.

Juvenile mussels were sampled every 2 or 3 wk to determine survival and growth rate. Thirty individuals per tank were randomly selected and measured for length using a micrometer and dissecting microscope to determine mean growth rate. Live juveniles were counted to assess survival rate and then returned to tanks after sampling. Water quality monitoring was the same as experiment *2.2.1*. Survival rates were the relative rates calculated as the percent of live juveniles collected in the previous sampling period. Survival rates and growth rates were tested for significant differences at $\alpha = 0.05$ using ANOVA (SAS 9.3).

2.2.3 Effectiveness of bio-filter media in juvenile mussel culture

Water quality improvement using bio-filter media in juvenile mussel culture systems (AMB Bio Media) was tested. The bio-filter media was prepared and coated with bacteria for use (1 L bio media/bucket). The concentrations of NH_3-N and NO_2-N were monitored before and after bio-filter media were added into the bucket. DO, pH and water temperature were also measured during the experimental period.

3. Results

Results of the first experiment (*2.2.1*) indicated that juveniles of *L. fasciola* fed on food in pond water and commercial algae exhibited significant differences in survival and growth ($p < 0.05$). Juveniles in pond water had significantly higher survival and faster growth than those fed commercial algae. After 40 days, only a few juveniles remained in the buckets supplied with commercial algae, while over 60% juveniles survived in those buckets filling with pond water

(Figure 1). Juveniles in pond water exhibited different growth rates among buckets. Juveniles reared in Bucket 1 grew significantly faster than those in the other two buckets since the second sampling occasion ($p < 0.05$) (Table 1), while juveniles in Bucket 2 and Bucket 3 had similar growth ($p > 0.05$) (Figure 1).

Results of the second experiment (*2.2.1*) showed that growth rates of juveniles were not significantly different between treatments of two types of mixed algae during all 3 sampling events ($P > 0.05$). However, survival rates differed after the second sampling occasion ($P < 0.05$). Juveniles fed multiple species of commercial algae (MCA) had a significantly higher survival rate than those fed multiple algae supplied by White Sulfur Spring Hatchery (MAWSS) (Table 2).

Results from the experiment to test culture systems (*2.2.2*) for rearing *E. capsaeformis* showed that juveniles in the TS had significantly higher survival rates than those cultured in the JCU ($p < 0.05$). Survival rate of juveniles in the JCU decreased to 0.2% after 2 mo (8 wk), but remained at 11.7 % in TS (Figure 2-1.). After 10 wk, relative survival rate in the TS remained as high as 90% (right on Figure 2-1). Juveniles in both culture systems had similar growth rates during the early 2 mo (8 wk) with an incremental length increase from 0.45 to 0.75 mm. However, juveniles in the JCU ceased growth thereafter. Juveniles in the TS grew continuously and reached 1.6 mm after 4 mo (17 wk) (Figure 2- 2). Flatworm predation was observed in both culture systems, and flatworms were more abundant in JCU.

The bio-filter media efficiently reduced high concentrations of NH_3 and NO_2^{-1}, and improved water quality. After bio-filter media was added into BBS for 6 hr, the NH_3 concentration changed from 0.22 mg N L^{-1} to 0.19 mg N L^{-1}, while the NO_2^{-1} concentration changed from 0.14 mg N L^{-1} to 0.23 mg N L^{-1}. After 3 days, NH_3 concentration declined to 0.04 mg N L^{-1}, and the NO_2^{-1} concentration lowered to 0.02 mg N L^{-1} (Figure 3). The other water quality parameters were measured and recorded, and were stable during the experimental period. The mean value of DO was 7.0 ± 0.3 mg L^{-1}; pH was 8.0; temperature was at 21.8 ± 2.0^{o}C; and alkalinity ranged from160 – 210 (mg L^{-1} as $CaCO_3$) (Table 3).

Juveniles initially reared in BBS and JCU were moved to TS units once they reached about 3 mm to receive pond water for further growth.

4. Discussion

Feeding behavior of juvenile mussels has been studied extensively. Algae, bacteria, organic materials and zooplankton were identified as suitable food, which are obtained by through ciliary feeding and pedal-feeding mechanisms (Yeager and Cherry, 1994; Gatenby et al.,1997, Silverman et al., 1997; Hua et al., 2013;). However, essential nutritional requirements of mussels remain undetermined. Algae have been used to feed juvenile mussels in early life stages (1-2 mo) (Gatenby et al. 1997, 2003; Barnhart, 2006), but we were not successful in growing juvenile mussels to sub-adults using algae alone, until pond water was added to our culture systems. Nutritional components in natural waters are their primary food source, to provide essential nutrients throughout their life cycle (Hua et al., 2001). Our results confirmed that natural food from a pond significantly outperformed algae diets in culturing juvenile mussels. The juvenile mussels of *L. fasciola* only survived slightly over 1 mo relying on the algal diet alone. Similarly, juveniles of *V. iris* fed algae alone survived for 2 mo. Likely, algal diets alone does not meet the nutritional requirements of juvenile mussels. Although the advantages of pond water have been recognized in rearing juvenile mussels (Hua and Neves, 2007) the quality and quantity of essential nutritional elements in pond water are still unknown. Some amino acids and their metabolites are considered to be important regulators of larval metamorphosis for fish growth (Li et al., 2009), and mineral elements in natural water are also essential to fish survival and growth (Steffens, 1989; Hepher, 1990). Seemingly, mussels also demand essential elements that natural water can provide, but are absent in algal diets. Additionally, food availability is also critical to juvenile growth. Juveniles of *L. fasciola* reared in Bucket 1 grew faster than those in Bucket 2 and 3, might due to the location, which facilitated to receive sufficient food support entangled in pond water. Bucket 1 was the first to receive pond water, and received relatively more water and suspended nutrients than the other two buckets. The relationship between juvenile growth and food supply in pond water also appeared in juveniles reared in different

chambers within the same bucket. For example, juveniles in chamber 1 and 2 grew faster than those in chamber 3, perhaps because chamber 1 and 2 were located under the water supply device that received pond water and suspended food first, compared to those reared in chamber 3.

The content of protein, carbohydrate, and lipid reserves in algal diets for mussels differed among algae species (Gatenby et al., 2003), and algal growth stage and ambient environment (Harrison et al., 1977; Morris et al.,1983). In our study, juveniles of *V. iris* fed with different algal diets had significantly different survival rates that juveniles fed MCA, and had higher survival rates (88.6%, 82.8%) than those fed MAWSS (68.9%, 59.2%) at sample events of 4 wk and 6 wk, respectively. Apparently, nutrient composition in MCA was more suitable to juveniles of *V. iris* when compared to that in MAWSS. Results in our study validated the previous findings.

Juvenile mussels reared in fine sediment achieve significantly higher survival and growth rates than those cultured in sand, gravel or without sediment (Hudson and Isom, 1984; Hua, 2005; Wang et al., 2011; Hua et al., 2013). Hence, sediments were added in our experiment with *E. capsaeformis* in the two culture systems (TS and JCU). Juveniles reared in TS had significantly higher survival rates than those reared in JCU, although the growth rates were not significantly different in the first 2 months. Juveniles reared in JCU experienced dramatically high mortality at this early life stage and only a few juveniles were alive after 8 wk (2 mo). During each sampling occasion, flatworms were found in both juvenile culture systems, and more abundantly in JCU. They either ate juvenile mussel tissues or swallowed the juveniles, shell and all. Zimmerman (2003) reported that flatworms preyed on young mussels and caused high mortality, and our results confirm that. Juveniles reared in TS were also influenced by flatworms and experienced high mortality during the first 2months. However, live individuals exhibited high survival rate thereafter. Likely, flatworms had less influence on the survival of larger (> 1.5 mm) and older (> 2 mo) juvenile mussels. The first 2 months of life is a critical time to culture newly transformed juveniles. Obviously, a protocol to prevent and control the predation by flatworms is needed. By investigating the source of the flatworms, we determined

that they entered the culture systems through host fishes or the pond water used for juvenile culture. Hence, a protocol was developed to quarantine of fish hosts, disinfect culture system and sediment, filter pond water, and change sample frequency have been applied as effective methods to restrict flatworm entry into in juvenile culture systems. Flatworm predation on juvenile mussels was not observed after applying this protocol. Our recent research confirmed that juvenile mussels reared in TS and JCU with no flatworms had high survival and fast growth.

Ammonium ion (NH_4^+), ammonia (NH_3), nitrite (NO_2^-) and nitrate (NO_3^-) are common inorganic nitrogen forms and are naturally present in aquatic ecosystems (Durand et al., 2011). The forms of NH_3 and NO_2^-, even at low concentrations, are considered toxic to aquatic animals (Russo, 1985; Camargo and Alonso, 2006). Juvenile mussels are more sensitive to ammonia than other aquatic organisms (Wang et al., 2011; USEPA, 2013). Recent long-term (28-day) chronic tests on the sensitivity of juvenile mussels to ammonia showed that a chronic toxicity value of 0.36 mg ammonia-N L^{-1} influenced survival and biomass in a water-only treatment, 0.66 mg ammonia-N L^{-1} affected survival, and 0.20 mg ammonia-N L^{-1} affected biomass in a water and sediment treatment (Wang et al., 2011). Hence controlling ammonia under these thresholds is critical to juvenile mussels as well as host fishes, because ammonia and nitrite are inevitable nitrogen forms produced in aquaculture operations. Bio-filter media provide large surface areas that can be colonized by bacteria, and have been commonly used in aquaculture to adjust water quality (Nelson, 2008). We applied this type of bio-filter in our BB to reduce NH_3 concentration nitrification of NH_3 to NO_2^{-1}, and then final oxidation to much less toxic NO_3^{-1}.The efficiency of bio-filter media was obvious; NH_3 concentration declined immediately after pre-prepared (bacteria colonized) bio-filter media were added in the BBS. The concentration of NO_2^{-1} expectedly increased initially and then declined as expected. The entire process of transformation of the three N forms (NH_3, NO_2^{-1}, NO_3^{-1}) took 3 days to reach an endpoint such that NH_3 was less than 0.05 mg N L^{-1} and NO_2^{-1} was less than 0.03 mg N L^{-1}. Thereafter, bio-filter media continuously maintained water quality within the range of 0.01 to 0.02 mg N L^{-1} that is suitable for juvenile mussels. Wang et al. (2011) showed that juvenile mussels at a concentration of 0.06 mg ammonia-N L^{-1} had the highest survival and maximum biomass. Although NO_2 ions are

considered a major toxicant to aquatic animals (Russo, 1985; Camargo and Alonso, 2006), the toxicity test on the sensitivity of nitrite to juvenile mussels was limited, with highly different results from existing data (Myers-Kinzie's, 1998; USEPA, 2010). We controlled NO_2^{-1} concentration to less than 0.02-0.03 mg N L^{-1} in all systems to minimize risk to juveniles.

In addition to water quality and predation by flatworms, filamentous algae were the other factor that caused mussel mortality. Some filamentous algae were able to colonize the periostracum of mussels and culture tanks. We prevented algae proliferation and photosynthesis using a layer of black film to cover the juvenile mussel culture systems to eliminate sunlight and filamentous algae growth.

Juvenile mussels are very active and can exhibit high escapement rates during their early life (Liberty et al., 2007). We used simple nylon filter bags potentially prevented escaping juveniles (Hua et al., 2013). Juvenile mussels of multiple species have been grown to sub-adult size at FMCC since 2006. Juvenile mussel culture has achieved great success that millions of juvenile mussels have been produced in our laboratory (Appendix A-1). All the advancements in juvenile culture are included in the protocol in the section 'Conclusions and Recommendations'.

5. Conclusions and Recommendations

Natural food in pond water significantly outperformed commercial algae diets to support survival and growth of juvenile mussels. The effects of algae diets to juvenile mussel culture varied among algae species. Sediments greatly supported growth and survival of juvenile mussels, especially in their early stages. Water quality is also critical to juvenile mussel culture. Concentrations of ammonia and nitrite under 0.03 mg N L^{-1} and 0.02 mg N L^{-1}, respectively, were considered safe and achievable for juvenile mussels. Bio-filter media effectively reduced the NH_3 and NO_2^{-1} concentrations and maintained stable water quality. Additionally, filamentous algae build-up in juvenile culture systems can be controlled using a cover of black plastic film. Applying sieve bags to drains can prevent escapement of juvenile mussels.

Further study on the requirements of essential nutrients and food requirements in the early life stages is needed. Establishment of a laboratory protocol for juvenile mussel culture is

important and essential to increase production capabilities and improve culture techniques. This initial protocol for culturing juvenile mussels was developed after many years of experience in optimizing our laboratory conditions to provide advancement in the methodology for propagation of juvenile mussels, to assist in the recovery of the many endangered species in this taxonomic family.

Protocol for culture of juvenile mussels under laboratory conditions

5.1. Gravid mussel and fish collection

Gravid female mussels can be collected from their source rivers by snorkeling and using view scopes during their periods of gravidity. Gravid mussels can be held in a cooler and transported to a laboratory or a facility for propagation. They can be held in a living stream system supplied with pond water or natural water and returned to their original collection sites after extraction of glochidia.

Host fishes can be collected using a Smith-Root backpack electro-fisher from various stream locations and transported to the mussel propagation facility. Fishes can be held in a 190 L cooler with controlled aeration (>7 mg L^{-1}) and temperature (15-18 °C) during transport. Salt should be applied at 0.7 ppt to reduce fish stress. Fishes should be quarantined at a salinity of 3-5 ppt for 2 days before the infestation, to eliminate flatworms and other intruders. Fishes can be then placed in recirculating aquaculture systems after infestation. Water temperature can be maintained at 20 ± 2^{o}C. Fishes can be fed live blackworms, frozen bloodworms, and glassworms.

5.2. Water quality in fish and mussel systems

Recirculating aquaculture systems can be prepared to establish suitable water quality 4 wk prior to receiving fish hosts. Systems can be filled with conditioned water, and Aquacoat, Bacta-pur probiotics and ammonium chloride should be added at suggested ratios. Hereafter, ammonium chloride should be added into systems weekly to meet the requirements for bacterial growth. Meanwhile, healthy and uninfected fishes can be reared in the fish system to maintain bacteria production, until they are replaced by glochidia infested host fishes. Water quality

parameters including ammonia (NH_3), nitrite (NO_2^-), pH, dissolved oxygen (DO), and temperature should be tested daily. Bio-filter medium must be used to control ammonia and nitrite concentration. Aeration should be applied to each system to maintain DO > 7.0 mg L^{-1}. Water temperatures in fish holding systems can be maintained at 20° C by heaters and chillers. Water exchange happens when the parameters exceeds safety levels (NH_3-N > 0.06 mg N L^{-1} and NO_2^-N > 0.02 mg N L^{-1}).

In juvenile culture systems, NH_3, NO_2^-, DO, pH and temperature should be measured every other day to monitor water quality. The concentrations of NH_3 and NO_2^- should be maintained within safety levels (0.06 mg N L^{-1} and 0.02 mg N L^{-1}). The DO, pH and temperature values can be around 7.0 mg L^{-1}, 8.0 and 25^oC, respectively.

5.3. Juvenile mussel propagation

Glochidia can be extracted from the gill marsupia of gravid mussels by injecting water into the gills using a syringe and hypodermic needle. To determine viability of glochidia, a small sample of 20-30 glochidia can be tested by exposure to a dilute salt solution. Matured glochidia shut both valves immediately. Fish and glochidia can be placed together in a container with a suitable amount of water under constant aeration for infestation. The infestation time can be determined from the monitored density of encysted glochidia on fish. Host fish should be then transported to recirculating aquarium systems and reared until juvenile mussels complete metamorphosis and excystment. Newly metamorphosed juveniles should be collected daily through siphoning, counted and measured using a dissecting microscope under 40 X magnification before they are placed in various juvenile culture systems for grow-out.

5.4. Juvenile mussel culture

5.4.1 Protocol for juvenile culture

A. Juvenile Culture Unit (JCU): Juveniles can be fed with food in pond water filtered through a 5 µm nylon monofilament filter bag to eliminate flatworms. Fine sediment (<150 μm in particle size) should be autoclaved, oxidized, and placed on the bottom of the JCU in a fine layer (1-2 mm). Juvenile mussels should be continuously fed a commercial algae mixture -

Nannochloropsis oculata 3600 and Shellfish Diet 1800 as a supplement. A continuous feeding scheme can be controlled by an electronic auto-off Timer (H3CR-F8-300, OMRON Corporation) and solenoid valve (Evolutionary Concepts Inc.). Pond water in the systems should be exchanged weekly. Algal feeding ratios are showed in Table 4. Heaters can be used to maintain the water temperature around 20 ± 2^{o}C in the winter.

B. Barnhart Bucket Systems (BBS, Barnhart, 2006): juveniles can be placed in enclosed chambers (up to 1000 individuals/chamber). Mesh size should be changed from 100 to 500 μm to accommodate the growth of juvenile mussels. Water supply and continuously feeding devices are the same as those in JCUs except for algal feeding ratios (Table 3). Feeding ratios are adjusted seasonally along with the fluctuation in food availability and water quality of pond water. Aeration and bio-filter media can be applied in the bucket systems to facilitate nitrification. Buckets can be placed in a water bath to maintain a water temperature around 20 ± 2^{o}C.

C. Trough Systems (TS): juveniles can be placed in four 15 L plastic containers at a density of approximately 2000-5000 individuals per container, once they reach 2-3 mm in length. Preparation of fine sediment is as the same as that in *4.4.1-A.* Trough systems use recirculating pond water throughout the spring, summer, and fall. Juveniles can be fed only natural foods in pond water during these seasons. Water temperature in TS fluctuates along with pond water during these seasons. Once the water temperature drops below 15^{o}C, TS should be then disconnected from the pond and only recirculating pond water used within the system. Juveniles are then continuously fed with the commercial algal mixture as a supplement along with a partial pond water exchange (~10%) daily. Heaters can be used to maintain the water temperature around 20 ± 2^{o}C to promote the growth of mussels over winter. Aeration should be applied to the system.

5.4.2 Juvenile mussel sampling and maintenance

Newly-metamorphosed juveniles should be sampled weekly until achieving a size greater than 2 mm in length, to prevent flatworm eggs hatching up if they entered into the culture

systems. Thereafter, they can be sampled every other week to monitor growth and survival. Juvenile culture systems should be completely dry for 1 wk before they are reused to eliminate flatworms and other undesireable biota.

One-year-old mussels can be moved to containers filled with limestone gravel (2-3 mm in size) at a depth of 10-20 mm for the post-stage culture in trough systems. Juveniles are supplied pond water and associated foods through continuous feeding systems recirculating between the pond and laboratory. Temperature at this stage is not controlled, fluctuating with pond water to simulate natural conditions for mussels before being released into rivers.

Although the culture of juvenile mussels in a natural water body has reached the stage for commercial production (Hua et al., 2001), food components and the essential nutrients in natural foods for mussels are still not understood. Hence, essential nutrition requirements at different life stages of mussels need to be qualitatively and quantitatively analyzed such that all dietary requirements are known. Without understanding these dietary requirements, mussel culture indoors or outdoors, and environmental influences will continue to promote great variability in propagation attempts. Developing a series of diets to meet the dietary requirements of mussels at various life stages is necessary to establish a rigorous protocol for indoor mussel culture, as has been developed for controlled fish culture. The components of natural waters that make them suitable for the survival and growth of juvenile mussels need further study, such that the use of laboratory methods for propagation can achieve comparable results and eventually increase the survival and growth of juvenile mussels to aid recovery of endangered species.

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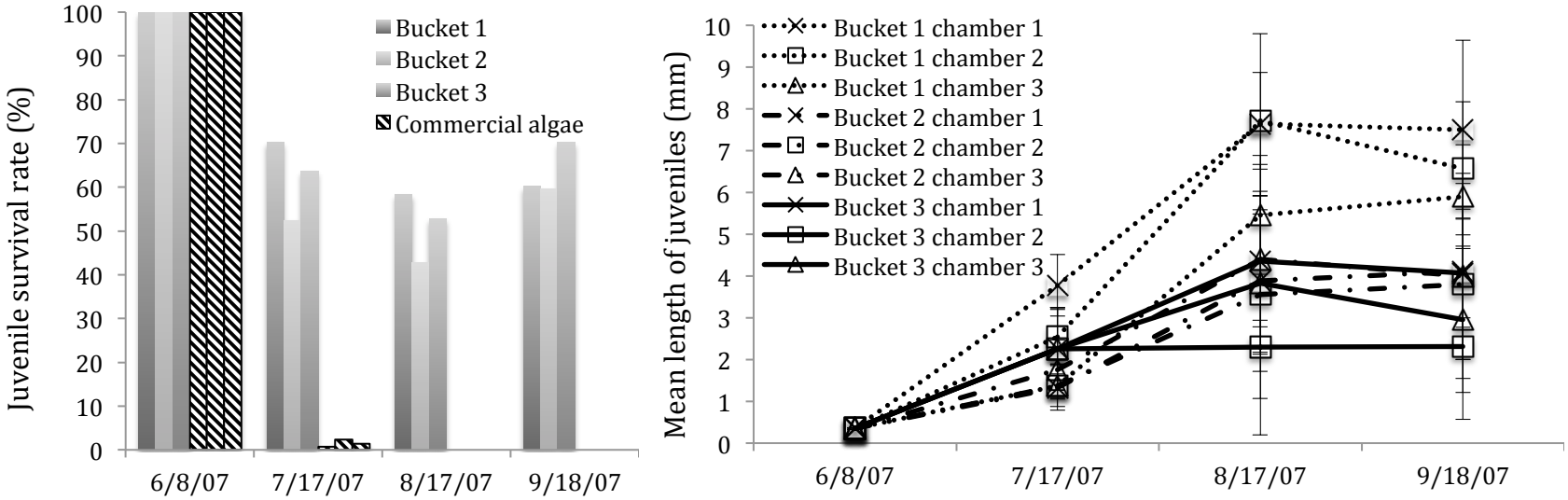


Figure 1. Comparison of survival (left) and growth (right, Mean ± SD) of juvenile mussels of *L. fasciola* fed commercial algal diets and natural foods in pond water.

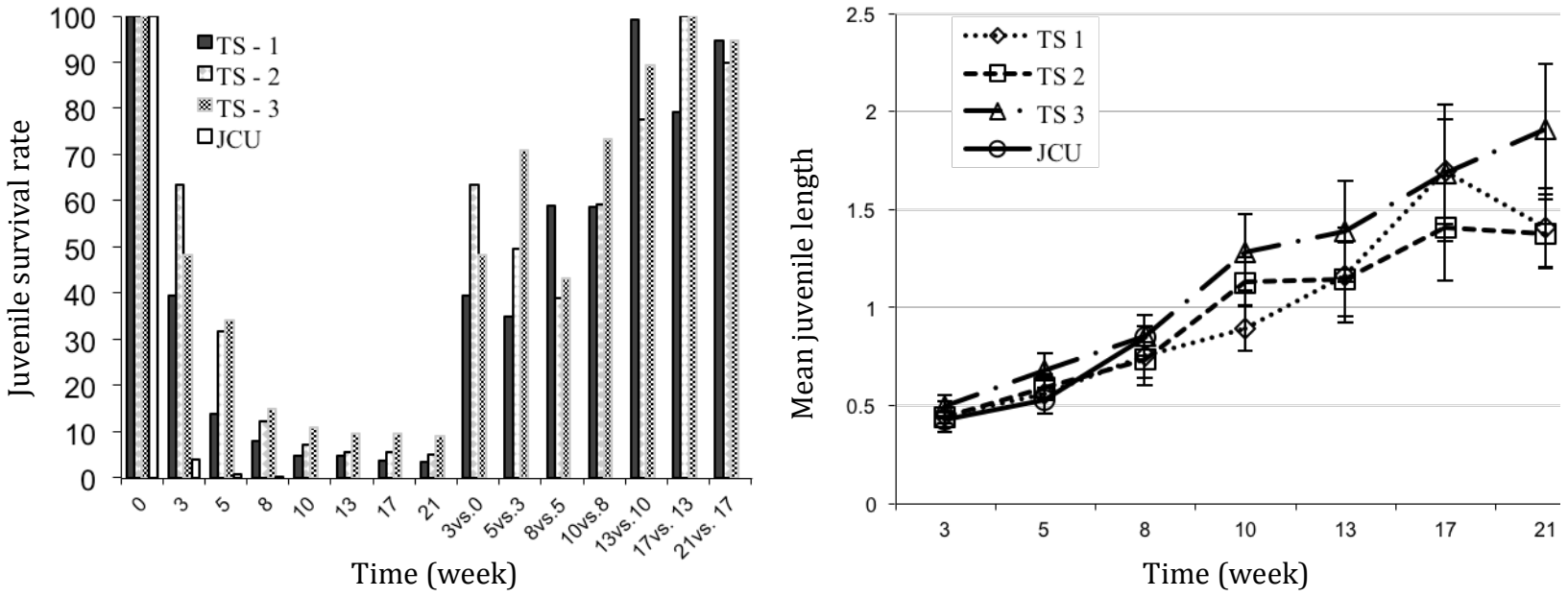
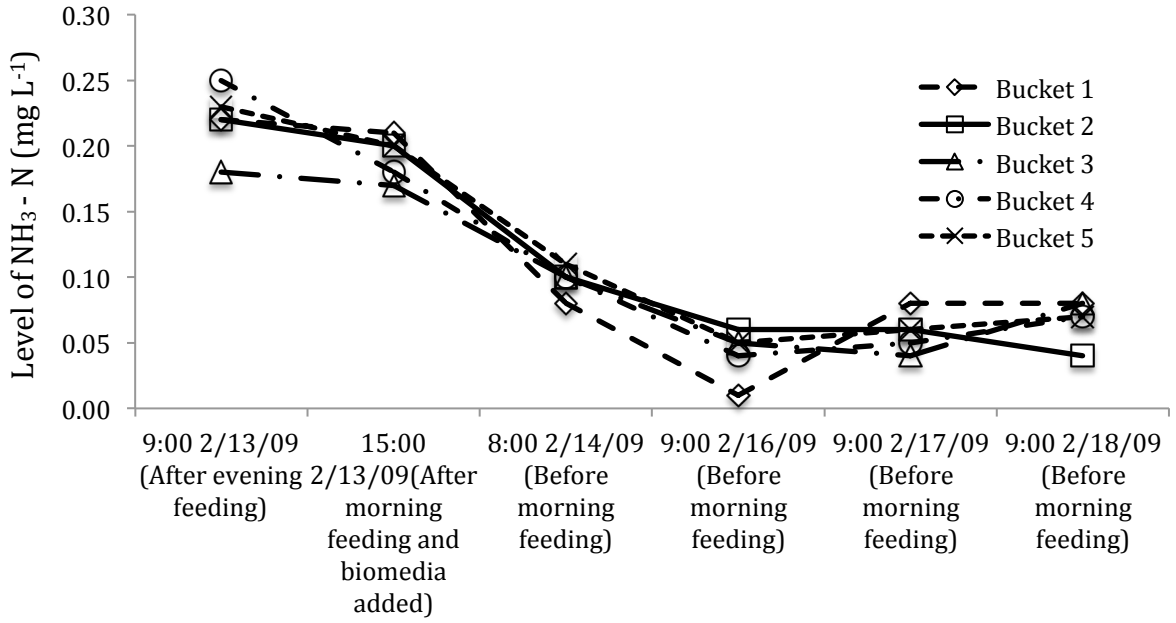
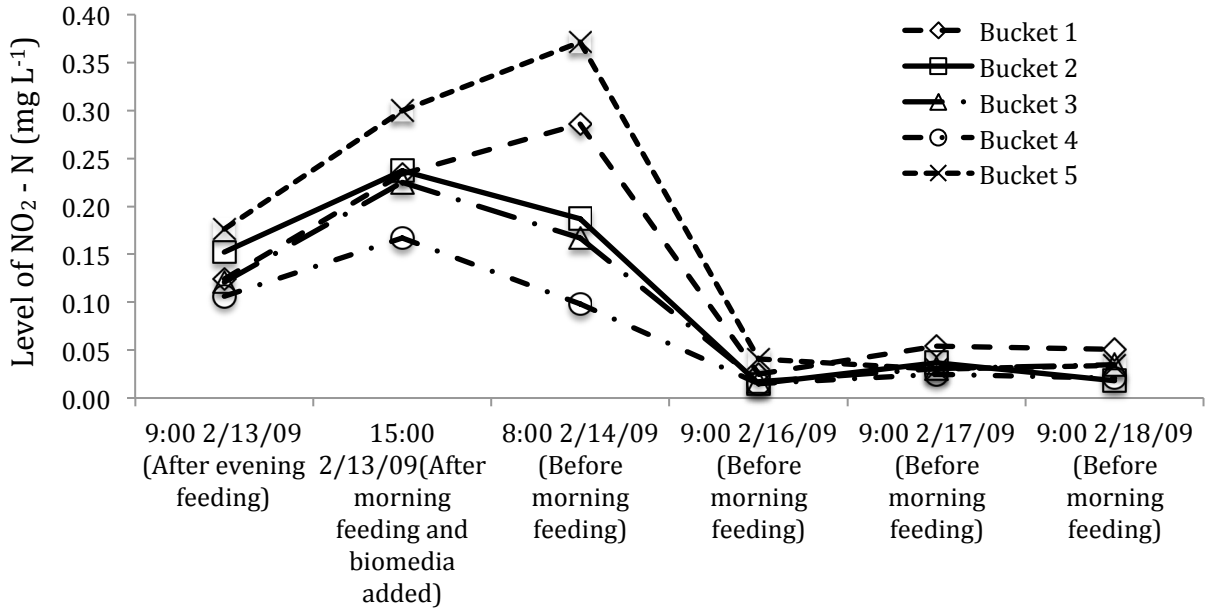


Figure 2. Comparison of survival (left) and growth (right, Mean ± SD) of juvenile mussels of *E. capsaeformis* between trough systems (TS) and juvenile culture units (JCU).


Level of NH_3 - N (mg L^{-1})
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Bucket 1
Bucket 2
Bucket 3
Bucket 4
Bucket 5
9:00 2/13/09 (After evening feeding)
15:00 2/13/09(After morning feeding and biomedia added)
8:00 2/14/09 (Before morning feeding)
9:00 2/16/09 (Before morning feeding)
9:00 2/17/09 (Before morning feeding)
9:00 2/18/09 (Before morning feeding)


Level of NO_2 - N (mg L^{-1})
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Bucket 1
Bucket 2
Bucket 3
Bucket 4
Bucket 5
9:00 2/13/09 (After evening feeding)
15:00 2/13/09(After morning feeding and biomedia added)
8:00 2/14/09 (Before morning feeding)
9:00 2/16/09 (Before morning feeding)
9:00 2/17/09 (Before morning feeding)
9:00 2/18/09 (Before morning feeding)

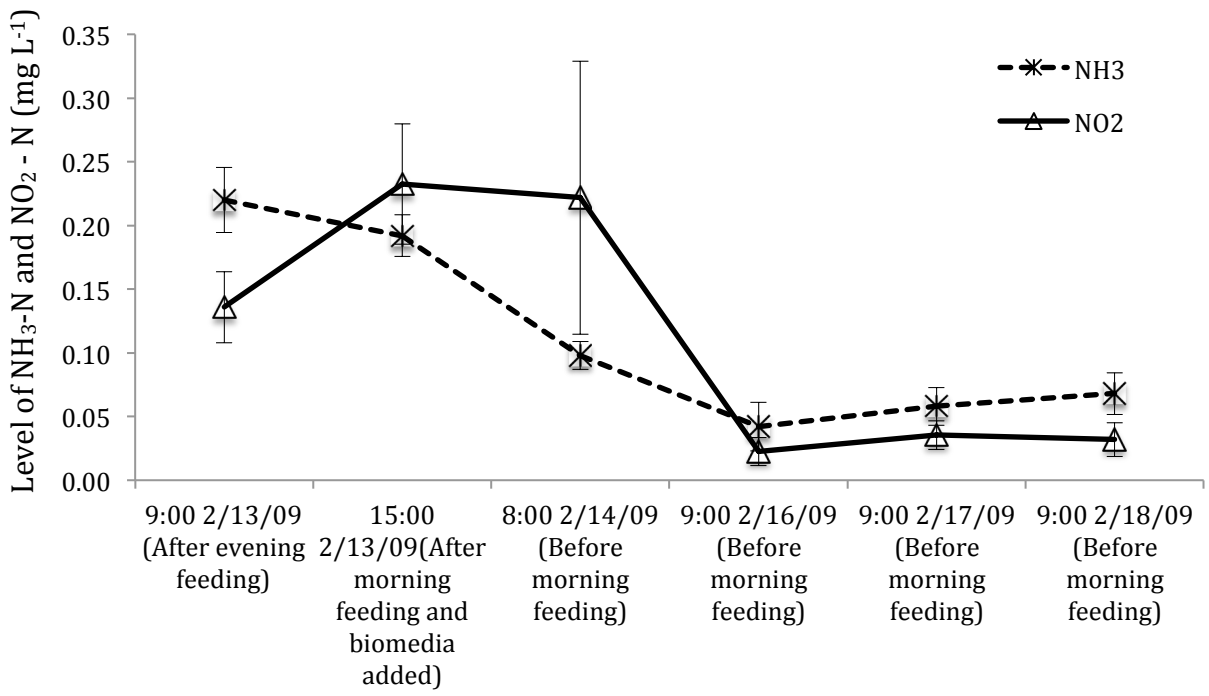


Figure 3. NH_3 (top), NO_2^{-1} (middle), change (bottom) in bucket systems after using bio-filter media.

Table 1. ANOVA results of growth in length (p values) of juvenile mussels of *L. fasciola* fed natural foods in pond water among buckets from June 8 to September 18, 2007 ($\alpha = 0.05$).

Date	p value of all	p-value between compared buckets		
		Bucket 1 - Bucket 2	Bucket 1- Bucket 3	Bucket 2 - Bucket 3
6/8/07	-	-	-	-
7/17/07	0.4092	-	-	-
8/17/07	0.0102	0.0118	0.0065	0.6000
9/18/07	0.0018	0.0034	0.0011	0.2105

Table 2. Comparison of algae diets for growth and survival of juvenile mussels of *V. iris* ($\alpha = 0.05$).

Growth rate (%)

	After 2 weeks			After 4 weeks			After 6 weeks		
Algae type	Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
MAWSS	21.5	8.8	0.28	54.7	10.4	0.94	86.1	24.2	0.98
MCA	26.3	9		55.2	17.1		85.8	17.6	

Survival rate (%)

	After 2 weeks			After 4 weeks			After 6 weeks		
Algae type	Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
MAWSS	93.3	4.7	0.74	68.9	8.8	<0.001	59.2	14.9	<0.001
MCA	92.8	1.5		88.6	7		82.8	6.8	

*MAWSS: Multiple algae produced by White Sulfur Spring hatchery , MCA: Multiple commercial algae

Table 3. Parameters of water quality and measured values during culture experiment at FMCC.

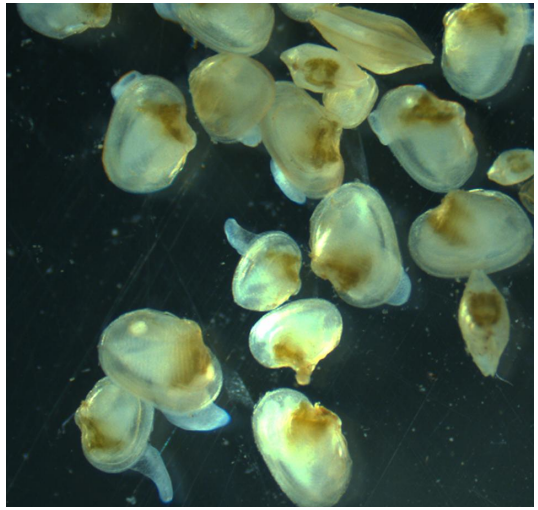
Parameter	Range
NO_2^- (mg N L^{-1})	0.002-0.008
NH_3^+ (mg N L^{-1})	0.001-0.009
Temperature (^oC)	19-23
pH	7.5-8.3
DO (mg L^{-1})	7.8-8.5
Alkalinity (mg L^{-1} as $CaCO_3$)	160 - 210

Table 4. Daily feeding values for juvenile mussels reared in each culture system (seasonal alternation in BBS).

Algae diets	BBS			JCU	TS
	Winter	Summer	Spring and Fall	All seasons	All seasons
Nanno 3600 (ml)	0.050	0.013	0.025	0.550	0.250
Shellfish 1800 (ml)	0.250	0.067	0.125	1.70	0.750

Juvneile mussels of *V. iris* rearing in the TS

Juvenile mussels of *V. iris* (9 mo.) from TS



Juvenile mussels of *E. capsaeformis* (4 mo.) rearing in the JCU

Juveniles of *E. capsaeformis* reared in the TS with byssus threads

Sampling of juvenile mussels of *L. fasciola* reared in the JCU

Appendix A-1: Juvenile mussels produced from FMCC

CHAPTER 3: An assessment of site suitability for restoring laboratory-reared juvenile mussels to historic river reaches in Virginia and Tennessee

Abstract

Laboratory-reared juveniles of the rainbow mussel (*Villosa iris*) and wavy-rayed lampmussel (*Lampsilis fasciola*) were released at three sites in the Clinch and Powell rivers based on their habitat characteristics and conservation interests using three types of release methods (untagged, tagged and caged) to test habitat suitability for restoration of these species. Mark-recapture methodology was used to monitor the survival and growth of released mussels during a 4-yr period. Exponential models and individual survival models estimated the natural mortality of released mussels. Growth rates of released mussels were significantly different ($p < 0.05$) among the 3 sites. Mussels released at Horton Ford, Clinch River, exhibited significantly faster growth than those released at Fugate Ford, Powell River ($p < 0.001$). Mussels released at Fugate Ford had slower growth than those at the other two sites ($p < 0.05$); however, mussels kept in cages and deployed at Fugate Ford and Payne Property, Clinch River, exhibited slightly different trends in growth rates. Concentrations of Al, Cu and Zn were significantly different in mussels collected from the 3 sites ($p < 0.05$), while the concentration of Fe was not significantly different in specimens collected ($p > 0.05$). Body burdens of Al concentration were significantly (3-10 times) higher in specimens of *V. iris* collected from Payne Property. The concentration of Cu was significantly lower in mussels from Freshwater Mollusk Conservation Center (FMCC) pond, and Zn level was significantly higher in mussels from FMCC pond than those at Horton Ford. Estimated annual mortality rate (M) of untagged mussels was significantly less than that of tagged mussels of *V. iris*. The estimated catch probability was higher in untagged mussels than that of tagged mussels. Estimates of M for *L. fasciola* were much less in caged mussels than those for tagged and free-released mussels. Evaluation of site suitability from in-situ survival and growth of juveniles indicated that Horton Ford is the most suitable site for

mussel restoration, while environmental conditions at Fugate Ford are deemed unsuitable for mussel restoration and recovery.

Keywords: rainbow mussel (*Villosa iris*), wavy-rayed lampmussel (*Lampsilis fasciola*), habitat suitability, mark-recapture, natural mortality, growth.

1. Introduction

Overview of freshwater mussels in North America

Freshwater mussels serve ecologically and economically important roles by removing particulates, detritus, and plankton as suspension-feeders. The benefit of suspension-feeders at high densities can be extremely significant and has been employed in the successful cleaning of previously polluted Dianchi Lake in China (World Bank project 2008). They are also recognized as indicators of water quality and health of aquatic ecosystems because of their sensitivity to contaminants (Wang et al. 2007; Clearwater et al. 2014). Their disappearance from river reaches with historic populations provides ample evidence that habitat suitability has been compromised by previous or ongoing degradation of water quality or other habitat perturbations.

Research on mussel propagation was initiated by the U.S. Bureau of Fisheries in 1914, in response to the over-harvest of mussel shells (Neves 2008). However, conservation efforts were unsuccessful until the Endangered Species Act of 1972 and subsequent recovery plans for those species recognized as federally threatened or endangered. In the recovery plans, a strategy of habitat restoration, followed by propagation and release of young mussels to natal rivers is recommended to augment existing populations and to reintroduce populations into historic habitats (Neves 2008).

Many factors impact the survival and growth of mussel populations (McMahon 1991); of these, ambient habitat quality is considered the most direct and primary factor (Strayer 2008). Unfortunately, knowledge of habitat requirements of mussels is limited, vague and untested (Stayer 2008), although the influence of detrimental factors such as exotic species, chemical pollutants, coal mining, wastewater discharge and predation has

been documented (Horne & McIntosh 1979; Neves & Odom 1989; McCann 1993; McKinney & Wade 1996; Moulton et al. 1996; Vaughn & Taylor 1999). Chemical contamination is another negative impact on mussels. Salinity inputs can almost reach sea water levels in the runoff from coal refuse piles which can greatly reduce the feeding rate of mussels (Soucek 2008). Aluminum and iron are major impurities in coal mine drainage waters (Sams III & Beer 2000) and sediments (Cherry et al. 2001). Mussels exposed to contaminated water and substrate cannot respond quickly to escape adverse conditions. They filter and accumulate contaminants in their tissue if they reside in an area with contaminated materials such as heavy metals (Bolognesi et al. 1999). The most practical test for environmental suitability is the release of juvenile mussels to seemingly desirable sites, and to monitor their growth and survival for evaluating survival and subsequent restoration strategies.

Modeling population dynamics of freshwater mussels

Population dynamics models in fisheries have been developed for more than 100 years (Ricker 1975; Quinn & Deriso 1999). Of those, population growth models are the basis for understanding how a population grows or shrinks over time. Natural mortality (M) is an important parameter used in such models to indicate the decline in population size, which significantly affects model results, biological conclusions, and management recommendations. However, M is one of the most difficult parameters to estimate (Hewitt et al. 2007). A change of 0.1 or even 0.05 (per yr) in M can significantly change stock abundance and target harvest rates (Clark 1999; Williams 2002). Hence, it is important to precisely evaluate the values of M in order to provide recommendations for management of any biological resource.

Monitoring of freshwater mussel populations is a recent phenomenon, although their life histories have been studied since the early 1900s. They have a complicated life cycle, and populations are largely influenced by environmental factors, which can change over time and greatly influence the population dynamics of these long-lived animals. To

save time and avoid errors in age estimation using shells (Anthony et al. 2008), mark-recapture models were introduced to estimate survival, recruitment and population growth of native mussels such as *Elliptio complanata*, *E. fisheriana*, and *Lampsilis cariosa* (Villella et al. 2004), *Lampsilis siliquoidea*, and *Pyganodon grandis* (Anthony et al. 2008), and *Elliptio dilatata* (Hart et al. 2001). The advantage of mark-recapture is that it allows repeated sampling of a targeted population through marked individuals to minimize modeling uncertainties.

Historical and current mussel and fish assemblages in Clinch and Powell rivers

The Clinch River is one of the most biologically diverse rivers in the United States, supporting a varied assemblage of aquatic life and the greatest number of endangered mussel species (Ahlstedt et al. 2005). The river is about 483 km long, originating in southwest Virginia near Tazewell and flows generally southwest to the state of Tennessee. An important tributary of the Clinch River is the Powell River, which arises in southwest Virginia and flows approximately 185 km through Virginia into Tennessee. Most land cover in the Clinch River watershed is forest (69 %) with some agriculture (28 %) (Diamond et al. 2002). Besides agriculture and farming, the coal mining industry and associated coal processing activities have been active many decades in the watershed. The coal-mining region is mainly located in the western portion of the Clinch watershed and very prevalent in the Powell River watershed (Zimmerman 2003).

Historically, the Clinch River contained 55 mussel species and nearly 120 species of fish (Ortmann 1918). Currently, 5 mussel species are extinct and 24 mussel species are now listed as federally endangered (Ahlstedt et al. 2005). In the Powell River, 41 mussel species have been documented historically, and 3 of those are extinct. Although more than 30 mussel species are still extant, several species are nearing extirpation, and 7 species are listed as federally endangered (Ahlstedt et al. 2005). Reduction in species diversity and abundance has been associated with dams, coal mining, sewage wastes and

agricultural run-off and urbanization (Williams et al. 1993). In addition to the above, toxic spills have had catastrophic impacts on these rivers. For example, a recent tanker-truck spill in August 1998 released chemicals into the Clinch River 0.8 km upstream of Cedar Bluff, Tazewell County, Virginia. The spill killed most aquatic invertebrates and fishes for approximately 11.3 km downstream (USFWS 2004).

Density of mussel populations varies widely in sections in the Clinch and Powell rivers. Ahlstedt et al. (2005) monitored mussel populations at 12 fixed-station sites in these rivers from 1979 to 2004 and indicated that populations in the Clinch River have declined by 70 % at sites in Virginia, while population abundance increased 2.5 times at sites in Tennessee. Population abundance at sites along the Powell River in Tennessee and Virginia decreased by 63% over the same time period. The data revealed longitudinal variability in mussel population changes at sites in both rivers. To provide information on the suitability of reaches in both rivers for mussel restoration, this study assessed site suitability using laboratory-reared mussels in Clinch and Powell rivers, and demonstrated how mark-recapture methods can improve population parameter estimations.

2. Methods and Materials

Juvenile propagation and culture

Juvenile mussels of *V. iris* and *L. fasciola* were propagated and cultured in 2007 at the Freshwater Mollusk Conservation Center (FMCC), Virginia Polytechnic Institute and State University, Blacksburg, VA. Juveniles of each species were from one propagation effort. Gravid females of *V. iris* were collected from Copper Creek, Russell County, VA and females of *L. fasciola* were collected from Indian Creek, Tazewell County, VA. Propagated juveniles were reared in indoor recirculating aquaculture systems using pond water. In winter, a mixed algal diet (Shellfish Diet 1800 and Nanno 3600 purchased from Reed Mariculture, Inc.) was used as supplemental food for juveniles, and fed at a mean concentration of 35,000 cells ml-1. Newly transformed juveniles were cultured in tanks with fine sediment (< 200 um) at a depth of 1-2 mm, and

were moved to a limestone sand substrate (2-5 mm) for grow-out when juveniles reached 3 mm in length, until reaching a suitable size for tagging (> 15 mm) and release.

Mussel Release

Horton Ford (36°34'23.0"N, 82°56'14.4"W) is located in a relatively undeveloped area, with low human density and anthropological disturbance in Hancock County, TN (Figure 1). Horton Ford at Clinch River Kilometer (CRK) 320 is a mid-reach of the Clinch River with high species richness and abundance of mussels. Payne Property (37°04'53.4"N, 81°46'43.8"W) is located in a zone (CRK 518) off the upper-reach of the Clinch River affected by the chemical tanker spill in 1998 in Tazewell County (Figure 1). Hence, this location was proposed as a restoration site by USFWS and Virginia Department Game and Inland Fisheries (VDGIF). Fugate Ford (36°35'03.8"N 83°20'11.9"W) is located in a remote area of the Powell River in Hancock County, TN (Figure 1), but is directly impacted by upstream coal mining that has deposited coal waste in the river substrate. Fugate Ford was in the mid-reach of the Powell River at Powell River Kilometer (PRK) 180, where the critical endangered Cumberland monkeyface (*Quadrula intermedia*) occurs. Hence, these 3 sites were selected as release sites in this study based on their habitat characteristics and conservation interests. All 3-release sites are in flowing-water reaches in forested landscape and vegetated riverbanks. River substrates are predominately sand, gravel, and cobble. At the Powell River site, coal-washer debris of various particle sizes is present in the river bottom. Prior to release of juveniles, mussel surveys at each site confirmed the occurrence of live mussels.

Nine-month old juveniles of *V. iris* and *L. fasciola* (ages of 5 mo and 15 mo) from FMCC were released at the 3 sites in October 2007 to determine site suitability. Area of release site was decided by similar habitat characteristics at location. A total of 1,960 untagged *V. iris* were evenly distributed within an area of 72 m2 (9 m x 8 m) at Horton Ford and an additional 100 mussels were tagged with Hallprint tags (Hallprint Manufacturers, Australia) and placed at the 1 m2 center of the release site. Location of

the release site was marked using 4 rebar stakes, one at each corner for both release areas of untagged mussels and tagged mussels. Similarly, a total of 2,806 untagged and 100 tagged mussels of *V. iris* were released in an area of 120 m2 (10 m x 12 m) and the central 1 m2, respectively, at Payne Property. The remaining Fugate Ford site received 977 untagged and 100 tagged mussels of *V. iris* within an area of 56 m2 (8 m x 7 m) and the central 1 m2, respectively. Prior to release, untagged mussels were randomly sampled to provide mean length, and all tagged mussels were measured individually to determine initial lengths (Table 1). Mussels of *L. fasciola* were released twice, October 2007 and September 2008, at ages of 5 and 15 mo, respectively. Mussels were distributed centrally in an area of 1 m2 for the first release and 2 m2 for the second release at the sites, adjacent to the area for tagged specimens of *V. iris* but without overlap. All mussels of *L. fasciola* were tagged with Hallprint tags and measured before release (Table 1).

A cage was fabricated to retain 100 mussels at each release site. The cage was built from a plastic storage container (13 cm x 38 cm x 53 cm). The lid and bottom of the container were removed and replaced by two sheets of plastic mesh screening (square mesh 1.3 cm) and fastened by zip ties to allow flow-through of water. Each cage was filled with one-third volume of substrate collected at the release site. A total of 100 juvenile mussels of *L. fasciola* were placed in each cage, and 2 cages deployed into the substrate at the 3 sites in June 2008. Each caged mussel had a Hallprint tag and was measured before release (Table 1).

Mussel sampling and heavy metal analysis

Because systematic sampling is preferred over random sampling for mussel surveys (Pooler & Smith 2005), systematic sampling was used to sample the juveniles at the release sites in the Clinch and Powell rivers (Figure 2). The mussel-sampling device is a square quadrat with an area of 0.25 m2 (0.5 m x 0.5 m). A target numbers of quadrats (n) was estimated from the area required to sample approximately 25% of total release area, and was consistent for all sampling events at each site. The population area *N* was

the total number of quadrats within the release area. The parameter *K* of the sampling interval is calculated as: $K = \frac{N}{n}$ and resulted in an approximate value of 6. The systematic sampling was initiated with a random start from the first row, then every sixth quadrat was selected as the sampling unit. Surface observation and then excavation using snorkeling or view–scoping were conducted within each quadrat to ensure maximum likelihood of capturing released mussels. Recaptured mussels were recorded and measured to calculate survival and growth rates. Untagged mussels of *V. iris* could be distinguished from resident mussels of that species through beak sculpture, periostracum color and body shape. Laboratory-reared mussels exhibited continuous growth during the indoor culture period, such that no first year growth annulus was evident on the shell. Additionally, released mussels were greater in ratio of height / length than resident juveniles of *V. iris*. Captured mussels were returned to the quadrat location or the 1m2 square area after they were counted and measured. Monitoring of released mussels was conducted in June, September, and October of 2008; May and September of 2009; June and September of 2010; and August of 2011, for a total of 8 sampling events.

Mussels of *L. fasciola* placed in cages were also sampled to calculate survival and growth in August and October of 2008, and June and September of 2010, respectively.

Metal analysis was implemented under the EPA- approved method (Atomic Absorption Spectrometry Analysis) to determine body burden concentration of aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg) and lead (Pb), selenium (Se) and zinc (Zn) (Bolognesi et al. 1999) in specimens. Mussels of *V. iris* released 3 sites were sampled during the last week of September 2010 after a residency of 3 yr for metal analysis and compared to those cultured in FMCC pond. The sample preparation followed the EPA approved method 200.3 (McDaniel 1991). The entire soft body of each mussel was removed and rinsed with metal-free water and blotted dry, and then transferred to a flask for acid digestion. Each sample contained the bodies of 2-3 mussels with a blotted dry weight of up to 5 g. A total of 4 replicates from each site was obtained. The tissues were digested with nitric acid (HNO3), hydrogen peroxide

(H2O2), and hydrochloric acid (30% HCl) with the alternatively heat and cooling process of 18 steps. Samples were transferred to a 125 ml flask and sealed for the atomic absorption spectrometry analysis. Samples were sent to the Soil Testing Laboratory, Virginia Polytechnic Institute and State University, for metal analyses using inductively coupled plasma atomic emission spectrometer (ICP-AES) (Spectro Analytical Instruments, Inc.). All reagents used in these tests were ultra-high grade to eliminate elemental impurities, and standard laboratory protocols were followed. The ASTM type I water was used for sample preparation and dilutions.

Statistics and Models

Mussel growth at each sampling event was analyzed using ANOVA and HSD (Tukey's Honest Significant Difference) through software R (Version 3.1.0) to determine differences in growth of juveniles among the 3 release sites, at a significant level of $\alpha = 0.05$. Mussel lengths of untagged specimens of *V. iris* at each sampling event were used to compare growth differences, since mussels of equal lengths were randomly assigned to the 3 release sites. Incremental lengths (length at sampling event minus initial length at release) for tagged mussels were compared to determine differences in growth among sites.

Mortality and catch probability of released mussels were estimated by monitoring the released aggregation over time using an exponential model widely used to assess mortality and survive of aquatic invertebrate species (Ricker 1975, Quinn & Deriso 1999). An individual model was developed for the tagged individuals. All models were constructed with the following assumptions:

- Labeled laboratory-reared mussels retained their tags throughout the study.
- Variations in mortality (or survival) rates followed a certain random distribution.
- Sampling protocol and study area were constant.
- No migration out of the study area.

The formulation for Model 1: $N_{t+\Delta t} = N_t \cdot e^{-(M)\Delta t}$

where N_t is the population size (number of mussels) at time t, M is instantaneous natural mortality rate.

$I_t = q \cdot N_t$

where I is catch per unit effort, or number of recaptured mussels from each sampling at time t, and q is catch probability.

A log-normal distribution for parameters was adapted in all 3 models, and Bayesian methodology was applied in model structures.

$$E[log(N_{i,j})] = log\ (N_{i,1}) - M_i(\Delta t)$$

$$I_{i,j} \sim LN(log\ \bar{I}_{i,j}\ , 1/\sigma^2)$$

$$E[log\bar{I}_{i,j}] = log(q_i) + log\ (N_{i,j}) \qquad (1)$$

$$M_i \sim U\ (0,1)$$

$$q_i \sim U\ (0,1)$$

$$\sigma \sim U(0,10)$$

where j represents the j^{th} capture event (or j^{th} sampling measurement, j = 0, 1, 2…8, j = 0 denotes time of mussel release without capture); i represents ith type and site of release; $E[log(N_{i,j})]$ denotes expected mussel number at capture event of j^{th}; M_i is mussel mortality of ith release site; Δt represents sample interval from release time; $I_{i,j}$ is CPUE at ith release method and jth recapture event, which assumed a log-normal distribution with mean of $\bar{I}_{i,j}$ and σ^2; then M_i, q_i and σ are assumed to follow uniform distribution with described upper and lower boundary in equation (1).

Model 1 was constructed to estimate parameters of released juveniles of *V. iris*, as the combination of release types and sites incorporating untagged and tagged mussels. Model 2 was built for untagged mussels of *V. iris*, while Model 3 was only for tagged mussels of *V. iris*. Model 5 was built to estimate parameters for tagged juveniles of *L. fasciola* released at 3 sites, while Model 6 was for tagged mussels placed in cages at 3 release sites in two rivers.

An individual model was applied to estimate the parameters incorporating individual variation of those tagged mussels of *V. iris*. Formulation for Model 4 was as follows:

$$
\begin{aligned}
&Pl_{i,k,j} = S_{i,j}^{\Delta t} L_{i,k-1,j} \\
&L_{i,k,j} \sim Bern(Pl_{i,k,j}) \\
&Cl_{i,k,j} = Pc_{l\,i,j} L_{i,k,j} \\
&Ol_{i,k,j} \sim Bern(Cl_{i,k,j}) \\
&S_{i,j} \sim N(\overline{S_j}, \sigma_{S_j}^2) I(0,1) \\
&\overline{S_j} \sim U(0,1) \\
&\sigma_{S_j} \sim U(0,1) \\
&Pc_{l\,i,j} \sim N\left(\overline{Pc}_{l\,j}, \sigma_{Pc_{lj}}^2\right) I(0,1) \\
&\overline{Pc}_{l\,j} \sim U(0,1) \\
&\sigma_{Pc_{i,j}} \sim U(0,1)
\end{aligned}
\qquad (2)
$$

where *i* represents the i^{th}individual; *k* represents the k^{th} capture event (*k* = 0, 1, 2…9, *k* = 0 denotes time of mussel release and 0th capture); *j* represents release sites (*j* = 1 denotes Horton Ford, *j* = 2 denotes Payne Property, and *j* = 3 denotes Fugate Ford) ; Δt represents time between adjacent capture events. $L_{i,k,j}$ represents live status of a individual mussel at the k^{th} capture event of site *j*. S_j is survival rate of *V. iris* among 3 sites. $Pl_{i,k,j}$ denotes probability of a live individual at capture event k^{th} that depends on survival status at $(k-1)^{th}$ recapture event and survival rate S_j. $Cl_{i,k,j}$ denotes probability of recapturing a live mussel based on recapture rate Pc_{lj} and its survival status at k^{th} capture event. $Ol_{i,k,j}$ is observation of live individual that follows Bernoulli distribution; $S_{i,j}$ and $Pc_{l\,i,j}$ were assumed to follow normal distributions described as $N\left(\overline{S_j}, \sigma_{S_j}^2\right)$ and $N\left(\overline{Pc}_{l\,j}, \sigma_{Pc_{lj}}^2\right)$, respectively. The $\overline{S_j}$ and $\overline{Pc}_{l\,j}$ are mean values of $S_{i,j}$ and $Pc_{l\,i,j}$ among individuals and were assumed to follow uniform distributions as well as standard deviations of σ_{S_j} and $\sigma_{Pc_{i,j}}$. *I* denotes boundary of distribution in WinBUGS.

Multi-level priors incorporating individual variations were tested to estimate survival and catch probability and their associated uncertainty through a Bayesian approach. WinBUGS package (version 14) associated with R software (Version 3.1.0) was used to process Bayesian analysis of Markov Chain Monte Carlo (MCMC) methods (Spiegelhalter et al. 1996). Three Markov chains were applied to determine convergence of the posterior distribution (Spiegelhalter et al. 2004; Jiao et al. 2008, 2009). A burn-in iteration of 50,000 and thinning interval (300) were determined based on convergence criteria (Jiao et al. 2008). The Bayesian inference was generated through random draws from the posterior distribution after convergence of the three chains.

3. Results

During the 4-yr study period, growth rates of released specimens of *V. iris* among the 3 sites were significantly different ($p < 0.05$) at each sampling event. Untagged mussels of *V. iris* released at Horton Ford exhibited significantly greater growth (Table 2) than those released at Fugate Ford ($p < 0.001$), and significantly faster growth than those released at Payne Property at the first two and last two sampling events ($p < 0.05$); other sampling events were not significantly different ($p > 0.05$). Mussels of *V. iris* released at Fugate Ford had significantly slower growth than those released at the other two sites ($p < 0.05$). All released mussels of *V. iris* exhibited continuous growth at the 3 sites (Figure 3). However, growth rates slowed when mussels were 41 mo old (32 mo after release) at the Payne Property, although these mussels out-performed those at Fugate Ford.

Similar results were observed in juveniles of *L. fasciola*; mussels at Horton Ford had significantly greater growth than those released at Payne Property and Fugate Ford for the first-released cohort (($p < 0.05$, Figure 4) as well as the second-released cohort ($p < 0.05$, Table 3). Incremental growth in length of the second-released mussels at Payne Property grew significantly faster ($p < 0.05$) than those released at Fugate Ford before the fourth sampling event (age < 37 mo), but not significantly different after that period ($p >$

0.05, Table 3). Mussels at Payne Property exhibited slower growth (Figure 5) after 37 mo of age (22 mo after release).

One cage at each release site was lost during the study period. Therefore, data were analyzed based on the 100 tagged *L. fasciola* retained in one cage at each release site. Mussels in the cage at Horton Ford grew significantly faster than those in cages at Payne Property and Fugate Ford ($p < 0.05$, Table 4). Incremental lengths were not significantly different ($p > 0.05$) in mussels between Payne Property and Fugate Ford at the first sampling event (2.5 mo in cages), but were significantly different thereafter ($p < 0.05$). Mussels in the cage at Payne Property exhibited greater growth than those at Fugate Ford ($p < 0.05$) at the second sampling event (4 mo after release), but not thereafter (Figure 6).

Dispersal of free-released mussels was observed in untagged and tagged mussels. Untagged mussels tended to move downstream, and tagged mussels originally released within the central area of 1 m^2 or 2 m^2 were captured in quadrats outside of the larger grid boundary (Table 5). Vertical movements of mussels also were recorded; they were recaptured at the surface to a depth of 30-35 cm within the substrate. Mussels moved to the surface when water temperatures exceeded 15 ℃ and positioned themselves sub-surface when water temperatures were below 10 -12 ℃. During the study period, morphological differences in mussels were apparent; mussels at Horton Ford and Payne Property appeared healthy and with thicker shells, while those released at Fugate Ford were thin and fragile with shell discoloration.

A total of 10 metals were tested in the bodies of *V. iris*. Only the 4 elements of Al, Cu, Fe and Zn were detectable. The concentrations of As, Cd, Cr, Hg, Pb and Se were less than the instrument detection limits of 0.017 ug L^{-1}, 0.004 ug L^{-1}, 0.007 ug L^{-1}, 0.011 ug L^{-1}, 0.016 ug L^{-1}, and 0.022 ug L^{-1}, respectively, and excluded from data analysis. Concentrations of Al, Cu, Fe and Zn were highly variable in mussels collected from the 3 sites in Clinch and Powell rivers and from the FMCC pond (Figure 7). The ANOVA test showed that concentrations of Al, Cu and Zn were significantly different in mussels ($p <$

0.05), while the concentration of Fe was not significantly different in specimens collected ($p > 0.05$) (Table 6). The Al concentration was significantly (3-10 times) higher in specimens of *V. iris* collected from Payne Property than those from other sites, while no significant difference was found in the other samples. The concentration of Cu was significantly lower in mussels from FMCC pond compared to those from Payne Property and Fugate Ford. Conversely, Zn level was significantly higher in mussels from FMCC pond than those at Horton Ford.

Estimated mortality rates (M) and catchability (q) of released mussels of *V. iris* varied among 4 models (Table 7). In Models 1, 2, and 3, M of mussels released at Fugate Ford was higher than those at Horton Ford and Payne Property, while Model 4 resulted in an inverse output. However, q of mussels released at Fugate Ford was higher than q of those released at Horton Ford and Payne Property in all 4 models. Among all 4 models, M of untagged specimens of *V. iris* estimated from Model 2 had the lowest values, followed by Model 1, Model 3 and Model 4; while q from Model 2 was higher than that for Model 1 and Model 3. The posterior density distributions of those estimated parameters from all 4 models are summarized in Appendices B-1 to B-4. The posterior density distribution curves of M and q from Model 4 exhibited a normal shape (Appendix B-4), while those of Model 3 showed a relatively skewed curve (Appendix B-3).

Estimated mortality rates of released mussels of *L. fasciola* between the 2 models differed greatly (Table 8). The M of mussels released in the larger area (Model 5) ranged from 0.5037 to 0.8136 (yr^{-1}), while those placed in cages at the 3 sites ranged from 0.0690 to 0.1610 (yr^{-1}) (Model 6). Mussels of *L. fasciola* released at Fugate Ford had the lowest mean mortality rate, followed by those at Payne Property and Horton Ford in both models. The posterior density distribution curves of M from Model 5 were skewed (Appendix B-5), while those from Model 6 exhibited a normal shape (Appendix B-6).

4. Discussion

In this 4-yr study, environmental suitability for the species *V. iris* and *L. fasciola*

was determined through the assessment of survival and growth. Mussels had overall significantly faster growth at Horton Ford, indicating that conditions at this location appear more suitable for the released mussels. Results of growth from untagged individuals of *V. iris,* distributed within the larger-scale area, those tagged and placed in the central area, those free-released, and those caged specimens of *L. fasciola* support this conclusion. Metal analysis showed that concentrations of Al, Cu, Fe and Zn were less in bodies of *V. iris* from Horton Ford when compared to those from Payne Property and Fugate Ford, although only the body burden of Al was significantly different in bodies of mussel among release sites, indicating elemental differences in water or substrate among the release sites. Mussels are sensitive to environmental change (Smith & Jepsen 2008), and their growth can decrease or stop if environmental conditions are unfavorable (Sansom et al. 2013). At the first sampling event, mussels of *V. iris* and *L. fasciola* released at Horton Ford had immediate and consistent incremental growth, while those released at Payne Property and Fugate Ford exhibited growth cessation or slow growth.

Between Payne Property and Fugate Ford, mussels of *V. iris* and *L. fasciola* did better at Payne Property, with significantly faster growth than at Fugate Ford, although some variation occurred. Growth of young *L. fasciola* freely released at the sites were not significantly different between these sites after 37 mo, and mussels placed in cages at Fugate Ford had relatively greater incremental growth than those caged mussels at Payne Property after 39 mo. Apparently, conditions at Payne Property were more suitable for growth for both species, with greater growth for a certain time (4 to 22 mo), when compared to Fugate Ford, but this site did not consistently support mussel growth. Bolognesi et al. (1999) indicated that mussels filter and accumulate contaminants in their tissues if they reside in an area with contaminated materials. The results from metal analyses indicated that Al concentration in *V. iris* from Payne Property was significantly higher (3 times) than those samples collected from other sites, including Fugate Ford, after mussels resided at sites for 3 yr. The body burdens of Cu, Fe and Zn in *V. iris* from Payne Property were higher than those from Fugate Ford, although difference levels were

not significant. Laboratory toxicity tests have confirmed that Cu and Zn are toxic to juvenile mussels. Chronic toxicity levels of Cu range from 8.5 to 9.8 ug $L^{-1}$and may affect survival of juveniles, while a range of 4.6 to 8.5 ug L^{-1} can affect growth (Wang et al. 2007). In this study, body burden of Cu were not significant different in mussels of *V. iris* released at 3 sites, probably due to the dilution or concentration mechanism of mussels (Absil et al 1996). Hummel et al (1997) also indicated that the Cu concentration was almost constant in bodies of some bivalves, while was negatively related to condition. Agreed with previous results, mussels of *V. iris* at Horton Ford exhibited fastest growth with lowest body burden of Cu. Cherry et al. (2001) reported that sediment in creeks in mined land areas with acid mine drainage had high concentrations of Fe (~10,000 mg kg^{-1}) and aluminum (~1,500 mg kg^{-1}), with very few organisms or no benthic macroinvertebrates. Mussels cultured in FMCC (control group) exhibited lowest body burden of Al and Cu, however highest concentration of Zn. Probability the values of Zn concentration (136.5 ug $L^{-1}g^{-1}$ to 190.4 ug $L^{-1}g^{-1}$) in mussels of *V. iris* reared in FMCC has not reached critical level to mussel growth. In contrast, EC_{50} values of Zn to glochidia were from 229 to 337 ug L^{-1} in an acute toxic test (Clearwater et al. 2014).

Habitat characteristics are significant factors that influence mussel population size (Strayer 2008). Quantitative surveys showed that the decline in mussel density and diversity in the Powell River has been linked to coal mining and abandoned mine lands (Wolcott & Neves 1994; Ahlstedt et al. 2005; Johnson 2011). Black water from the release of coal fines periodically occurred in Powell River in the 1980s (Ahlstedt 1986), and mussel mortality was observed between PRK 104.8 and 230.9 in 1983 (Jenkinson & Ahlstedt 1988). The release site of Fugate Ford was located within the river section where this die-off occurred, and residual coal fines were readily visible in the substrate during study duration. Laboratory testing of coal wastes in sediments showed that mussels reduced filtration times and increased movements when coal fines were present, and occurrence of mussels was inversely correlated with coal fines in the substrate of Powell River (Kitchel et al. 1981). The negative effects of coal-related waste on juvenile

mussels in the Powell River also have been documented (McCann & Neves 1992). In my study, mussels released at Fugate Ford exhibited a stressed appearance, with thin shells and brown color on the periostracum (Figure 8), different in appearance from mussels at Horton Ford, Payne Property and FMCC pond. The environmental conditions at Fugate Ford for recovery of mussel species therefore are unsuitable as judged by the results of this study.

Payne Property is near Cedar Bluff, Virginia, where active underground coal mining occurs in the headwaters of the Clinch River (Hampson et al. 2000). Aluminum is one of the main elements commonly present in mine waste water and sediments in rivers (Sams III & Beer 2000; Cherry et al. 2001). Kádár et at. (2002) indicated that aluminum ions can be released by acid mine drainage, and the freed aluminum can bind to the gills of mussels such that respiration is obstructed. Acid mine drainage and urban runoff were determined to limited mussel populations in the Clinch River (Zimmerman 2003). In addition, the chemical spill near the Payne Property on August 27, 1998, directly eradicated nearly all mussels and one of the last remaining populations of the endangered tan riffleshell (*Epioblasma florentina walkeri*) (USFWS 2004). After 10 yr of habitat regeneration and animal recolonization, some individuals of mussels including *V. iris*, *Pleurobema oviforme* and *Ptychobranchus fasciolaris* were found during periodic sampling. Payne Property was selected as a mussel restoration site by Virginia Department of Game and Inland Fisheries (VDGIF) and USFWS because it seemingly is capable of supporting recolonization of the mussel species in this study. Although positive evidence of environmental suitability was observed at Payne Property, a longer-term relocation and monitoring program is certainly needed. The delayed decline in growth of released mussels may affect a long-term program of mussel restoration.

Population analyses are crucial to freshwater mussel conservation, and determination of population growth rates is one of the key components (Matter 2013). A proper model is an abstract of the population of interest because no model can perfectly recreate the object being modeled (Haddon 2001). In this study, the exponential growth

model was applied for untagged and tagged mussels, because the cohort introduced at each site was the initial population of low density. However, results from untagged and tagged mussels differed; estimated *M* of *V. iris* was higher in tagged mussels than untagged mussels. The *M* in Model 2 incorporating untagged mussels was 0.5076, 0.4520 and 0.7523 for the populations of *V. iris* at Horton Ford, Payne Property and Fugate Ford, respectively, which were about 0.2 less than the values from Model 3, incorporating only tagged mussels (except for at Fugate Ford, 0.07 less). Theoretically, the mortality of untagged and tagged mussels of *V. iris* at the same release site should be same, because the small Hallprint tags should have no influence on mortality. I then designed Model 1 combining both untagged and tagged mussels to test the variation in parameter estimation. Results showed that *M* values of Model 1 at all 3 sites were larger than those from Model 2, but less than those from Model 3. Similar results were found for the parameter *q* among the three models. The estimated parameters of *M* and *q* seemingly confounded and regulated the process of mathematic modeling results. In other words, small *q* probably over-estimated *M*. Hence, I think Model 2 is a more proper model that outperformed the other 2 models in estimation of *M* and *q*. I also developed an individual model (Model 4) based on available information from tagged mussels. With the tag, each individual could be tracked at each recapture event, by response of "recapture" or "no recapture" Some individual mussels experienced "recapture" after several occasions of "no recapture" resulting in larger *q* values than those estimated from Model 3. The *M* values from Model 4 were very close to those from Model 3, except for mussels at Fugate Ford.

Overall, estimated *M* values were highest at Fugate Ford, followed by Horton Ford and Payne Property from Model 1 to Model 3, while Model 4 exhibited different results with lowest *M* at Fugate Ford, which might be due to algorithmic errors caused by classification algorithm and the data-gathering process (Fielding et al. 1997). In this study, the emigration of untagged and tagged mussels was observed. Untagged specimens of *V. iris* were recaptured downstream of the release site, and tagged mussels were

recaptured from quadrat sampling outside of the original release area of 1 m^2. To prevent the sampling errors caused by emigration of untagged mussels, I modified sampling method to shift 3 m downstream beyond the original area of the release sites, to meet the requirement of model assumption. However, the emigration of those tagged mussels were not prevented and might result in greater errors in parameter estimation.

Caging of mussels was used to verify mortality estimation. Mussel capture probability is 1 since all caged individuals were recaptured at each sampling event. However, mortality due to predation in the cage was excluded. During the 4 yr monitoring program, mussel predation was observed only once, at Horton Ford, when a pile of mussel shells including native mussel shells and laboratory-reared specimens of *L. fasciola* was found. Therefore, the demographic analyses from caged mussels are still capable of use as a reference to those free-released mussels. The estimated *M* values of caged mussels of *L. fasciola* at Horton Ford, Payne Property and Fugate Ford were 0.1610, 0.1516 and 0.0690 (yr^{-1}), respectively, while values were 0.7231, 0.6805 and 0.6514 (yr^{-1}) for the free-released mussels, respectively. The high *M* values for free-released mussels was likely due to the low capture probability caused by emigration. The capture records of tagged mussels of *L. fasciola* within quadrats (outside of original release area) demonstrated the downstream movement of released mussels.

A Bayesian approach associated with Markov Chain Monte Carlo (MCMC) algorithms was applied to the mathematical process to reduce model uncertainty (Wade 2000; Gimenez 2008). The posterior distribution function of estimated *M* for untagged mussels exhibited a reasonably symmetrical curve; however, *M* for tagged mussels was evidently skewed perhaps due to small sample size. The individual model (Model 4) incorporating individual variation resolved this problem, resulting in a normal-shaped curve for both *M* and *q,* with a small standard deviation. The results from caged mussels indicated that *M* was similar for mussels at Horton Ford and Payne Property, but lower in mussels placed at Fugate Ford. This result implies that habitat conditions at Fugate Ford were problematic to mussel survival. The quadrat survey data revealed that there were

some native species at this site, including *Actinonaias pectorosa*, *Actinonaias ligamentina*, *Medionidus conradicus*, *Ptychobranchus fasciolare*, *Amblema plicata*, *Elliptio dilatata*, and the two endangered *Quadrula intermedia* and *Dromus dromas*. However, these resident mussels were old adults; juveniles were not collected at this location. During previous surveys, little to no recruitment was reported in mussel populations of the Powell River (Wolcott & Neves 1994). In contrast, Horton Ford readily supported released mussels as well as resident species of mussels. A total of 13 common species resided at this site: *A. plicata*, *A. pectorosa*, *A. ligamentina*, *Cyclonaias tuberculata*, *E. dilatata*, *Hemistena lata*, *Lasmigona costata*, *L.fasciola*, *L. ovate*, *M. conradicus*, *P. fasciolare*, *P. subtentum*, *Villosa vanuxemensis,* and the endangered *Fusconaia cor*, *F. cuneolus*, *Epioblasma brevidens*, *E. capsaeformis* and *Plethobasus cyphyus*. Moderate densities were found during the quadrat surveys at this release site. Additionally, juvenile mussels of multiple species were also detected, indicating population recruitment. Thus, Horton Ford can be classified as a suitable site to support source populations of freshwater mussels as well as a suitable site to restore other populations through laboratory-produced juvenile mussels.

5. Conclusions and Recommendations

Periodic monitoring efforts greatly facilitated the evaluation of site suitability for mussels. Although only two common mussel species were tested in this study, results likely can be applied to co-existing species that prefer conditions similar to that of *V. iris* and *L. fasciola*. It is critical to test environmental suitability using common species before any attempt is made to restore endangered species of mussels. This study demonstrated a protocol of evaluating site suitability for mussel restoration through a mark-recapture study. Use of cages was the most effective method to determine site suitability because the free-release of mussels (untagged, tagged) had low catchability. Released mussels exhibited slower growth and higher mortality at Fugate Ford in the Powell River. Therefore, this site is deemed unsuitable for the restoration of endangered

species based on the results of this study. The mark-recapture method needs to be refined to increase catchability, and a longer-term monitoring project is needed to investigate population recruitment for risk assessment, and to evaluate the success of restoration efforts using laboratory- produced mussels.

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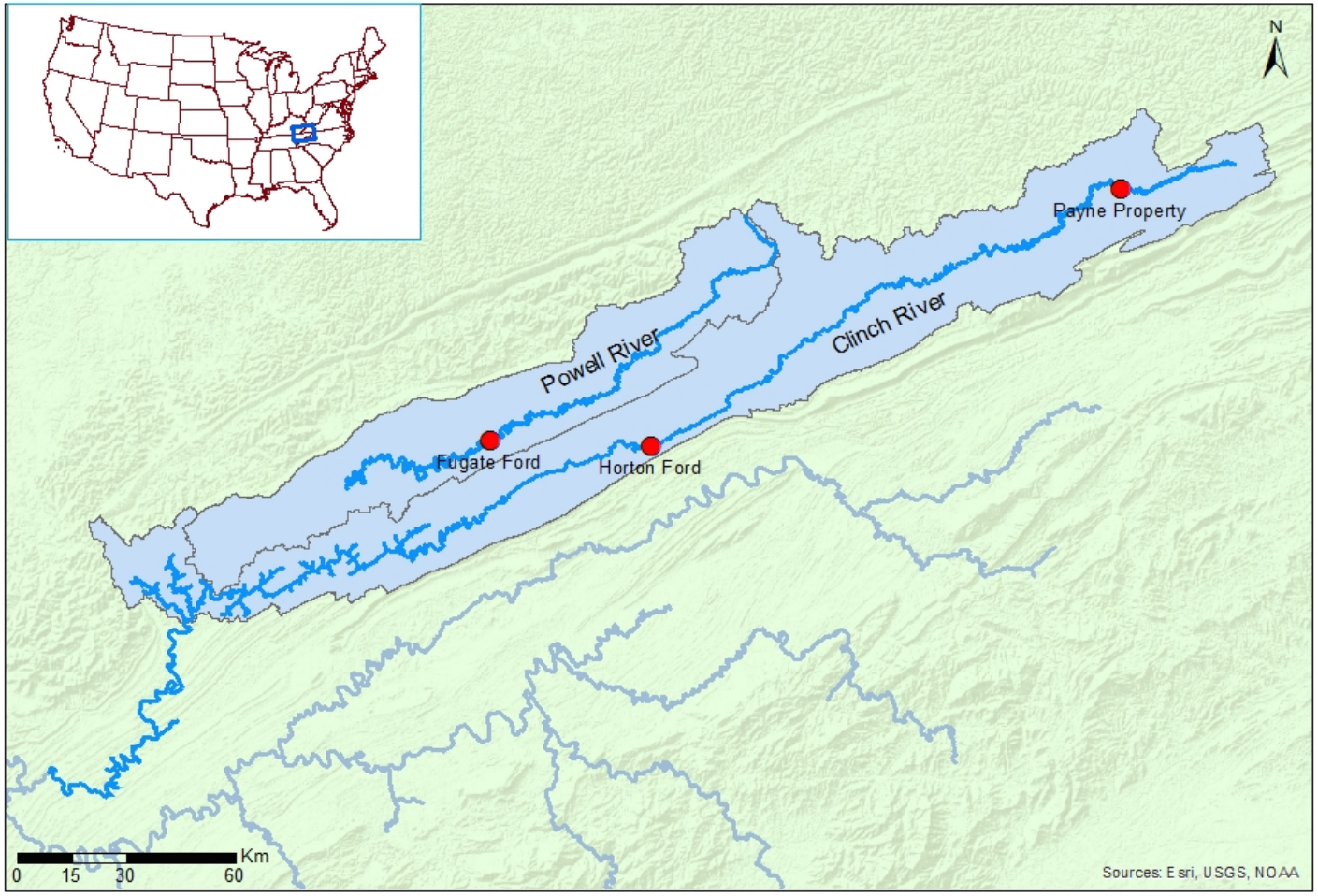


Figure 1. Release of young mussels of *V. iris* and *L. fasciola* at Horton Ford and Payne Property, Clinch River, and Fugate Ford, Powell River, Virginia and Tennessee.

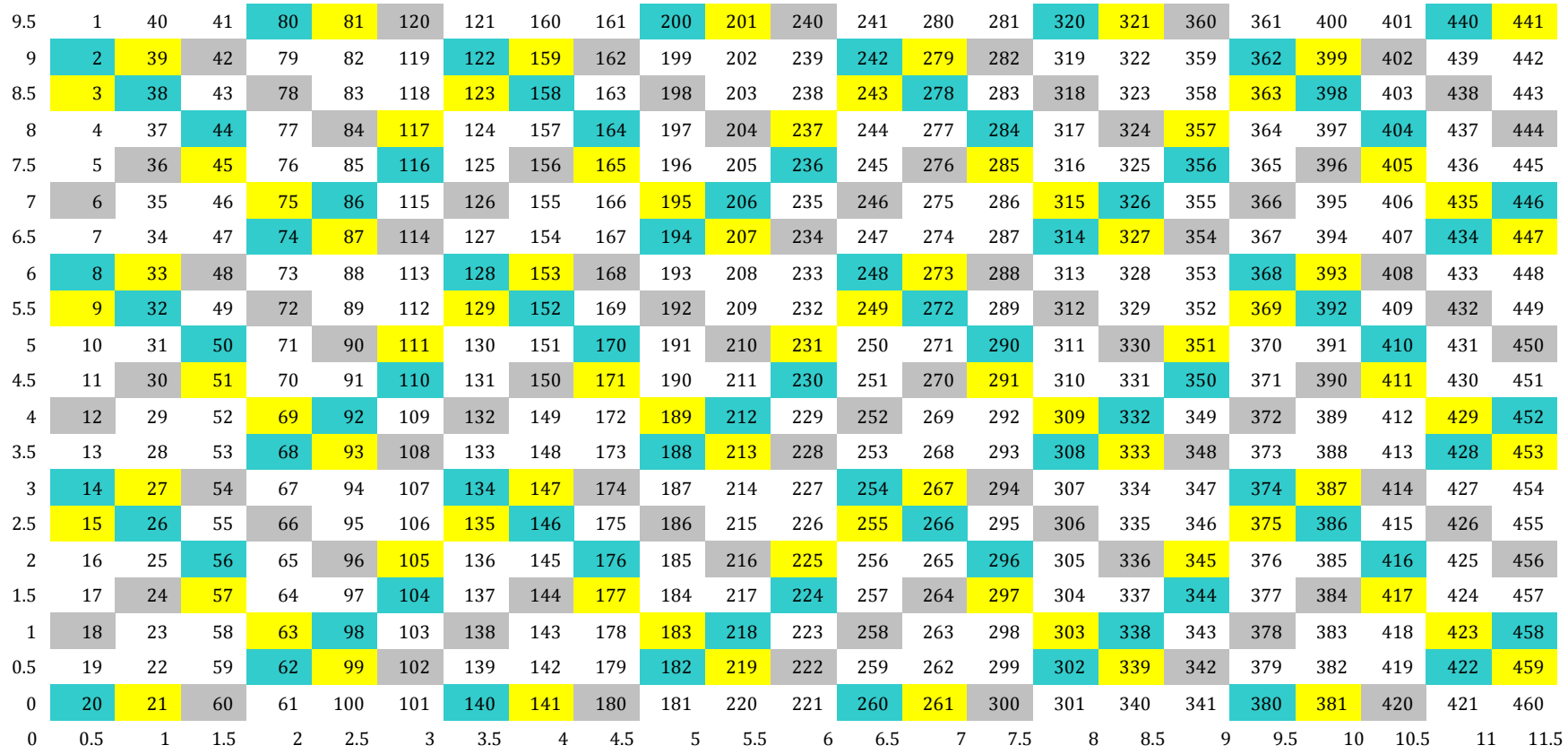


Figure 2. Diagram of systematic survey method. Sampling unit (quadrat) was placed once every 6 quadrats with an initial random start at each sampling event. Each color represents a sampling unit

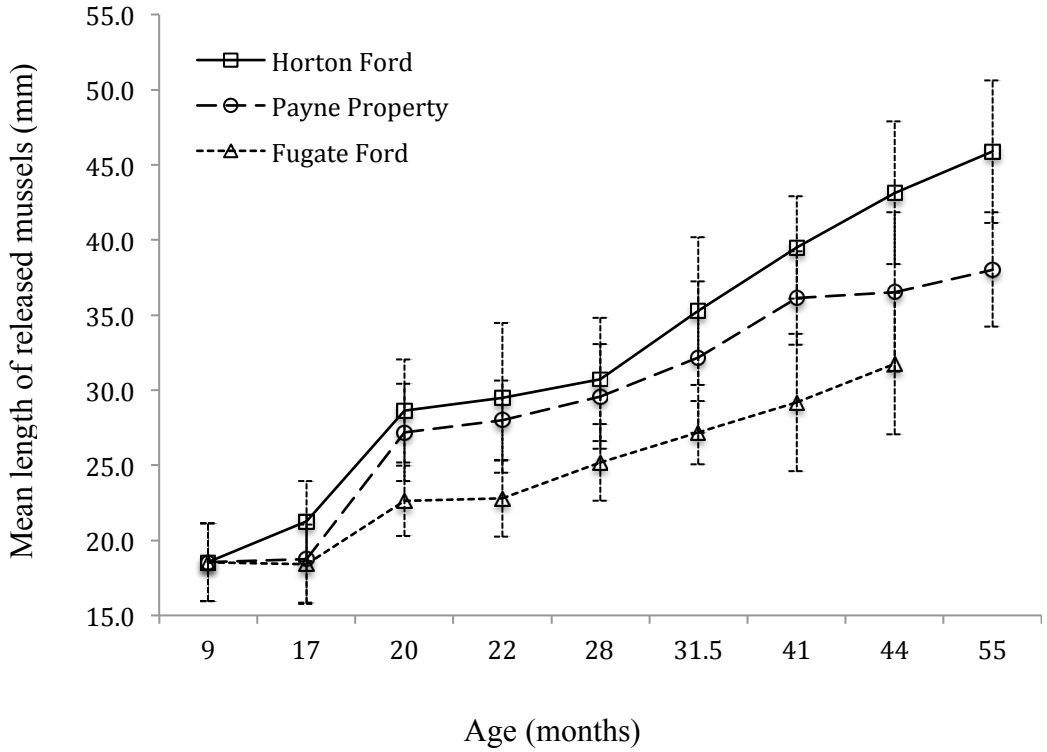


Figure 3. Growth (length ± SD mm) of released young mussels of *V. iris* at Horton Ford and Payne Property, Clinch River, and Fugate Ford, Powell River from October 1, 2007 to August 17, 2011.

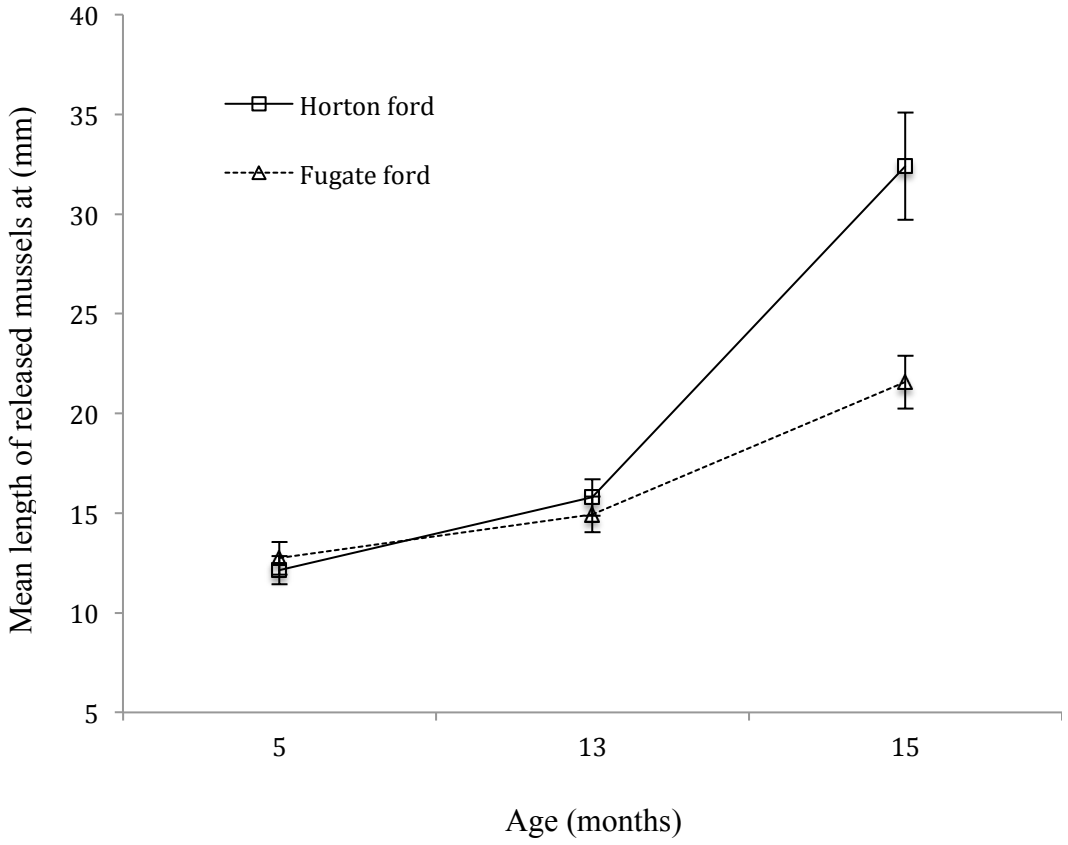


Figure 4. Growth (length ± SD mm) of released young mussels of *L. fasciola* at Horton Ford, Clinch River and Fugate Ford, Powell River, from October 1, 2007 to September 4, 2008.

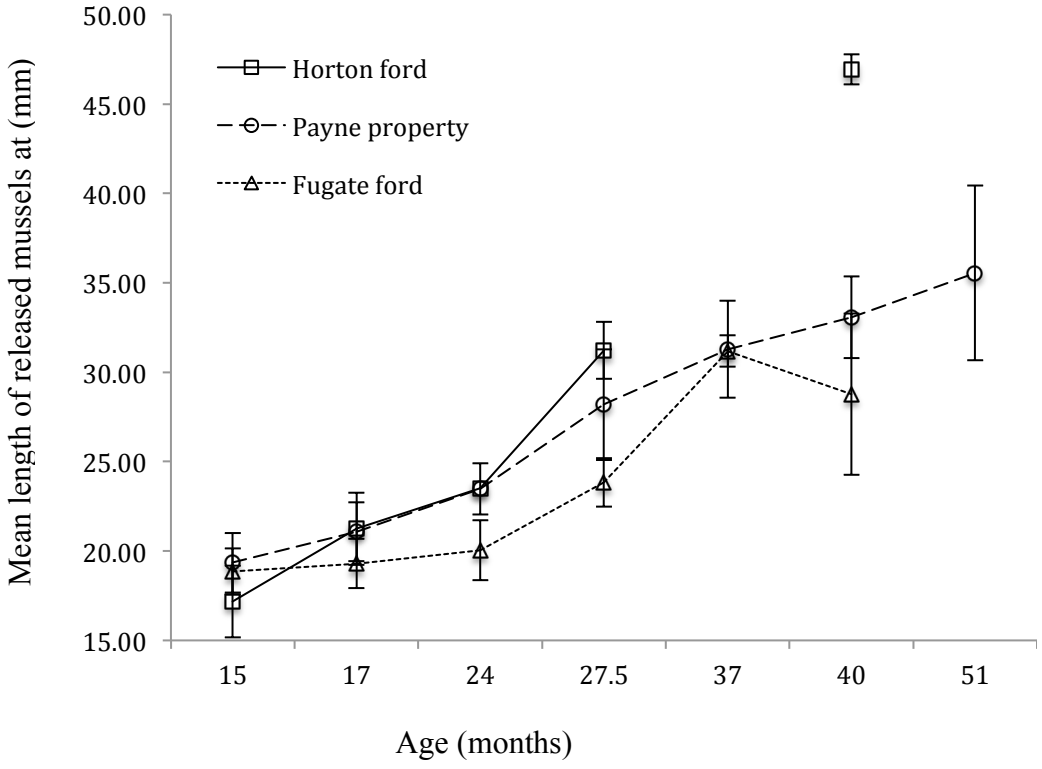


Figure 5. Growth (length ± SD mm) of released young mussels of *L. fasciola* at Horton Ford and Payne Property, Clinch River, and Fugate Ford, Powell River, from September 4, 2008 to August 17, 2011.

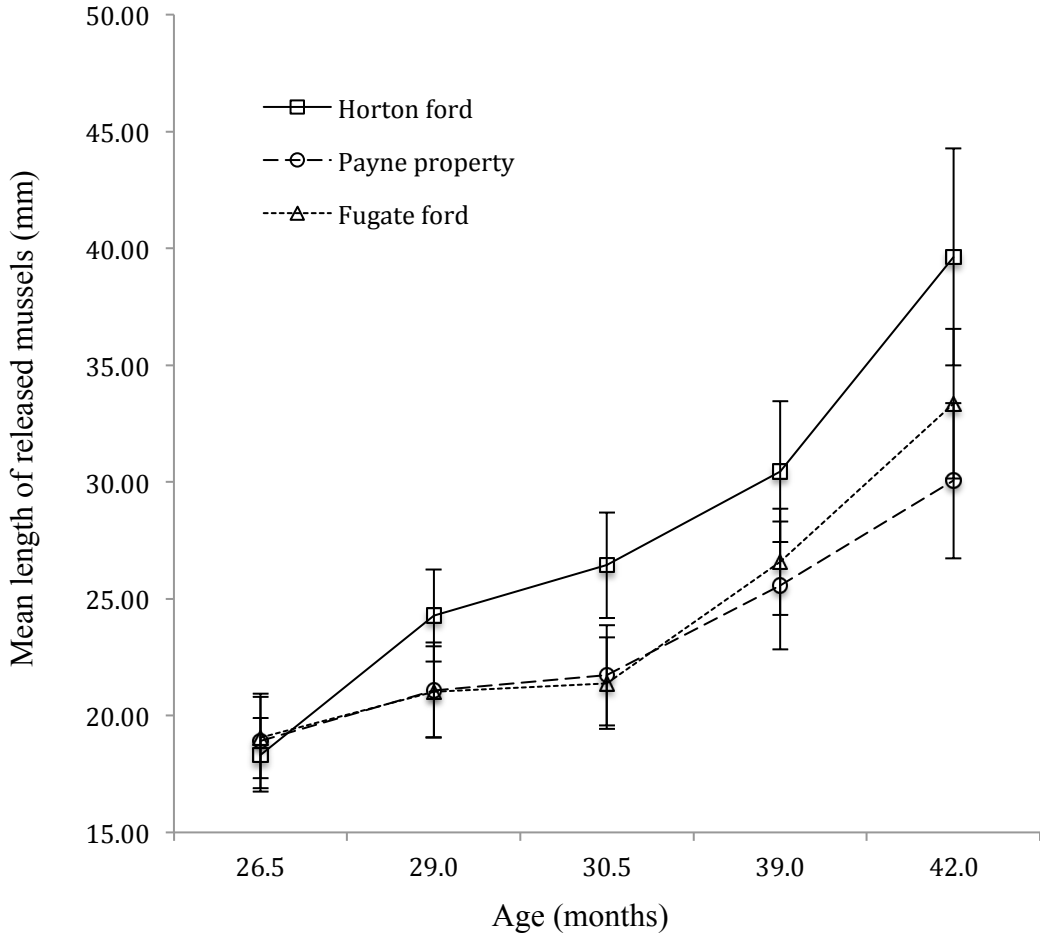


Figure 6. Growth (length ± SD mm) of released young mussels of *L. fasciola* in cages at Horton Ford and Payne Property, Clinch River, and Fugate Ford, Powell River, from June 9, 2009 to September 18, 2010.

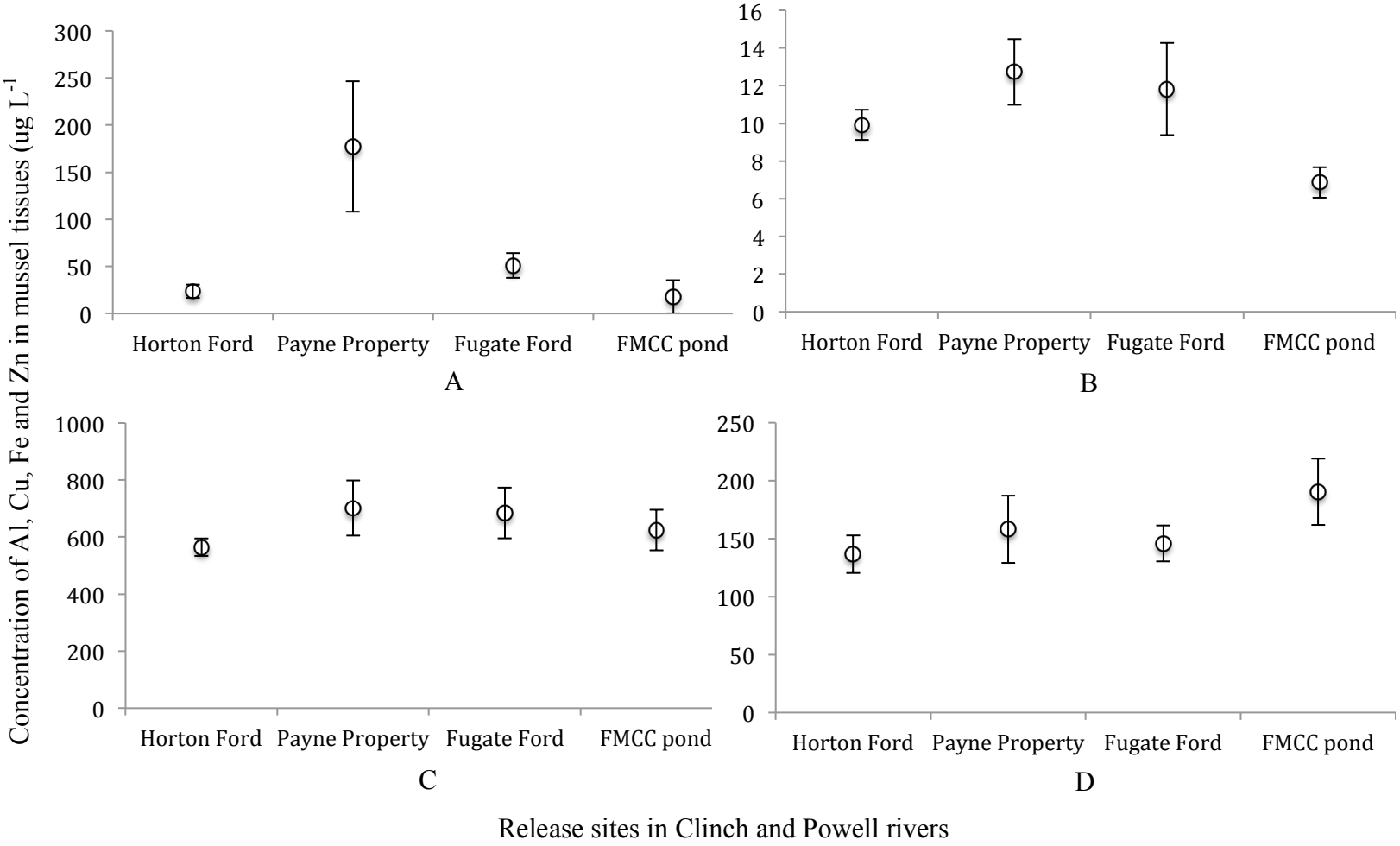


Figure 7. Comparison of concentration ($\pm$ *SD*) of metals in mussels of *V. iris* released at Horton Ford and Payne Property, Clinch River, Fugate Ford, Powell River and reared in FMCC pond (A. Aluminum, B. Copper, C. Iron, D. Zinc

A

B

C

D

Figure 8. Recaptured mussels from release sites in Clinch and Powell rivers and mussels from FMCC pond (A. Horton Ford, B. Fugate Ford, C. Payne Property, and D. FMCC pond. Mussels released at Fugate Ford exhibited a stressed appearance, with thin shells and brown color on the periostracum, different in appearance from mussels at Horton Ford, Payne Property and FMCC pond.).

Table 1. Release of laboratory-reared mussels of *Villosa iris* and *Lampsilis fasciola*.

Species	Released mussels	Tag		Size at release Mean length ±SD (mm)	Age at release (month)	Area of release site (m^2)	Release site
		No	Color				
V. iris	1960	-	-	18.5±2.6	9	72	Horton Ford,
	100	A846-A962	Red	23.1±1.6	9	1	Clinch River,
L. fasciola	100	Bee tag 00-99	Yellow	12.1±0.7	5	1	Hancock, TN
	299	X890-X999, Y00-Y099	Red	17.2±2.0	15	2	
	200	S201-S400	Red	18.3±1.6	26.5	cage	
V. iris	2806	-	-	18.5±2.6	9	120	Payne Property,
	100	A592-A691	Red	20.9±1.6	9	1	Clinch River,
L. fasciola	115	A946-A960, Bee tag 00-99	Red	14.0±1.3	5	1	Tazewell, VA
	302	X299-X600	Red	19.4±1.7	15	2	
	200	S000-S200	Red	18.9±2.0	26.5	cage	
V. iris	977	-	-	18.5±2.6	9	56	Fugate Ford,
	100	A692-A791	Red	22.2±1.8	9	1	Powell River,
L. fasciola	100	Bee tag 00-99	Pink	12.7±0.8	5	1	Hancock, TN
	11	Bee tag 01-11	Green	18.9±1.3	15	2	
	290	X601-X889, Y117	Red	18.9±1.3	15	2	
	200	S401-S600	Red	19.1±1.7	26.5	cage	

Table 2. ANOVA results of growth in length (p value and adjusted p-value) of young mussels of *V. iris* at 3 release sites in the Clinch and Powell rivers, from October 1, 2007 to August 17, 2011.

Date	Age	Mean length ±SD (mm)			p value	Adjusted p-value		
		Horton	Payne	Fugate Ford				
	(months)	Ford (HF)	Property (PP)	(FF)		PP-FF	HF-PP	HF-FF
10/1/07	9	18.5±2.6	18.5±2.6	18.5±2.6	-	-	-	-
6/3/08	17	21.3±2.7	18.8±2.9	18.4±2.6	2.94E-08	0.8243	0.0001	0.0000
9/4/08	20	28.6±3.4	27.2±3.2	22.6±2.3	2.00E-16	0.0000	0.0463	0.0000
10/28/08	21	29.5±5.0	28.0±2.6	22.8±2.5	3.01E-13	0.0000	0.1055	0.0000
5/26/09	28	30.7±4.1	29.6±3.5	25.2±2.6	2.63E- 4	0.0024	0.5357	0.0002
9/10/09	31.5	35.3±4.9	32.2±5.0	27.2±2.1	8.12E- 4	0.0118	0.1483	0.0006
6/24/10	41	39.5±3.4	36.1±3.1	29.2±4.6	2.62E-06	0.0001	0.1108	0.0000
9/19/10	44	43.1±4.8	36.5±5.3	31.8±4.7	1.48E-07	0.0121	0.0002	0.0000
8/17/11	55	45.9±4.8	38.0±3.8	-	1.41E-06	-	0.0000	-

Table 3. ANOVA results for increase in incremental lengths (p value and adjusted p-value) of young mussels of *L. fasciola* at 3 release sites in the Clinch and Powell rivers, from October 1, 2007 to August 17, 2011.

Date	Release	Age	Mean incremental length±SD (mm)			p value	Adjusted p- value		
			Horton Ford (HF)	Payne Property (PP)	Fugate Ford (FF)		PP-FF	HF-PP	HF-FF
		(months)							
10/1/07	1st	5	-	-	-	-	-	-	-
6/3/08	1st	13	3.6±0.5	-	2.2±0.4	7.73E-14	-	-	-
9/4/08	1st	15	19.1±2.7	-	8.9±1.3	-	-	-	-
10/28/08	2nd	17	4.2±1.6	1.7±0.5	0.4±0.6	<2e-16	0.0000	0.0000	0.0000
5/26/09	2nd	24	-	3.6±0.9	1.0±0.4	4.93E-10	-	-	-
9/10/09	2nd	27.5	13.8±1.4	8.4±2.8	4.3±1.8	1.19E-4	0.0366	0.0014	0.0001
6/24/10	2nd	37	-	20.4±1.3	19.5±1.1	0.2599	-	-	-
9/19/10	2nd	40	28.8±1.4	12.3±2.4	9.4±5.5	2.49E-05	0.4032	0.0000	0.0000
8/17/11	2nd	51	-	-	-	-	-	-	-

Table 4. ANOVA results of increase in incremental lengths (p value and adjusted p-value) of young mussels of *L. fasciola* in cages at 3 release sites in the Clinch and Powell rivers, from June 9, 2009 to September 18, 2010.

Date	Age	Mean incremental length±SD (mm)			p value	Adjusted p- value		
		Horton Ford	Payne Property	Fugate Ford				
	(months)	(HF)	(PP)	(FF)		PP-FF	HF-PP	HF-FF
6/9/09	26.5	-	-	-	-	-	-	-
8/25/09	29.0	6.0±1.2	2.2±0.7	2.0±0.6	<2e-16	0.3193	0.0000	0.0000
10/6/09	30.5	8.1±1.5	2.8±0.8	2.3±0.6	<2e-16	0.0136	0.0000	0.0000
6/24/10	39.0	12.1±2.5	6.6±2.0	7.5±1.7	<2e-16	0.0281	0.0000	0.0000
9/19/10	42.0	21.3±4.1	10.9±2.7	14.3±2.5	<2e-16	0.0000	0.0000	0.0000

Table 5. Distribution of recaptured, tagged mussels of *V. iris* inside (In) the original release site and outside (Out) the original release site during mark-recapture sampling from 2007 to 2011.

Date	10/1/07	6/3/08		9/4/08		10/28/08		5/26/09		9/10/09		6/24/10		9/19/10		8/17/11	
Age (months)	9	17		20		22		28		31.5		41		44		55	
Release site	In	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out
Horton Ford	100	8	11	4	3	7	5	3	1	3	0	1	1	0	0	0	0
Payne Property	100	9	1	7	3	7	2	4	1	1	0	0	0	0	0	0	1
Fugate Ford	100	30	7	9	9	17	3	1	5	0	2	1	1	0	1	0	0

Table 6. ANOVA results of concentrations of aluminum, copper, iron and zinc (p value and adjusted p-value) in bodies of *V. iris* at 3 release sites in the Clinch and Powell rivers and reared in FMCC pond.

Metallic elements	Mean contents ±SD (ug L^{-1} g^{-1})				p value	Adjusted p- value					
	Horton Ford (HF)	Payne Property (PP)	Fugate Ford (FF)	FMCC pond (Fp)		PP-FF	HF-PP	HF-FF	PP-Fp	HF-Fp	FF-Fp
Al	23.6±7.1	177.2±69.3	50.9±13.1	17.9±17.7	0.000	0.002	0.000	0.721	0.000	0.996	0.592
Cu	9.9±0.8	12.7±1.7	11.8±2.4	6.9±0.8	0.001	0.850	0.113	0.375	0.001	0.081	0.004
Fe	564.4±30.4	702.0±96.6	684.0±88.4	624.5±71.1	0.093	-	-	-	-	-	-
Zn	136.5±16.2	158.1±29.0	145.8±15.5	190.4±28.6	0.032	0.877	0.573	0.940	0.253	0.029	0.078

Table 7. Estimated survival rate and detection probability of released mussels of *V. iris* from 2007 to 2011. Model 1 was constructed from the release of untagged mussels evenly distributed at sites, and tagged mussels placed within an area of 1 m^2 at the middle of release sites. Model 2 was constructed from the release of untagged mussels, and Model 3 was only for tagged mussels. Model 4 was a hierarchical model incorporating individual variations. The σ_s and σ_p represent the standard deviations of mean values of survival rates and recaptures rates in Model 4 incorporating individual variations.

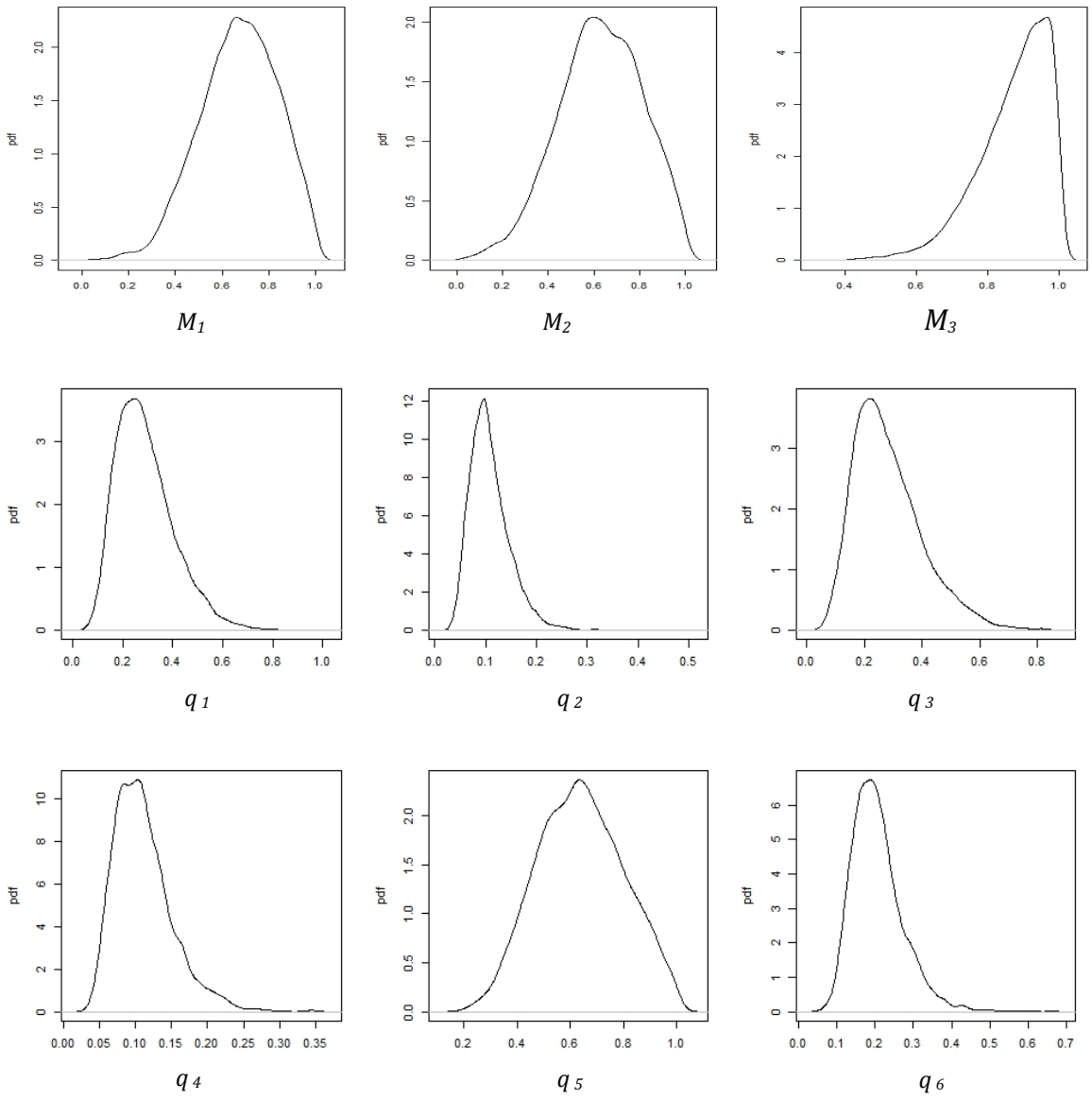
Parameter	Mortality (yr^{-1})		Recapture rates			
			Untagged mussels		Tagged mussels	
	Mean	SD	Mean	SD	Mean	SD
Model 1						
Horton Ford	0.6727	0.1653	0.2914	0.1164	0.1082	0.0395
Payne Property	0.6283	0.1838	0.2833	0.1196	0.1108	0.0408
Fugate Ford	0.8710	0.1005	0.6403	0.1606	0.2055	0.0671
Model 2						
Horton Ford	0.5076	0.1662	0.2049	0.0803	-	-
Payne Property	0.4520	0.1659	0.1934	0.0774	-	-
Fugate Ford	0.7523	0.1397	0.5185	0.1572	-	-
Model 3						
Horton Ford	0.7139	0.2272	-	-	0.1281	0.0660
Payne Property	0.7123	0.2369	-	-	0.1396	0.0713
Fugate Ford	0.8185	0.1715	-	-	0.2124	0.0996

Model 4	Survival rate (yr^{-1})		Recapture rate					
	Mean	SD	Mean	SD	σ_s	SD of σ_s	σ_p	SD of σ_p
Horton Ford	0.2628	0.0487	0.3797	0.0701	0.0052	0.0044	0.0112	0.0087
Payne Property	0.2177	0.0445	0.4136	0.0743	0.0050	0.0039	0.0182	0.0112
Fugate Ford	0.3891	0.0433	0.4730	0.0507	0.0065	0.0057	0.0161	0.0108

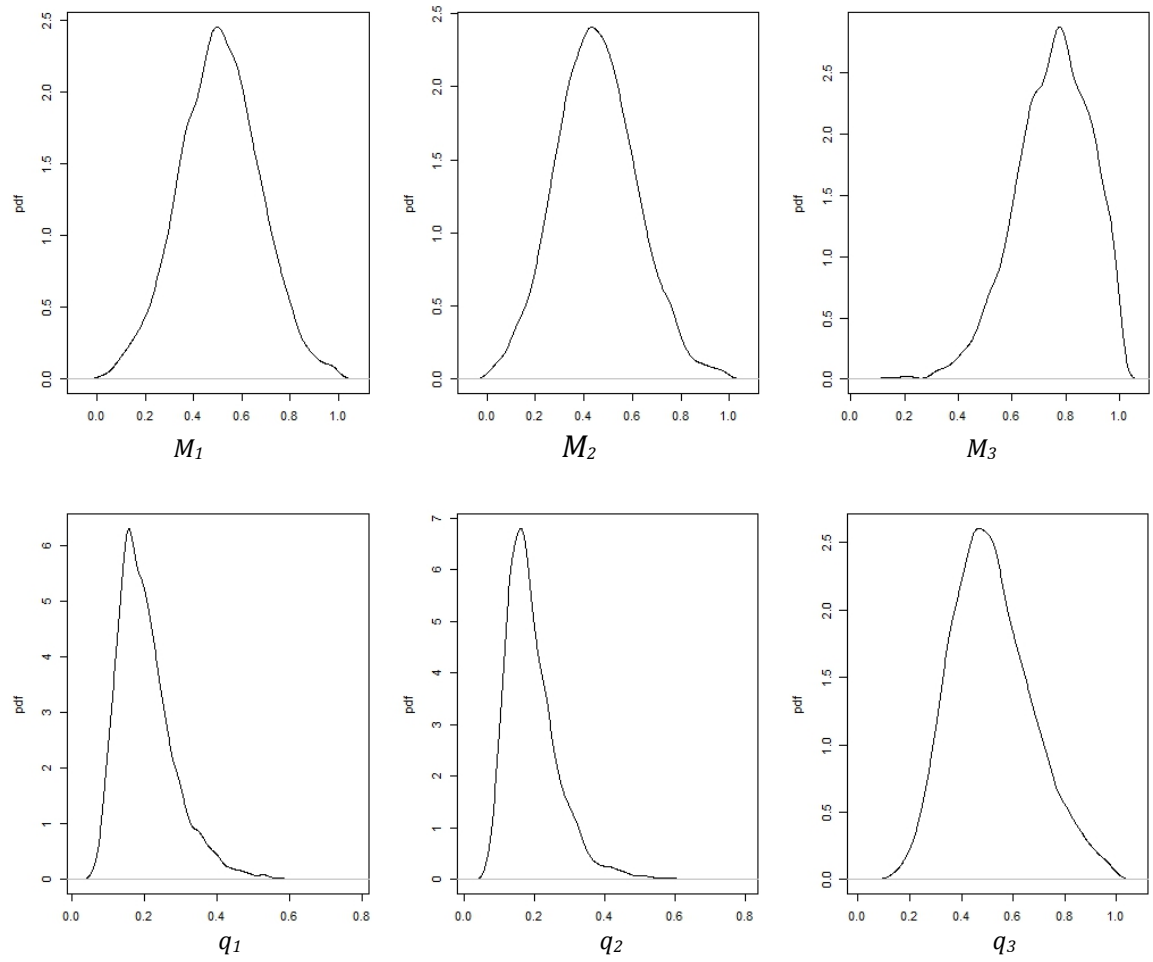
Table 8. Estimated survival rate and detection probability of released mussels of *L. fasciola* from 2007 to 2011. Model 5 was constructed for tagged mussels placed within an area of 3 m^2 located at the middle of release sites. Model 6 was constructed for tagged mussels placed in cages.

Parameter	Mortality (yr^{-1})		Recapture rates	
	Mean	SD	Mean	SD
Model 5				
Horton Ford	0.7231	0.2232	0.1166	0.1162
Payne Property	0.6805	0.2499	0.2871	0.2045
Fugate Ford	0.6514	0.2654	0.2426	0.2148
Model 6				
Horton Ford	0.1610	0.0347	-	-
Payne Property	0.1516	0.0346	-	-
Fugate Ford	0.0690	0.0316	-	-

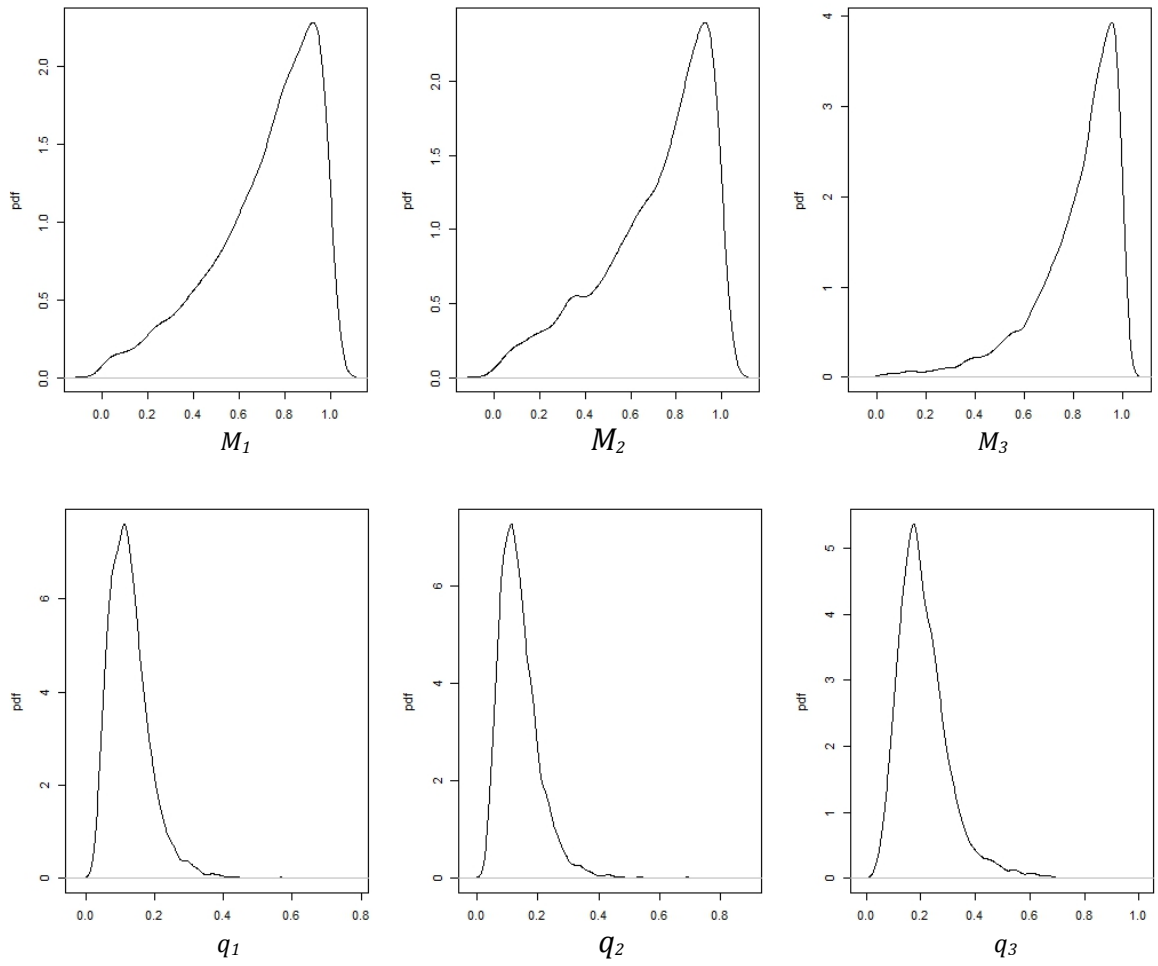
Appendix



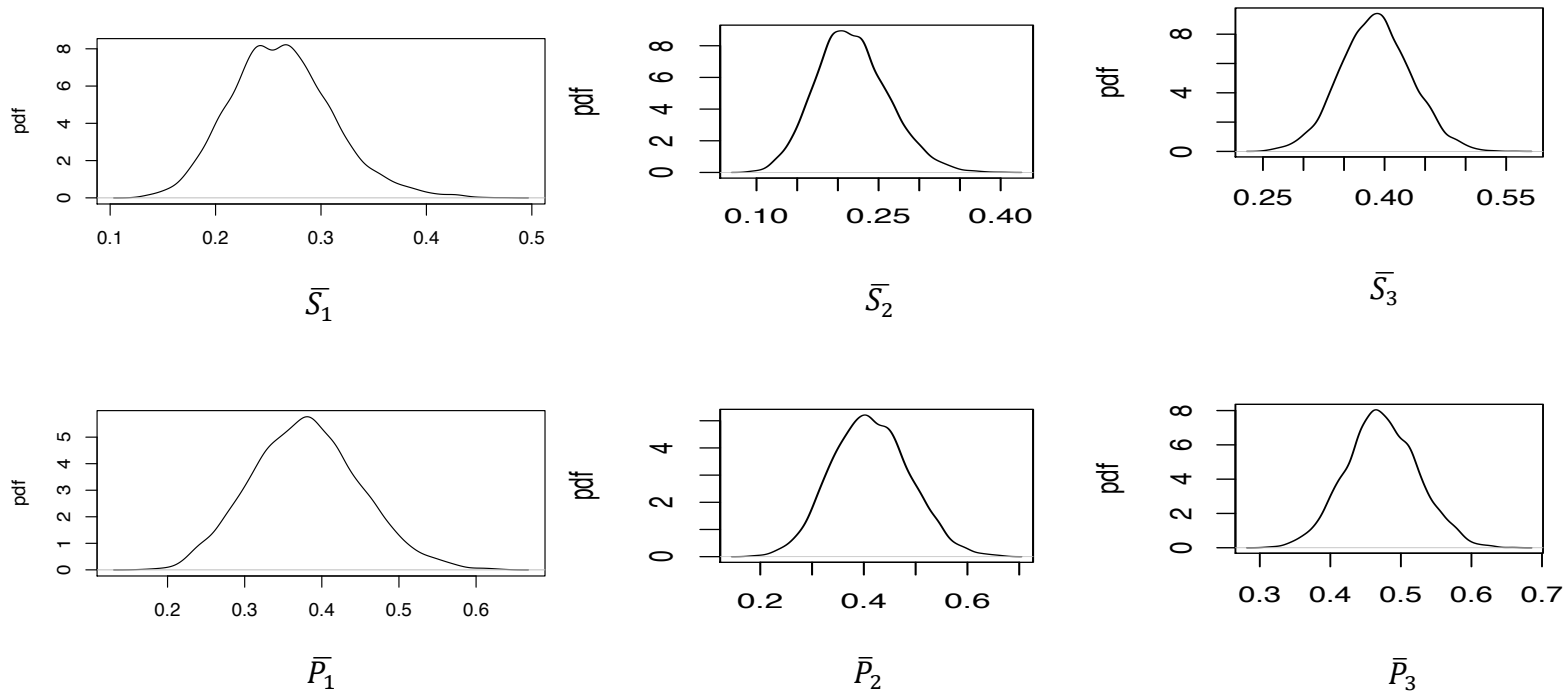
Appendix B-1. Posterior density function of parameters in Model 1. The M_1, M_2 and M_3 denote annual mortality of *V. iris* released at Horton Ford, Payne Property and Fugate Ford, respectively. The q_1 and q_2 are recapture rates of untagged and tagged mussels released at Horton Ford; q_3 and q_4 are recapture rates of untagged and tagged mussels released at Payne Property; and q_5, and q_6 are recapture rates of those untagged and tagged mussels released at Fugate Ford, respectively.



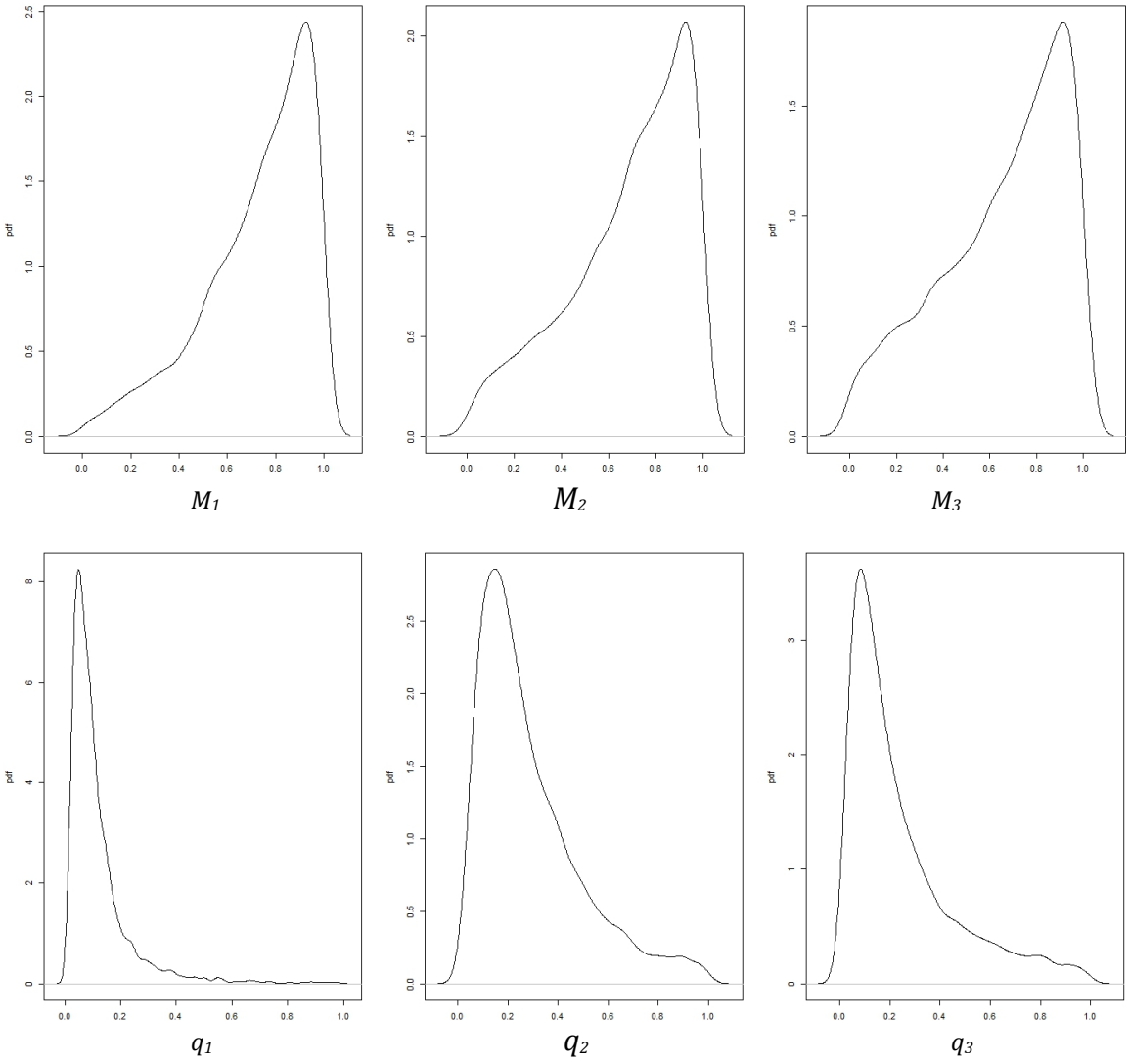
Appendix B-2. Posterior density function of parameters in Model 2. The M_1, M_2 and M_3 denote annual mortality of untagged *V. iris* released at Horton Ford, Payne Property and Fugate Ford, respectively. The q_1, q_2 and q_3 are recapture rates of untagged mussels released at Horton Ford and Payne Property, Clinch River, and Fugate Ford, Powell River, respectively.



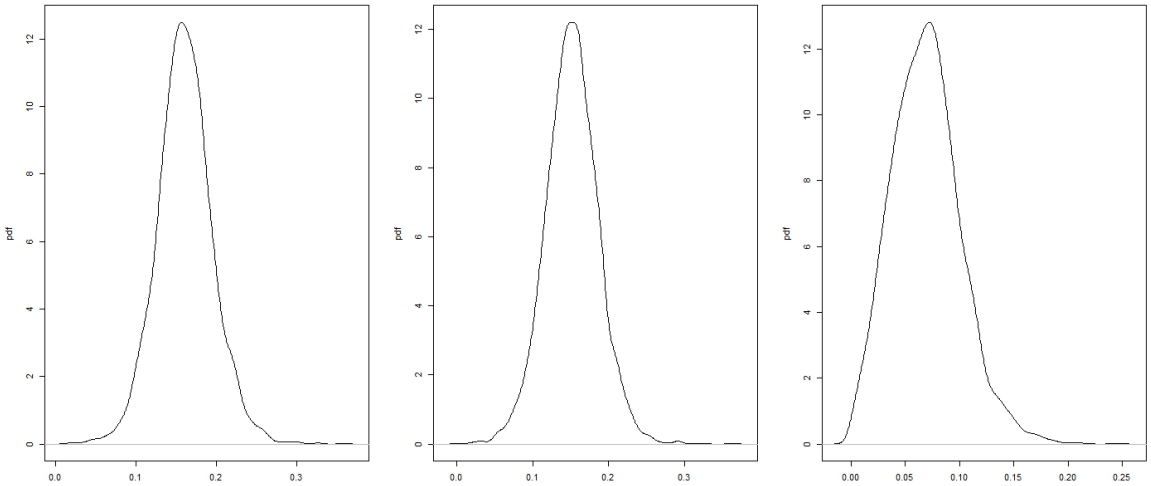
Appendix B-3. Posterior density function of parameters in Model 3. The M_1, M_2 and M_3 denote annual mortality of tagged *V. iris* released at Horton Ford, Payne Property and Fugate Ford, respectively. The q_1, q_2 and q_3 are recapture rates of tagged mussels released at Horton Ford and Payne Property, Clinch River, and Fugate Ford, Powell River, respectively.



Appendix B-4. Posterior density function of parameters in Model 4. The $\overline{S_1}$, $\overline{S_2}$, $\overline{S_3}$ and $\overline{P}_1$, $\overline{P}_2$, $\overline{P}_3$ denote annual survival rate and probabilities of recapture rates for tagged mussels of *V. iris* at Horton Ford, Payne Property, Clinch River, and Fugate Ford, Powell River, respectively.



Appendix B-5. Posterior density function of parameters in Model 5. The M_1, M_2 and M_3 denote annual mortality of tagged *L. fasciola* released at Horton Ford, Payne Property and Fugate Ford, respectively. The q_1, q_2 and q_3 are recapture rates of tagged mussels released at Horton Ford and Payne Property, Clinch River, and Fugate Ford, Powell River, respectively.



M_1 M_2 M_3

Appendix B-6. Posterior density function of parameters in Model 6. The M_1, M_2 and M_3 denote annual mortality of tagged *L. fasciola* placed in cages and deployed at Horton Ford, Payne Property, and Fugate Ford, respectively.

CHAPTER 4-1: Use of PIT tags to assess individual heterogeneity of laboratory-reared juveniles of the endangered Cumberlandian combshell (*Epioblasma brevidens*) in a mark-recapture study

Abstract

The federally endangered Cumberlandian combshell (*Epioblasma brevidens*) was propagated and reared to tagable size (5-10 mm), and released to the Powell River, Tennessee to augment a relict population. Methodology using Passive Integrated Transponder (PIT) tags on these mussels greatly facilitated the detection process. The overall mean detection probability and survival rate of released individuals reached 97.8% to 98.4% and 99.7% to 99.9% (per month), respectively, during 9 successive recapture occasions in the 2-year study period, regardless of seasonality. Nonhierarchic models and hierarchic models incorporating individual and seasonal variations through a Bayesian approach were compared and resulted in similar performance of prediction for detection probability and survival rate of mussels. This is the first study to apply the mark-recapture method to laboratory-reared mussels using PIT tags and stochastic models. Quantitative analyses for individual heterogeneity allowed examination of demographic variance and effects of heterogeneity on population dynamics, although the individual and seasonal variations were small in this study. Our results provide useful information in implementing conservation strategies of this faunal group and a framework for other species or similar studies.

Keywords: Cumberlandian combshell (*Epioblasma brevidens*), survival rate, detection probability, mark-recapture, PIT tag, heterogeneity, hierarchic model, Bayesian.

Hua, D., Jiao, Y., Neves, R. J. and Jones, J. 2015. Ecology and Evolution (in press).

1. Introduction

Global diversity of freshwater mussels (Unionoida) is estimated to be 860 species (Graf 2013). Approximately 37% of this fauna occurs in North America, which contains the world's greatest diversity (Neves 2008); 281 species and 16 subspecies (Williams et al. 1993). However, this order of mollusks is globally declining and has become the most imperiled group of animals in North America (Haag & Williams 2014). Of the United States taxa, only 70 species (23.6%) are considered stable, while 213 species (71.7 %) are considered imperiled, endangered, threatened, extinct, or of special concern to state or federal agencies (Neves & Williams 1994). Roughly 30 species are assumed extinct, disappearing in the last 100 years (Neves 2008), and 84 species are listed as federally endangered or threatened (USFWS 2013), with that number expected to increase (Shannon et al. 1993). The mussel declines are attributed to habitat degradation and destruction mostly due to water pollution, dams, sedimentation and urbanization effects (Parmalee & Bogan 1998; Neves 1999, 2008). Without immediate efforts to recover federally protected species in watersheds throughout the country, extinction of additional species is inevitable.

The Powell River is located in eastern Tennessee (TN) and southwestern Virginia (VA) and is a tributary to the Tennessee River system. Historically, the river contained up to 46 species of freshwater mussels (Ortmann 1918; Johnson et al. 2012), and remains one of the most diverse faunas in the United States, with at least 32 extant species. However, overall declines in mussel abundance and species richness have been chronicled, and the mean mussel density (mussels/m^2) has declined by 63% in this river from 1979 to 2004 (Ahlstedt et al. 2005). Numerous species are nearing extirpation, including the federally endangered Cumberlandian combshell (*Epioblasma brevidens*) (Johnson 2011). This species was listed as endangered under the federal Endangered Species Act of 1973. Globally, it is designated as critically endangered on the IUCN Red List of threatened species. Besides the Powell River, *E. brevidens* has been extirpated from most of its former range in the Cumberland and Tennessee river systems (USFWS

2003). Given the high degree of threat and low recovery potential of this species, a recovery plan for *E. brevidens* was prepared and approved by the U.S. Fish and Wildlife Service in 2004 (USFWS 2004). Recovery plans for endangered species proposed a strategy of propagation and release of young mussels to their natal rivers to augment remnant populations or to reintroduce populations into historic habitats (USFWS 2007; Neves 2008).

Monitoring of restored populations is an important and essential approach to evaluate the success of mussel release efforts, which has involved mark-recapture programs. Current methods using numbered glue-on tags (Kjos et al. 1998; Villella et al. 2004; Peterson et al. 2011) or direct curved labels on mussel shells (Hua 2005) are inefficient due to low recapture rate (Villella et al. 2004; Peterson et al. 2011, Rogers 1999). Hence, a reliable and efficient tagging methodology is desirable to effectively recapture released mussels. The passive integrated transponder (PIT) technology was originally developed in the 1980s to relocate animals (Fagerstone & Johns 1987; Schooley et al. 1993) and was rapidly expanded to track activities of a wide range of taxa including amphibians, birds, fish, and mammals (Germano & Williams 1993; Becker & Wendeln 1997; Burns et al. 1997, Zydlewski et al. 2001; Galimberti et al. 2000). As a mark-recapture tool, this novel technique was recently applied to monitor survival of freshwater mussels. Kurth et al. (2007) found that use of PIT tags doubled mussel recapture rates (72–80%) over visual observation (30–47%) at all sites, with a retention rate of 93%. On the basis of previous studies, we developed a protocol to apply electronic PIT tags on laboratory-produced juveniles of *E. brevidens* and evaluated effectiveness of the technique through recapture rates and demographic analysis.

Demographic modeling has been widely developed to estimate parameters of animal life history through capture-recapture protocols (Williams et al. 2002; Pledger et al. 2003). The traditional mark-recapture models often assume homogeneity in animal survival, capture probabilities and individual variability, which likely introduces bias into model selection and parameter estimation (Pledger et al. 2003), resulting in

misunderstanding of life-history traits (Cam et al. 2002) and high variability in small populations (Conner & White 1999). To estimate variability among individuals, models have been developed and constructed to allow individual heterogeneity using computer programs, and thus have enabled significant applications for understanding population biology (Pledger et al. 2003). Consequently, stochastic models with individual variation can capture the uncertainty of estimated parameters with that variability incorporated; hence reflecting biological reality to a better degree (Pfister & Stevens 2003; Gimenez & Choquet 2010; McVinish & Pollett 2013). Markov Chain Monte Carlo (MCMC) algorithms used in Bayesian statistical inference provide a mathematical framework to circumvent the problem of high-dimensional integrals and allow the likelihood function to be conditional on the unobserved variables in models, simplifying and expediting Bayesian parameter estimation (Gimenez 2008; Paap 2002: Wade 2000).

Effective conservation and restoration strategies of endangered mussel species will require knowledge of population dynamics and predictions of population growth. Our goals were to develop empirical models to estimate changes in mussel population after release to natal rivers, with the following objectives: (1) develop a recapture method to increase mussel recapture rates, (2) develop models incorporating the heterogeneity of individual variations and seasonal changes in survival and detection probabilities for live and dead mussels, and (3) demonstrate applications of stochastic analyses through a Bayesian approach to minimize bias in parameter estimation.

2. Methods and materials

Juvenile mussels

Juvenile mussels of *E. brevidens* were propagated and cultured at the Freshwater Mollusk Conservation Center, Department of Fish and Wildlife Conservation, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Tagging methods

Juvenile mussels were tagged upon reaching 5 mm in length using bee tags (The

Bee Works, Ontario, Canada), and FPN glue-on shellfish tags (Hallprint, Hindmarsh, Australia) for the larger individuals (>10 mm in length). Each bee tag was glued on the mussel umbo, and a FPN shellfish tag was adhered to the external valve using 'Loctite super glue'. Bulk PIT tags (TX1411SST, Biomark, Boise, Idaho, USA) were used in this study because of their high radio frequency identification performance and small size (12.5 × 2.1 mm). Each electronic PIT tag is coded with one of 10 trillion unique codes. The Portable BP antenna and FS2001F-ISO reader (Biomark, Boise, Idaho, USA) were used to detect mussels with attached PIT tags in our study. The PIT tag is activated when the Portable BP antenna reaches its detection area and immediately responds with sound, and the unique digital code is transmitted back to the reader. Mussels were affixed with PIT tags once they grew to sub-adults, at least 20 mm in length. We developed a tagging method to apply PIT tags on mussels. PIT tags were applied using a 3-step process. Shells were cleaned and dried, PIT tags were attached with cyanoacrylate glue, and tags were embedded with dental cement (Fuji Glass Ionomer Luting Cement, Japan). To minimize any negative effects, the whole process to conduct PIT tagging on each mussel was completed in < 2 min, and in the shade under field conditions to reduce stress.

Release and sample

A mark-recapture method was used to monitor released mussels. Sub-adult mussels of *E. brevidens* were tagged when they reached a minimum size of 20 mm (in length) and released to a site (36.535109, -83.441621) near Brooks Bridge, Powell River, Tennessee, USA (Figure 1). River width at the site is around 30 m. The release site is about 50 m^2 in area, with 25-40 cm depth of variable substrates, from clay and sand to infrequent cobbles and boulders. Discharge varies seasonally with precipitation levels, typical of rivers. Mussels were released at multiple times, on 1 July 2009, 26 August 2009, October 7 2009, 25 June 2010, and 11 October 2010 (Table 1), respectively, once they reached the tagable size at FMCC or in cages held at the release site. The in-situ cage was built from a plastic storage container (13 cm x 38 cm x 53 cm). The lid and

bottom of the container was constructed with a sheet of plastic mesh screening (square mesh size of 1.3 cm) to allow water flow The cage was filled to one-third with substrate collected at the site of release to receive the small juveniles, and then was deployed into the substrate. We developed this cage to retain young juveniles (prior to tagable size) to monitor their natural survival and growth rate. Later, this tagged and released group was relocated by PIT tag detector to determine survival, shell length and then returned to their captured locations. Recapture sampling occurred from July 1 2009 until October 11 2011, with a total of 8 sample periods (Table 1). We began sampling 5 m downstream of the release site and moved upstream to 5 m above the release site. Similarly, the sampling area extended 5 m to the left and right margins of the release site. Each PIT tag detected by the portable BP antenna and reader allowed determination whether an individual mussel was live or dead. Located mussels were excavated by snorkeling or view-scopes to record survival and shell length. To increase the likelihood of recapture, we scanned the site 3 times during each sampling event. More than 95% of mussels were captured during the first pass of scanning, and no mussels were detected during the third scan.

Modeling

An individual model was designed and used in this study with the following assumptions:

- Labeled sub-adult mussels retained their tags throughout the study. PIT tags were detectable with certain rate, without negative influence to their survival.
- Variation of survival rates among seasons followed a certain random distribution.
- Sampling protocol and study area were constant.

Data of observations and models

The capture history of individuals was recorded as a sequence of 0's and 1's to indicate that each individual was seen or not during the sampling periods. For example, the history (0 1 1 0 1 1 0 1 0) indicates that the tagged mussel with a unique code was captured for the first time at sampling occasions 2 and 3, not capture at time 4, captured

again at times 5, 6 and 8, but not at times 7 and 9. Use of PIT tags allowed us to recapture live mussels and dead ones (shells). Two data sets, including capture history of live and dead individuals, were used for analyses.

Formulation for Model 1:

$$
\begin{aligned}
Pl_{i,k,j} &= S_j^{\Delta t}\, L_{i,k-1,j} \\
L_{i,k,j} &\sim Bern(Pl_{i,k,j}) \\
Cl_{i,k,j} &= Pc_{lj}\, L_{i,k,j} \\
Ol_{i,k,j} &\sim Bern(Cl_{i,k,j}) \\
D_{i,k,j} &= 1 - L_{i,k,j} \\
Cd_{i,k,j} &= Pc_{dj}\, D_{i,k,j} \\
Od_{i,k,j} &\sim Bern\,(Cd_{i,k,j})
\end{aligned}
\qquad (1)
$$

where *i* represents the i^{th}individual, *j* represents the season of sampling occasions (*j* = 1 denotes summer from June to October, when mussels grow rapidly, *j* = 2 denotes winter from October to May, a slow growing season), *k* represents the k^{th} capture occasions (*t* = 0, 1, 2…7, *t* = 0 denotes as the time of mussel release and the 0th capture), *j* represents the season of sampling occasions, and Δt represents time (month) between adjacent capture occasions. $L_{i,k,j}$ represents the live statue of a individual mussel at the k^{th} capture occasions of the season *j*. S_j is the monthly survival rate of *E. brevidens* in the summer or winter and its prior is assumed to follow a uniform distribution between 0 and 1. $Pl_{i,k,j}$ denotes the probability of a live individual at the capture occasion k^{th} that depends on its survival status at the $(k-1)^{th}$ recapture occasion and survival rate S_j. $Cl_{i,k,j}$ denotes probability of recapturing a live mussel based on recapture rate Pc_{lj} and its survival status at the k^{th} capture occasion. $Ol_{i,k,j}$ is the observation of live individual that follows Bernoulli distribution. $D_{i,k,j}$ denotes as the status of a dead individual. $Cd_{i,k,j}$ denotes probability of recapturing a dead mussel based on recapture rate Pc_{dj} and its death status at the k^{th} capture occasion. $Od_{i,k,j}$ is the observation of dead individual that follows Bernoulli distribution. S_j, Pc_{lj} and Pc_{dj} represent population characteristics and were assumed to follow uniform distributions between 0 and 1.

Besides the above model, the Model 2 was constructed assuming the seasonal variations only in detection probability of dead mussels (Pc_{dj}), but ignoring them in survival rate (S) and detection probability of live mussels (Pc_l); Model 3 was constructed ignoring seasonal variation in all S, Pc_d and Pc_l; Model 4 only included the parameters of S and Pc_l without seasonal variation and records for dead mussels due to limited observations; Model 1-1, 2-1, 3-1 and 4-1 were constructed hierarchically considering the individual variations in the above correspondent models.

Formulation for Model 1-1:

$$Pl_{i,k,j} = S_{i,j}^{\Delta t} L_{i,k-1,j}$$

$$L_{i,k,j} \sim Bern(Pl_{i,k,j})$$

$$Cl_{i,k,j} = Pc_{l\,i,j} L_{i,k,j}$$

$$Ol_{i,k,j} \sim Bern(Cl_{i,k,j})$$

$$D_{i,k,j} = 1 - L_{i,k,j}$$

$$Cd_{i,k,j} = Pc_{d\,i,j} D_{i,k,j}$$

$$Od_{i,k,j} \sim Bern\,(Cd_{i,k,j})$$

$$S_{i,j} \sim N(\overline{S_j}, \sigma_s^2) I(0,1) \qquad (2)$$

$$Pc_{l\,i,j} \sim N\left(\overline{Pc_{l\,j}}, \sigma_{Pc_l}^2\right) I(0,1)$$

$$Pc_{d\,i,j} \sim N\left(\overline{Pc_{d\,j}}, \sigma_{Pc_d}^2\right) I(0,1)$$

where $S_{i,j}$, $Pc_{l\,i,j}$ and $Pc_{d\,i,j}$ represent population characteristics and were assumed to follow normal distributions $N(\overline{S_j}, \sigma_s^2)$, $N\left(\overline{Pc_{l\,j}}, \sigma_{Pc_l}^2\right)$ and $N\left(\overline{Pc_{d\,j}}, \sigma_{Pc_d}^2\right)$ respectively. The $\overline{S_j}$, $\overline{Pc_{l\,j}}$ and $\overline{Pc_{d\,j}}$ represent mean values of population characteristics of $S_{i,j}$, $Pc_{l\,i,j}$ and $Pc_{d\,i,j}$ among individuals and were assumed to follow uniform distributions between 0 and 1 because uniform prior distributions for variance out-performed in hierarchical models (Gelman 2006). I denotes the boundary of the distribution in WinBUGS.

Priors

Multi-level priors incorporating seasonal variations and individual differences were tested to estimate survival and recapture rates and their associated uncertainty. The Bayesian approach was used to calculate a posterior distribution from the observed data

and multi-level prior distribution.

WinBUGS is a numerically intensive software package for the Bayesian analysis of complex statistical models to implement Markov Chain Monte Carlo (MCMC) methods (Spiegelhalter et al. 1996). We used WinBUGS software associated with three Markov chains to determine the convergence of the posterior distribution by monitoring the trace plot, diagnosing the autocorrelation and Gelman and Rubin statistics (Spiegelhalter et al. 2004; Jiao et al. 2008, 2009). A proper burn-in iteration and thinning interval were determined based on the convergence criteria (Jiao et al. 2008). The Bayesian inference was generated from the samples of random draws from the posterior distribution after the three chains converged.

Model selection

Deviance information criterion (DIC) was used for the model selection for the Bayesian models and formulated as:

$$DIC = 2\overline{D}(\theta) - \widehat{D}(\theta) \; or \; \overline{D}(\theta) + P_D \quad (3)$$

$$P_D = \overline{D}(\theta) - \widehat{D}(\theta)$$

where D is the deviance to measure the predicted goodness-of-fit for all 8 models, P_D is the effective number of parameters in a Bayesian model, $\overline{D}$ is the posterior mean of the deviance, and $\widehat{D}$ is the deviance of the posterior mean. The DIC is particularly applied in Bayesian model selection using MCMC simulation to determine the posterior distributions of the models (Spiegelhalter et al. 2002). Model 4 and Model 4-1 were constructed by excluding the data for detection probability of dead mussels. The other 6 models were produced using the data including both detection probabilities of live and dead mussels. Thence, models were compared between Model 4 and Model 4-1, and among the other 6 models. Models were evaluated based on the DIC values, which model with the lowest DIC made the best prediction. Models within 5 DIC units of the 'best' model received the best consideration. Those within 5-10 DIC units of the 'best' model were considered substantially less well supported. Models more than 10 DIC units from

the 'best' model were definitely excluded (Spiegelhalter et al. 2002; Jiao et al., 2009).

3. Results

During the 2-year sampling period, 100% of recaptured mussels retained their PIT tags. A dead mussel was recaptured from the substrate at an approximate depth of 35 cm by the affixed PIT tag on one piece of broken shell (Figure 2). Vertical and horizontal movements of released mussels were observed as they positioned themselves from surface to a depth of 35 cm in the substrate, with seasonal variations. Mussels were detected and recaptured whether they burrowed in sand, clay, gravel, and even beneath cobbles and boulders.

Estimated mean survival rate of released mussels (*S*) among all models reached 0.997 to 0.999/mo during the 2-yr period, indicating that the release site in Powell River is suitable for this endangered species. The mean detection probability for live mussels (Pc_l) ranged from 0.978 to 0.984 (Table 2). Seasonal variation in Pc_l and *S* was not detected. The mean detection probability for dead mussels showed slight seasonal variations; Pc_d value ranged from 0.373 to 0.438 in summer and from 0.339 to 0.438 in winter with a relatively high standard deviation ranged from 0.155 to 0.199 in summer and from 0.169 to 0.243 in winter.

The distributions of posterior density function (pdf) for parameters *S*, Pc_l and Pc_d are illustrated in Figure 3. The pdf of *S* and Pc_l exhibited a reasonable symmetric curve that approaches a normal distribution; however, the pdf of detection probability for dead mussels was apparently skewed especially in winter, in those models incorporating individual variations (Figure 3-A). Variations among individuals in *S* ($< 10^{-5}$) and Pc_l ($<10^{-3}$) were very small, only exhibited in Pc_d with a noticeable value (Figure 3-B).

In our study, the hierarchical model 4-1that estimated survival and detection probability of live mussels incorporating individual variations, exhibited a slightly lower DIC value (69.05) compared to the non-hierarchical model 4 (70.22), a difference of 0.17. Among the other 6 models, the lowest DIC (76.29) identified Model 3 with the best

performance. However, the maximum difference in DIC values among the 6 models was 3.37 units (Table 3). Therefore, the overall differences in these 6 models are not significant. These results reflect the small estimated standard deviation from seasonal and individual variations in models 1-2, 2-2, 3-2 and 4-2. In this study, results showed that hierarchical models incorporating individual variations and non-hierarchical models performed similarly.

4. Discussion

Use of quadrats or transects have been applied for quantitative and qualitative mussel sampling (Smith et al. 2001; Strayer & Smith 2003; Ahlstedt et al. 2005). However, these traditional survey methods are inadequate to monitor population changes of rare species of mussels (Johnson 2011). Even for non-listed mussel species, recapture rates for *Elliptio complanata*, *E. fisheriana* and *Lampsilis cariosa* were only 3% to 19% during a 2-yr mark-recapture study (Villella et al. 2004). Low capture probability results in less precision in estimation of survival rate because that rate is estimated based on the probability of detecting individuals during subsequent sampling occasions (Burnham et al. 1987). The standardized protocol for mussel surveys is by visual observation and excavation of substrates. However, the vertical movement and horizontal dispersion of specimens often result in low catchability (Villella et al. 2004). Mark-recapture of *E. brevidens* using PIT technology in our study reached a high detection probability of 98%. The electronic tags detected through a portable BP antenna and reader greatly facilitated the relocation and identification of released individuals.

Kurth et al. (2007) stated that the non-captured mussels (20-28%) in their study might be due to loss of PIT tags. We noted that tagging method directly influenced the tag retention rates. High recapture rate (>98%) in our study benefited from a proper tagging method. Prior to tagging, the cleaning and drying of the shell surface, and prefixing of a glass-encapsulated PIT tag onto the valve using Loctite super glue are critical to the process. Viscosity of the dental cement mixture is also critical. A thin

mixture could be eroded by mussel movements, and swift currents can cause PIT tags to be exposed, making them vulnerable to detachment from mussels. A thick cement mixture dries too quickly to envelop the entire PIT tag. We have tagged mussels of multiple species and reared them in the laboratory for many years, and overall survival rates of nearly100% support the PIT tagging protocol in this study. Our results showed a high recapture probability and survival rate over 2 yr, indicating an effective protocol using the described PIT tagging method. However, inappropriate handling methods can cause PIT tag loss or mussel stress. Wilson et al. (2011) tested mussel behavior in an experiment of mussels with and without PIT tags, and indicted that mussel-burrowing activities were influenced by the additional weight of PIT tags. However, their results did not show significant differences in mussel activity, burrowing ability, burrowing time, and burrowing rate index between the two treatments. Conversely, mussels had a delayed response seemingly attributed to the long process of tagging (40 min) and impact from ethanol used in their study. Ethanol is used to preserve animal tissues and should be used with caution in tagging live mussels. The other concern of PIT tags is the potential of greater predation caused by visibility of white cement. Our released mussels were implanted into the substrates to eliminate this concern. Observations indicated that the coating cement became dark-brown after a few months in the river.

The traditional mark-recapture models often assume the homogeneity in animal survival, capture probabilities and individual variability, which can bias model selection and parameter estimation (Pledger et al. 2003). Computing resolution associated with mathematical development allows for analyzing the heterogeneity and constructing individual models on capture-recapture of animals. Heterogeneous models have been extended to detect the capture probabilities, survival rates, capture probability, population size, birth rates, lifetime growth in body mass, or variability in individual disease status (Pledger et al. 2002, 2003; Catchpole et al. 2001, Schofield & Barker 2011). We applied individual models to detect heterogeneity in survival rates, and detection probability for live and dead mussels associated with seasonal changes in the Powell River. In most

previous studies, the detection probability component for dead mussels was ignored due to limitation of shell recovery. Use of PIT tags also resolves the problem of low recapture rates and associated bias in estimating parameters. Catchpole et al. (2001) modeled the recovery rates of dead abalone through visual searching for shells, and the recovery rates varied from 0.05 to 0.56, influenced by animal visibility and the experience of divers. The detection probabilities of dead mussels in our study were relatively stable, ranged from 0.339 to 0.382, with associated standard errors at 0.199 to 0.239. The high standard errors might be due to the low mortality rate since only one mussel was collected dead and recovered by the PIT detector, excavated from a substrate depth of approximately 35 cm. Results showed that the detection probability for dead mussels differed slightly in summer compared to winter, while other parameters exhibited little difference from season to season. Use of the PIT method can significantly increase detection probabilities for both live and dead animals, regardless of visibility and experience of operators during the sampling process.

Capture-recapture models are now widely used in population biology with the advent of various computing programs; MARK is one of the most commonly used. However, the limitation of this program is that it does not incorporate individual effects, though it can implement a simple MCMC algorithm (White & Burnham 1999; Gimenez & Choquet 2010). Bayesian theory has greatly increased in applicability in population ecology (Gimenez 2008), and allows individual random effects to be modeled (Gimenez & Choquet 2010), particularly in conservation biology with alternative standard statistical procedures (Wade 2000). However, the Bayesian approach does require programming skills and could be time-consuming to process complex models because it uses extensive MCMC simulation to implement the program. We used WinBUGS (14) in R to generate three chains of length 100,000 (with the first 5,000 as burn-in) for non- hierarchical models from 1 to 4, where individual variations were not considered. However, we had to increase the iterations to 10,000,000 (with the first 50,000 as burn-in) to make 3 chains converged for models of 1-1, 2-1, 3-1, and 4-1 along with individual characteristics. We

applied these advantages in our individual models to detect the uncertainty of capture and survival rates associated with seasonal changes.

Seasonal variations in detection probability and survival rates of mussels occur in traditional survey methods through surface visual observation and excavation of substrates (Villella et al. 2004). We developed Model 1 to incorporate seasonal variations in the parameters of survival rate (*S*), detection probabilities for live (Pc_l) and dead mussels (Pc_d), but found that seasonal variations only existed in Pc_d, not in *S* and Pc_l. Hence, we applied Model 2 with the seasonal variations in Pc_d but not in others, while Model 3 did not incorporate seasonal variations in all parameters. The standard deviation of Pc_d decreased in Model 3 compared to Model 1 and 2; however, it was still as high as 55.1% of mean value of Pc_d.

Royle (2008) and Gimenez & Choquet (2010) indicated that models had the best performance which incorporated random effects. We developed 3 corresponding hierarchical models (1-1, 2-1 3-1) incorporating individual variations. The standard deviations of all parameters in hierarchical models were reduced compared to their non-hierarchical models, especially in Pc_d. The distribution of posterior density function for parameters *S*, Pc_l and Pc_d exhibited a reasonable symmetric curve (close to normal distribution), including the pdf of detection probability for dead mussels in winter, while it was skewed (mean is not near the center) in those non-hierarchical models. The standard deviation of Pc_d in Model 3-1 decreased 24.9% compared to it in Model 3; however, it was still as high as 32.4% of mean value of $\mathrm{Pc_d}$. In accordance with this result, we reconstructed Model 4 to ignore Pc_d, and Model 4-1to incorporate individual variations. The results showed that Pc_l and *S* agreed with those in other models. We found that heterogeneity of seasonal variations apparently occurred in the recapture of dead mussels but not in live mussels. Heterogeneity was not significant in the recapture of live mussels, likely due to the similarity among mussels since all derived from the same cohort of propagated mussels; and the high recapture rate using the PIT tag method that reduced variations among detected samples. Pit tag methodology allowed detection

of almost all tagged individuals, with equal probability of detection whether in summer or winter. Notwithstanding, the heterogeneity in recapture of dead mussels in this study might derive from low mortality since only one dead mussel was recaptured over 2 yr.

The Deviance Information Criterion (DIC) developed by Spiegelhalter et al. (1998) is one of several methods for comparing Bayesian models. Recently, it has been widely incorporated into WinBUGS and OpenBUGS to implement MCMC simulation. Note that the model with the smallest DIC indicates the estimated model with the best prediction, and models within 5 DIC units of the 'best' model need to be reported (Spiegelhalter et al. 2002, Jiao et al. 2009). Hence, model 3 is the best model with the minimum DIC value (76.29). Comparing other DIC values to that of model 3, the other 5 models (Models 1, 2, Model 1-1, 2-1,3-1) also performed well because the differences of DIC values were within 5 units. Similarly, the DIC difference between model 4 (70.22) and model 4-1 (69.05) is less than 1 unit. Royle (2008) and Gimenez & Choquet (2010) indicated that models using random effects had the better performance with lowest DIC values. In our study, the skewed distributions of posterior density function for Pc_d in models (1, 2 and 3) were adjusted to an asymptotically normal curve in their corresponding hierarchical models (1-1, 1-2 and 1-3), although the hierarchical models incorporating heterogeneity did not outperform those models without individual and seasonal variations. That is due to the small variation in seasons and individuals, with insignificant impacts on the DIC values. Hence, they are considered in this study. It seems to be explained reasonably well by the negligible value of σ_s and σ_{Pc_l} that the similarity of life history traits occurs in the same cohort of laboratory-produced mussels. Model complexity by adding individual variations might not reveal the advantage of hierarchical models incorporating individual variations in our study. Seasonal variations did not affect survival rates of released mussels, and the PIT tag method provides an equal opportunity for each individual to be captured and to adjust the accuracy of mussel survival rates. Explanations of the heterogeneity in survival rate and detection probability among seasonal and individual variations are still valuable in answering questions of our

objectives and providing the framework for other species or similar studies.

We conclude that the mark-recapture model using PIT tags is highly efficient, and advances our ability to monitor the restoration of this and other endangered species in rivers. High mortality was only exhibited in the early life stage. Laboratory- reared mussels can exhibit high survival rates where released into suitable habitat. Our methods and results provide optimism for the recovery of this faunal group and can be applied to other faunal groups that are difficult to collect and monitor for conservation and restoration.

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Table 1. Release and recapture of laboratory-produced juvenile mussels (*E. brevidens*).

Date	7/1/09	8/26/09	10/7/09	6/25/10	10/11/10	5/10/11	8/17/11	10/12/11
Age (months)	24.5	26.5	28	36.5	40	47	50	52
Number of released mussels	23	28	38	9	1	0	0	0
Number of recaptured live mussels		23	50	88	97	96	95	94
Number of recaptured dead mussels	0	0	0	0	1	0	0	0

Table 2. Estimated survival rate (monthly) and detection probability of released mussels in 8 models. The *j* represents the season of sampling occasions (*j* = 1 denotes summer; *j* = 2 denotes winter). The S_j is the monthly survival rate of *E. brevidens* in the summer or winter; Pc_{lj} and Pc_{dj} denote the recapture rate of live and dead mussel in the summer or winter, respectively. The $\overline{S_j}$, $\overline{Pc_{l\,j}}$ and $\overline{Pc_{d\,j}}$ represent mean values of monthly survival, the recapture rates of live and dead mussel among individuals in the summer or winter, respectively. The $\bar{S}, \overline{Pc_l}$ and $\overline{Pc_d}$ are mean values of monthly survival, the recapture rates of live and dead mussel among individuals without seasonal variations. The σ_s, σ_{Pc_l} and σ_{Pc_d} are standard deviations of $\overline{S_j}$, $\overline{Pc_{l\,j}}$ and $\overline{Pc_{d\,j}}$ in Models 1-1 and 2-1; and are the standard deviations of $\bar{S}$, $\overline{Pc_l}$ and $\overline{Pc_d}$ in Models 3-1 and 4-1. The seasonal variations among above standard deviations were not incorporated due to their extremely small values. The S, Pc_l and Pc_d represent the monthly survival rate and recapture rate for live and dead mussels without seasonal variations.

Model	Parameter	Summer		Winter		No seasonal variation	
		Mean	SD	Mean	SD	Mean	SD
Model 1	S_j	0.997	0.002	0.999	0.001	-	-
	$Pc_{l\,j}$	0.981	0.008	0.984	0.009	-	-
	$Pc_{d\,j}$	0.373	0.198	0.343	0.243	-	-
Model 1-1	$\overline{S_j}$	0.997	0.002	0.998	0.001	-	-
	$\overline{Pc_{l\,j}}$	0.978	0.008	0.978	0.010	-	-
	$\overline{Pc_{d\,j}}$	0.428	0.156	0.438	0.174	-	-
	σ_s	$3.34e^{-7}$	$4.43e^{-7}$	$3.34e^{-7}$	$4.43e^{-7}$	-	-
	σ_{Pc_l}	$2.43e^{-5}$	$2.64e^{-5}$	$2.43e^{-5}$	$2.64e^{-5}$	-	-

	σ_{Pc_d}	$6.48e^{-3}$	$5.77e^{-3}$	$6.48e^{-3}$	$5.77e^{-3}$	-	-
Model 2	S	-	-	-	-	0.999	0.001
	Pc_l	-	-	-	-	0.984	0.006
	$Pc_{d\ j}$	0.382	0.199	0.339	0.239	-	-
Model 2-1	S	-	-	-	-	0.999	0.001
	Pc_l	-	-	-	-	0.984	0.006
	$\overline{Pc}_{d\ j}$	-	-	-	-	0.437	0.169
	σ_{Pc_d}	-	-	-	-	$6.76e^{-3}$	$5.85e^{-3}$
Model 3	S	-	-	-	-	0.999	0.001
	Pc_l	-	-	-	-	0.984	0.006
	Pc_d	-	-	-	-	0.321	0.177
Model 3-1	$\bar{S}$	-	-	-	-	0.998	0.001
	$\overline{Pc}_l$	-	-	-	-	0.980	0.007
	$\overline{Pc}_d$	-	-	-	-	0.423	0.133
	σ_s	-	-	-	-	$5.75e^{-7}$	$7.62e^{-7}$
	σ_{Pc_l}	-	-	-	-	$4.45e^{-5}$	$4.60e^{-5}$
	σ_{Pc_d}	-	-	-	-	$1.44e^{-2}$	$1.13e^{-2}$
Model 4	S	-	-	-	-	0.999	0.001
	Pc_l	-	-	-	-	0.984	0.006
Model 4-1	$\bar{S}$	-	-	-	-	0.997	0.001
	$\overline{Pc}_l$	-	-	-	-	0.979	0.007
	σ_s	-	-	-	-	$7.88e^{-7}$	1.03e-6
	σ_{Pc_l}	-	-	-	-	$4.92e^{-5}$	5.16e-5

Table 3. Comparison of DIC values among the 8 models (survival rate is per month). The j represents the season of sampling occasions (j = 1 denotes summer; j = 2 denotes winter; $j_1 = j_2$ when parameters ignoring seasonal variations). The S_j is the monthly survival rate of *E. brevidens* in the summer or winter; Pc_{lj} and Pc_{dj} denote the recapture rate of live and dead mussel in the summer or winter, respectively.

Model	All Parameters	Parameters incorporation seasonal variations	Parameters ignoring seasonal variation	Parameters incorporating individual variations	DIC
1	S_j, $Pc_{l\,j}$, Pc_{dj}	S_j, $Pc_{l\,j}$, Pc_{dj}	-	-	76.72
1-1	S_j, $Pc_{l\,j}$, Pc_{dj}	S_j, $Pc_{l\,j}$, Pc_{dj}	-	S_j, $Pc_{l\,j}$, Pc_{dj}	79.66
2	S_j, $Pc_{l\,j}$, Pc_{dj}	$Pc_{d\,j}$	S_j, $Pc_{l\,j}$	-	77.04
2-2	S_j, $Pc_{l\,j}$, Pc_{dj}	$Pc_{d\,j}$	S_j, $Pc_{l\,j}$	$Pc_{d\,j}$	77.94
3	S_j, $Pc_{l\,j}$, Pc_{dj}	-	S_j, $Pc_{l\,j}$, Pc_{dj}	-	76.29
3-3	S_j, $Pc_{l\,j}$, Pc_{dj}	-	S_j, $Pc_{l\,j}$, Pc_{dj}	S_j, $Pc_{l\,j}$, Pc_{dj}	78.05
4	S_j, $Pc_{l\,j}$	-	S_j, $Pc_{l\,j}$	-	70.22
4-4	S_j, $Pc_{l\,j}$	-	S_j, $Pc_{l\,j}$	S_j, $Pc_{l\,j}$	69.05

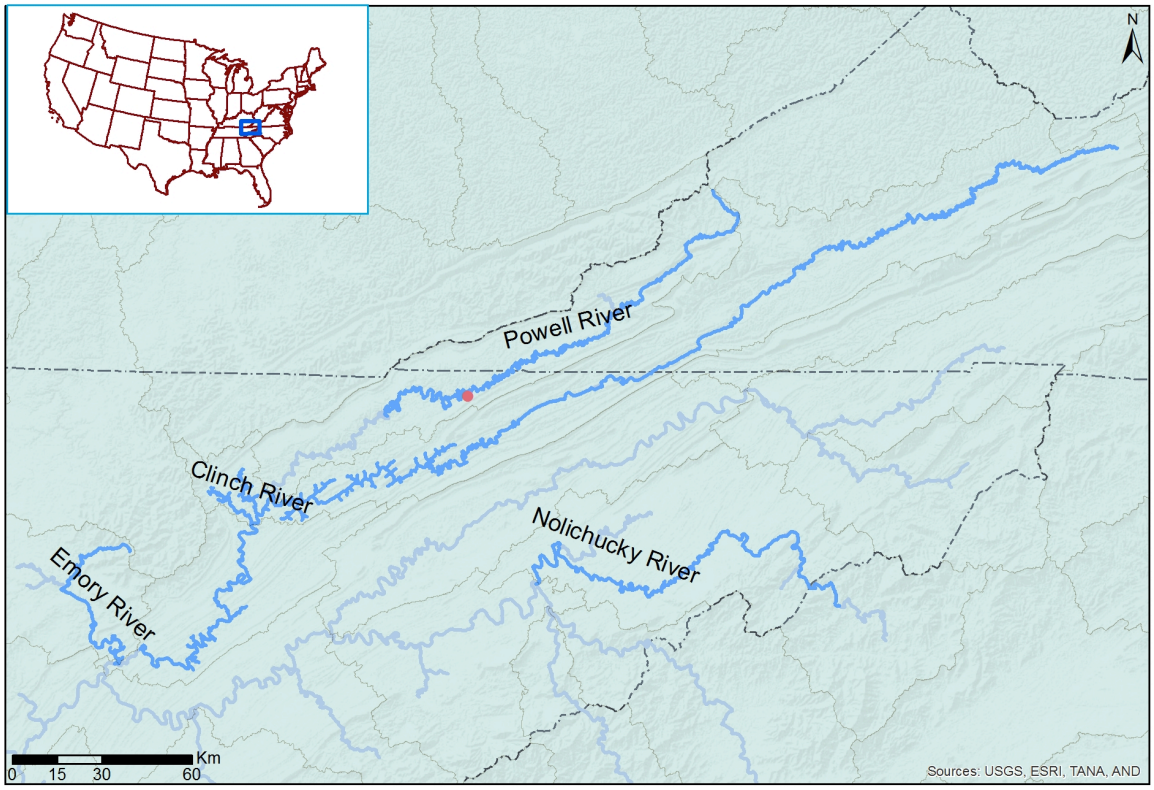


Figure 1. Release of juvenile mussels of *E. brevidens* at Brooks Bridge (36.534879, -83.441762) in the Powell River, Tennessee, USA.

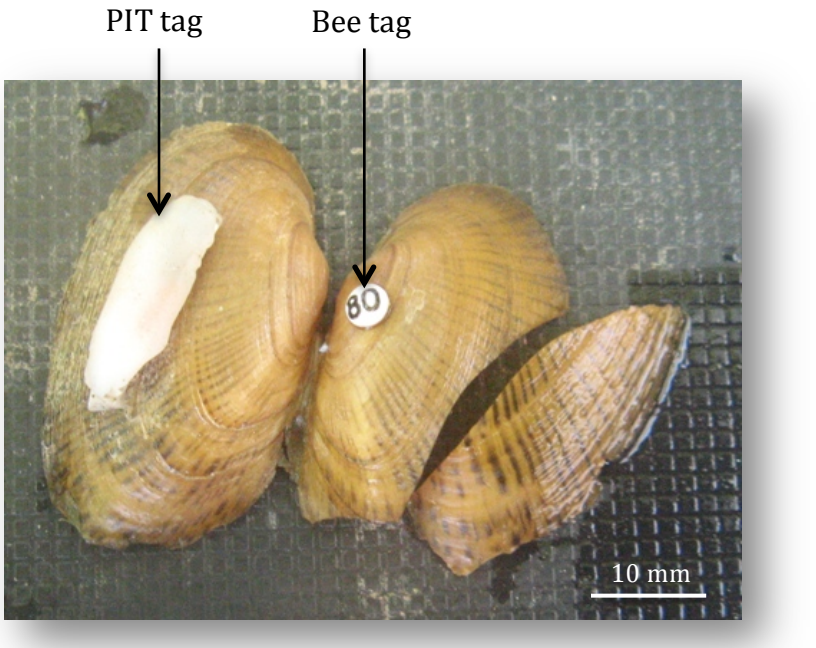
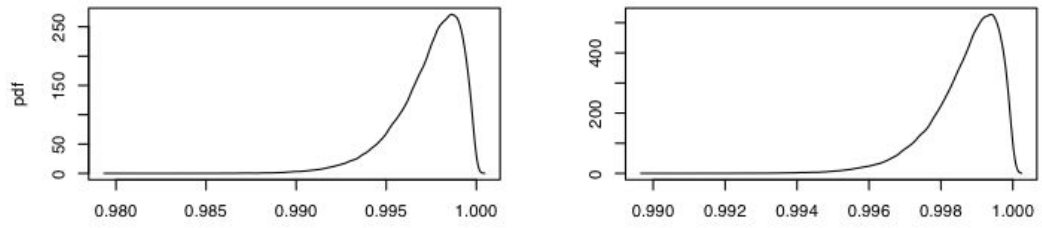
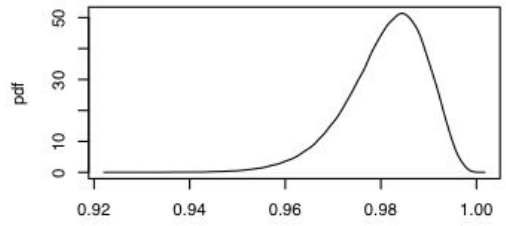


Figure 2. Dead mussel located and recaptured using PIT tag protocol.

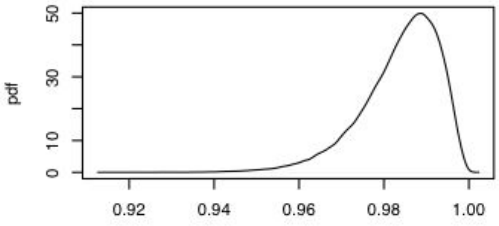


$S_j\ (j = 1)$

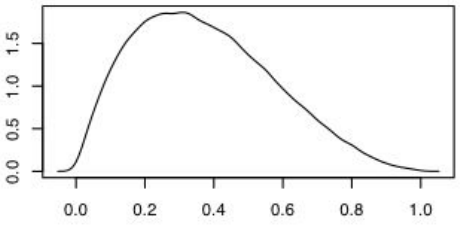
$S_j\ (j = 2)$



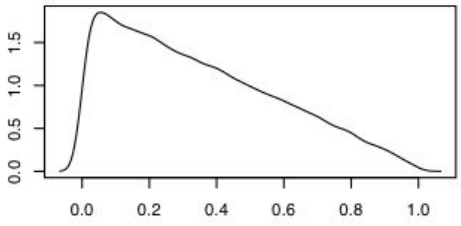
$Pc_{ij}(j = 1)$



$Pc_{ij}(j = 2)$



$Pc_{dj}(j = 1)$



$Pc_{dj}(j = 2)$

Figure 3-A. *(Model 1)*

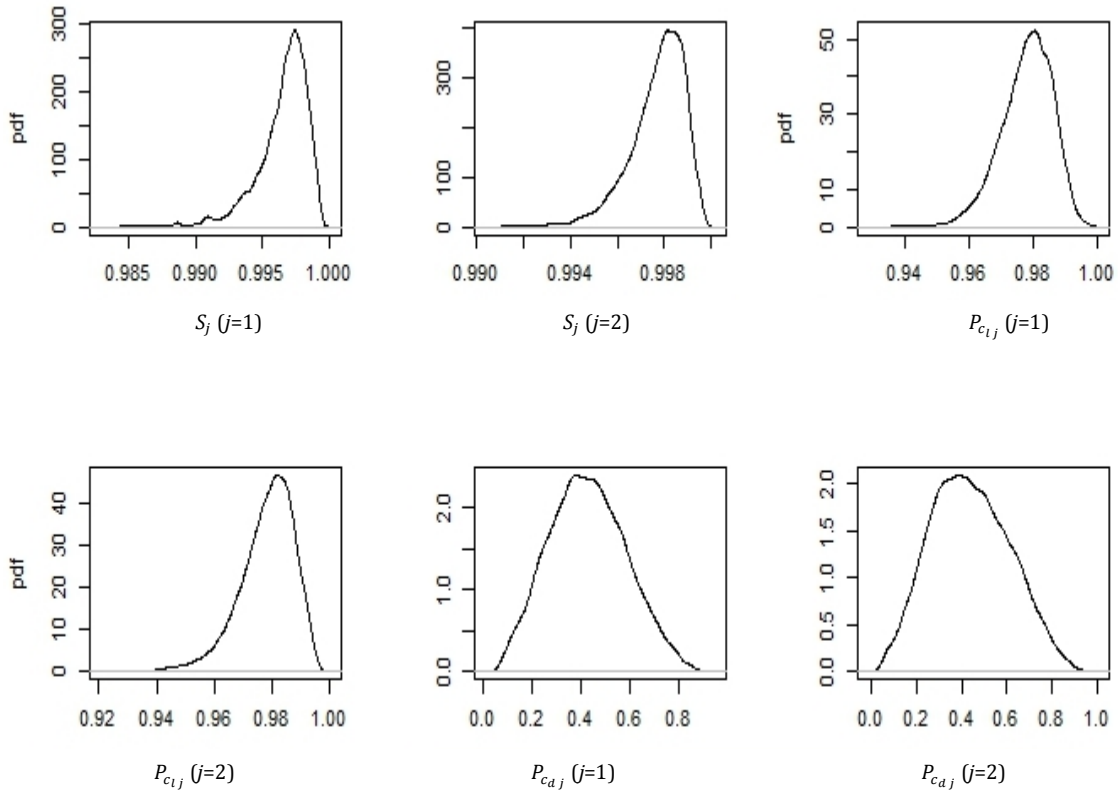
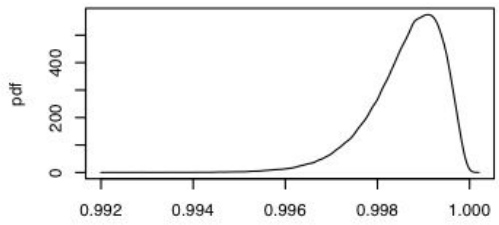
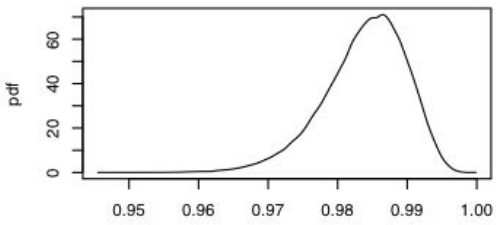


Figure 3-B. *(Model 1-1)*

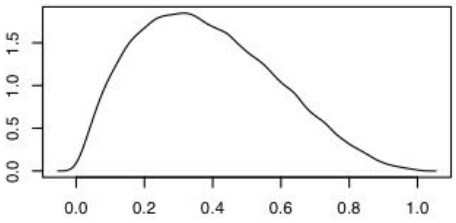
Figure 3. Posterior density function of parameters in models 1 and 1-1. S_j, Pc_{lj} and Pc_{dj} represent monthly survival rate, probabilities of recapture rates for live and dead *E. brevidens* (j = 1 denotes summer, j = 2 denotes winter).



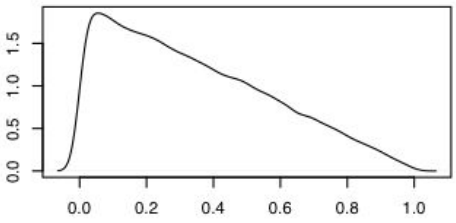
S



Pc_l

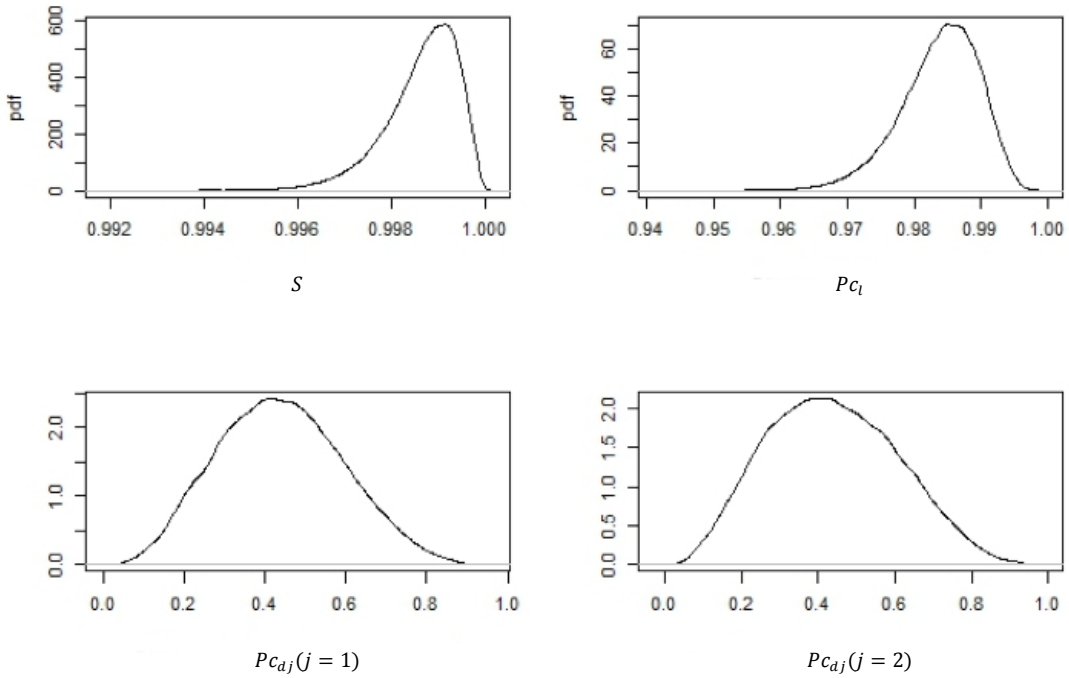


$Pc_{dj}(j = 1)$



$Pc_{dj}(j = 2)$

Model 2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1.00
Pc_t
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2.0
0.0
0.2
0.4
0.6
0.8
1.0
$Pc_{dj}(j=1)$
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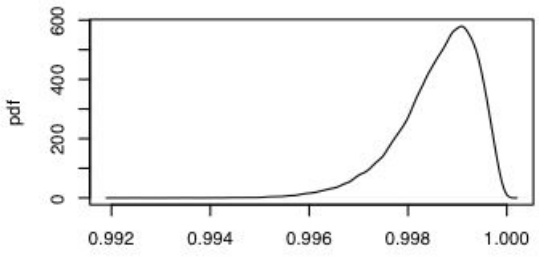
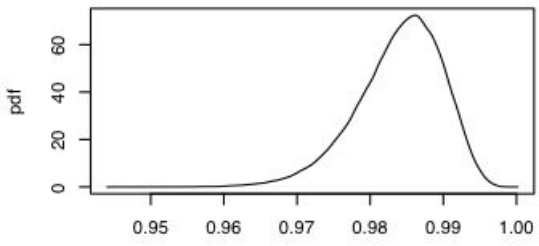
0.5
1.0
1.5
2.0
0.0
0.2
0.4
0.6
0.8
1.0
$Pc_{dj}(j=2)$

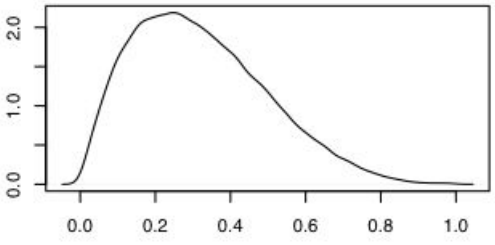
Model 2-1



S

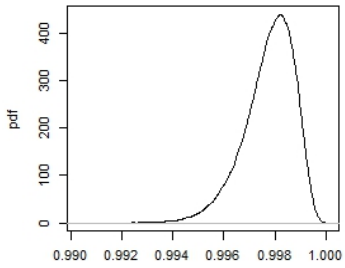


Pc_l

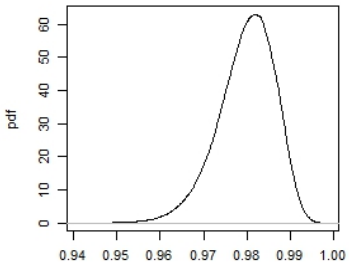


Pc_d

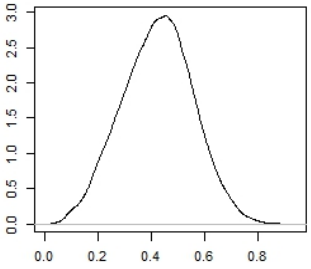
Model 3



S

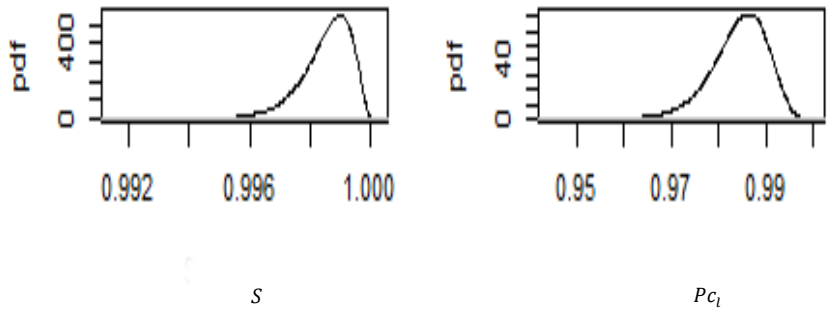


Pc_l



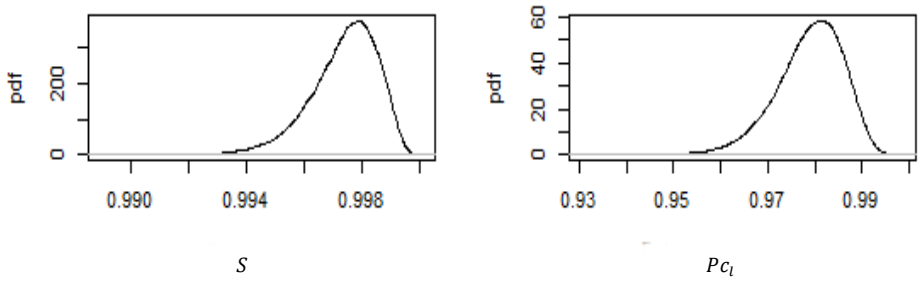
Pc_d

Model 3-1



S Pc_l

Model 4



S Pc_l

Model 4-1

Appendix C-1. Posterior density function of parameters in the other 6 models. S and Pc_l represent the monthly survival rate and probabilities of recapture rates for live *E. brevidens*, Pc_{dj} represents the probability of recapture rate for the dead mussel (j = 1 denotes summer, j = 2 denotes winter).

CHAPTER 4-2: Modeling of periodic growth and growth cessations in the federally endangered freshwater mussel Cumberlandian combshell (*Epioblasmabrevidens*) using a hierarchical Bayesian approach

Abstract

Two-year-old species of the endangered Cumberlandian combshell (*Epioblasma brevidens*) were produced in a laboratory and released to the Powell River in 2009 to augment a declining population. A mark-recapture monitoring approach using Passive Integrated Transponder (PIT) tags was used to assess survival and growth of released mussels. Hierarchical Bayesian growth models incorporating individual growth variations, periodic growth and growth cessations, along with multiple release occasions were developed and compared to the classic von Bertalanffy growth model. Our results showed that the hierarchical model that incorporated individual growth variation gave the best estimates of model parameters, yielding the lowest deviance information criterion value. Mussels exhibited different growth increments at various rates of (K), including 0.015, 0.026, 0.110 and 0.050 ($year^{-1}$), corresponding to the duration of laboratory culture, ages 2, 3 and 4 years old, respectively; and a growth cessation (GC) for 5.98 months. The other parameters of asymptotic length L_∞ and age at the length of zero (t_0) were 51.36 mm and -0.648 months, respectively. The flexible structure of Bayesian hierarchical models allowed us to examine growth characteristics of *E. brevidens* in a changing environment to better understand details of its growth and lifespan, and thus, provide useful data for conservation management. Additionally, the methodological implication of this model is that its construction, use and modification can be extended to other growth models and used under various environmental circumstances.

Keywords: Freshwater mussel, Cumberlandian combshell (*Epioblasma brevidens*), growth rate, asymptotic length (L_∞), growth cessation, periodic growth, von Bertalanffy growth model, Bayesian hierarchical model.

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1. Introduction

The freshwater mussel fauna (Unionoida) of North America contains the highest diversity in the world with 281 species and 16 subspecies (Graf 2013, Neves 2008, Williams et al. 1993). However, it is the most imperiled group of freshwater animals in North America (Ricciardi and Rasmussen 1999, Haag 2012). At least 29 species are considered to have gone extinct over the last 100 years (Haag and Williams 2014), and 75 species are listed as federally endangered, 13 species are threatened (Williams et al. In press). To reduce the risk of species extinction, various conservation management strategies have been implemented over the last 30 years, including reintroduction and augmentation of populations with hatchery propagated mussels (National Native Mussel Conservation Committee, 1998; Hua et al. 2013) and translocation of adults to sites and populations in need of restoration (Cope and Waller 1995; Hamilton et al. 1997).

The Powell River is located in southwestern Virginia and northeastern Tennessee and is part of the upper Tennessee River watershed, supporting a unique assemblage of >30 extant mussel species, 13 of which are listed as endangered species on the United States Endangered Species Act (ESA) of 1973. However, quantitative mussel surveys conducted in the Powell River from 1979 to 2004 document a 63% decline mean mussel density (mussels/m^2) (Ahlstedt et al. 2005). A more recent quantitative survey conducted from 2008 to 2010 revealed that mussel populations continue to decline in the river, including the Cumberlandian combshell (*Epioblasma brevidens*) (Johnson 2011). This species is listed as endangered under the ESA and also is considered globally critically endangered on the International Union for Conservation of Nature (IUCN) Red List. The recovery plan for *E. brevidens* recommends reintroduction of propagated mussels into previously occupied habitats (USFWS 2004). Hence, an effective approach for monitoring populations of this and other reintroduced mussel species is essential to evaluate population viability, life history traits, habitat suitability and success of conservation efforts.

Body-size, growth, and survival are important life history traits that have been

monitored and analyzed by fisheries scientists for decades (Hilborn and Walters 1992; Haddon 2001). In fisheries, the most commonly used model to describe fish growth was developed by von Bertalanffy (1938), which has been applied to analysis of growth in freshwater mussels (Neves and Moyer 1988; Hastie et al. 2000; Miguel et al. 2004; Jiao et al. 2008; Haag and Rypel 2011; Jones and Neves 2011). The von Bertalanffy growth model (VBGM) is expressed mathematically as a three-parameter equation $L_t = L_\infty\left(1 - e^{-k[t-t_0]}\right)$, where theoretical maximum body length is L_∞, growth rate coefficient is *K*, and hypothetical age at zero in length is t_0.The parameters in the equation are typically analyzed by combining data for all individuals in a population. Hence, the standard VBGM model assumes homogeneity of individuals in a population dynamic assessment, which can introduce bias into model construction and parameter estimation (Pledger et al. 2003). A result of introducing bias, is that estimates of model parameters are poorly characterized (Cam et al. 2002; Conner and White 1999). For example, growth rates of individuals can vary substantially with temperature, food availability, population density, and habitat location (Krohn et al., 1997; Swain et al., 2003; Kimura, 2008; Jiao et al., 2010). Hence, growth models that incorporate individual variations are considered more realistic and appropriate to describe the growth pattern of a population (James 1991; Smith et al. 1997; Alós et al. 2010; Tang et al 2014). These models include residual variance into the modeling process, so growth heterogeneity of individuals can be assessed to better understand a species population biology (Pledger et al. 2003). Bayesian approach using Markov Chain Monte Carlo (MCMC) algorithms is considered the most efficient mathematical framework to incorporate and reduce model uncertainty (Gimenez 2008; Wade 2000).

Growth of aquatic animals often reveals strong seasonal oscillations, mainly due to fluctuations of temperature, food supply, (Shul'man 1974), body reserves (Bacon et al 2005), and even social behavior (Alanara et al. 2001). Hence, the VBGM has been discussed and modified to allow for seasonal oscillations during specific growth periods. The seasonal growth model was originally proposed by Ursin (1963) and has been

modified by including a sine-wave function into the traditional VBGM to allow for seasonal oscillations (Pitcher & MacDonald 1973; Cloern and Nichols 1978; Pauly and Gaschultz 1979; Somers 1988; Hoenig and Hanumara 1990; Allision 1994). Freshwater mussels experience growth cessation (GC) during winter months in most North America geographic locations (Downing & Downing, 1993; Downing et al 1992; Anthony et al. 2001). However, earlier versions of the seasonal oscillating models may not be well suited for characterizing mussel growth because they could not accommodate growth cessation (Pauly et al. 1992). Further, other challenging issue in freshwater mussel conservation management is how to evaluate growth rates of populations exposed to a suite of environmental stressors, such as pollution and climate change. Because changes in environmental conditions can cause variations in growth rates of successive cohorts or stocks of same species (Rizvi et al. 2012). Overall, estimates of growth parameters (K and L_∞), have not incorporated individual variations, seasonal variations, and growth cessations for freshwater mussels, primarily due to absence of reliable data and advanced demographic modeling techniques.

Effective conservation and restoration management of endangered mussels will require a thorough understanding of species life history traits and population dynamics. Thus, the purpose of our study was to develop a new model incorporating individual variations and seasonal variations with growth cessation to model the growth of *E. brevidens*. We also demonstrate applications of stochastic analyses with modifiable models through a Bayesian approach to meet the various realistic environmental circumstances.

2. Methods and Materials

Laboratory propagation and tagging of mussels

Juveniles of *E. brevidens* were propagated and cultured at the Freshwater Mollusk Conservation Center (FMCC), Department of Fish and Wildlife Conservation, Virginia Polytechnic Institute and State University, Blacksburg, VA. All juveniles were

from one propagation effort in 2007. Propagated juveniles were reared in two culture systems utilizing pond water, including an open-water recirculating system and a closed recirculating aquaculture system. In winter, a mixed algal diet (Shellfish Diet 1800 and Nanno 3600 purchased from Reed Mariculture, Inc.) was used as supplemental food for juveniles fed at a mean concentration of 35,000 cells ml^{-1}. Newly transformed juveniles were cultured in tanks with fine sediment (< 200 um) at a depth of 1-2 mm, and were moved to a limestone sand substrate (2-5 mm) for grow-out when juvenile mussels reached a length of 3 mm. Juvenile size (length) was measured and counted on 12 sampling occasions during the 2-year culture period. Survival rates were calculated as percent of the number collected in the previous sampling period.

Juvenile mussels were tagged using Bulk PIT tags (TX1411SST, Biomark, Boise, Idaho, USA) to ensure the high recapture rates of each individual. FPN glue-on shellfish tags (Hallprint, Hindmarsh, Australia) were also tagged on the other side of valve. Double tagging on both valves of mussel secured the identification information during monitoring process. Tagged juvenile mussels were released at Brooks Bridge (36°32'05.5644"N, 83°26'30.3432"W), Powell River, Tennessee, USA on multiple occasions on 1 July 2009, 26 August 2009, October 7 2009, 25 June 2010, and 11 October 2010, respectively (Figure 1). They were then relocated using the PIT tag detector for the measurements of length and records of age, then returned to the release site. A total of 8 mark-recapture sampling events were implemented from July 1 2009 until October 11 2011 (Table 1) during this study.

Water quality measurement

Water temperature was determined using a YSI DO meter (Model: 55/12 FT, Yellow Springs, Ohio). Content of organic matter was measured through ash free dry weight (AFDW) following Standard Methods for the Examination of Water and Wastewater (Method 10300 C, D. 20th Ed. 1998. American Public Health Association, Washington, D.C.).

Models and equations

Two new growth models were developed and the VBGM was applied in this study with the following assumptions:

- Labeled juvenile mussels retained their tags throughout the study, and PIT tags were detectable without negative influence to their survival.
- Variation of mussel length followed a certain random distribution.
- Sampling protocol and study area were constant.

Formulation for Model 1, the VBGM was applied as:

$$L_t = L_\infty\left(1 - e^{-k[t-t_0]}\right) \quad (1)$$

where L_t represents the length of mussel at time *t* (month), L_∞ (asymptotic length) denotes the theoretical average maximum body length, *K* represents growth rate coefficient that defines growth rate toward the maximum, and t_0 represents the hypothetical age when mussels were at zero length.

Applying the Bayesian approach to Model 1, a traditional and nonhierarchical VBGM was constructed as:

$$E(L_j) = L_\infty\left(1 - e^{-K[t_j-t_0]}\right)$$

where *j* represents the j^{th} capture occasions (or j^{th} sampling measurement, j = 0, 1, 2…8, j = 0 denotes the time of mussel release and the 0th capture); $E(L_j)$ denotes the expected mussel length at the j^{th} capture occasion; t_j represents the mussel age (months) at the j^{th} occasion; L_∞, K and t_0 are corresponding parameters as those described above, and their priors were $U(45, 100)$, $U(0, 1)$ and $U(-2, 2)$ separately. L_j was assumed to follow a normal distribution $N(E(L_j), \sigma_L^2)$, and prior of σ_L^2 was assumed to be $U(0, 400)$.

Since released mussels revealed uneven or abnormal growth with a potential non-growth season and different annual growth rates during the sampling observations (Figure 1), two new models were developed based on the above preliminary diagnostics to explore the growth of *E. brevidens* for the purposes of propagation and conservation. The newly developed models were then compared with the commonly used VBGM for

model selection.

Formulation for Model 2 included two scenarios: (1) Juvenile growth during the culture period in laboratory was analyzed using the VBGM to evaluate the parameters of growth rate, t_0 and L_∞, and (2) Model 2 was reconstructed from the VBGM based on the time-based length increment of the tagged mussels (Fabens, 1965; Jiao et al., 2010) after they were released to the river:

$L_t = L_\infty\left(1 - e^{-k[t-t_0]}\right)$ before mussel release, (2-1)

$E(L_{t+\Delta t}) = L_t + (L_\infty - L_t)(1 - e^{-k\Delta t})$ after mussel release, (2-2)

where L_t represents mussel length at the time *t* (month); $L_{t+\Delta t}$ denotes mussel length at the time $t + \Delta t$; L_∞ (asymptotic length) denotes the theoretical average of maximum body length; *K* represents growth rate coefficient toward the maximum length; Δt is the duration between $t + \Delta t$ and *t*. The other parameters are the same as (1).

Applying the Bayesian approach, Model 2-2 incorporated variations in annual growth rates and considered the non-growth seasons, while Model 2-1 did not because all the mussels were cultured in the lab environment during this time period. Model 2, the Bayesian non-hierarchical model was constructed as:

$E(L_j) = L_\infty\left(1 - e^{-K_1[t_j - t_0]}\right)$ if $t_j <$ the age at release

$E(L_j) = L_{j-1} + (L_\infty - L_{j-1})(1 - e^{-K_g(\Delta t_{j-1} - GC)})$ if $\Delta t_{j-1} > GC$ after November 1st;

$E(L_j) = L_{j-1}$ if $\Delta t_{j-1} < GC$ after November 1st

where j represents the j^{th} capture occasions; $E(L_j)$ represents the expected mussel length at the j^{th} capture occasion; L_j denotes the mussel length at the j^{th} occassion, and follows a normal distribution with mean $E(L_j)$ and variance σ_L^2; K_1 denotes the growth rate of juvenile mussels cultured in laboratory. K_g denotes the growth rates of released mussels at various stages ($g = 2, 3, 4$ represents the time in 2009, 2010 and 2011 along with the ages of mussels, 2, 3 and 4 years, respectively); Δt_{j-1} denotes the duration (months) between the recapture occasion of $(j-1)^{th}$ and j^{th}; *GC* is the growth cessation (month), which counts from November 1st based on empirical growth data at the release site. Mussels were released on multiple occasions;

thence this model was constructed on the combinations of separated analyses of each release occasion to minimize uncertainties. Priors of L_∞, K , t_0 and σ_L^2 were assumed to be the same as in Model 1, and prior of GC here was U (2, 6).

Model 3 was generated from Model 2, additionally incorporating individual variations of released mussels since each mussel had an identical tag number. Model 3, the Bayesian hierarchical model was constructed as:

$E(L_j) = L_\infty\left(1 - e^{-K_1[t_j - t_0]}\right)$ if $t_j <$ the age at release

$E(L_{i,j}) = L_{i,j-1} + (L_{\infty,i} - L_{i,j-1})(1 - e^{-K_{i,g}(\Delta t_{j-1} - NGT)})$ if $\Delta t_{j-1} > GC$ after November 1st;

$E(L_{i,j}) = L_{i,j-1}$ if $\Delta t_{j-1} < GC$ after November 1st (3)

$L_{\infty,i} \sim N(\bar{L}_\infty, \sigma^2_{L_\infty})I(45, 100)$

$\bar{L}_\infty \sim U(45, 90)$

$\sigma^2_{L_\infty} \sim U(0, 400)$

$K_{i,g} \sim N(\bar{K}_g, \sigma^2_{K_g})I(0, 1)$

$\bar{K}_g \sim U(0.001, 0.3)$

$\sigma^2_{K_g} \sim U(0.0001, 0.1)$

$GC_i \sim N(T, \sigma_t^2)I(2, 6)$

$T \sim U(2, 6)$

$\sigma_t^2 \sim U(0.0001, 5)$

where i represents the i^{th}individual; j^{th}represents capture occasions; $E(L_{i,j})$ denotes the expected length of i^{th} mussel at j^{th} occasions; K_1denotes the growth rate of juvenile mussels cultured in the laboratory; $L_{\infty,i}$ and $K_{i,g}$ are corresponding parameters of mussel length and growth rate coefficient of individual i. They are assumed following normal distributions, described as $N(\bar{L}_\infty, \sigma^2_{L_\infty})$ and $(\bar{K}_g, \sigma^2_{K_g})$. In which, $\bar{L}_{\infty,i}$and $\bar{K}_g$ (mean values of each parameters) are assumed following uniform distributions with the variance of $\sigma^2_{L_\infty}$ and $\sigma^2_{K_g}$, respectively. I denotes the limited boundary of the parameter distribution; the other parameters describes same as those in Model 2. Priors of L_∞, K, t_0 and σ_L^2 were assumed to be the same as in Models 1 and 2; priors for hyper-parameters were listed in

equation 3 to help clarification of the hierarchical model.

The posterior distribution of the parameters were estimated using a Bayesian approach with software WinBUGS (version 14. MRC Biostatistics Unit and Imperial College School of Medicine; Spiegelhalter et al. 1996) and MATLAB (version 12b, The MathWorks, Inc.). Three Markov chains were used in the analysis and Gelman Rubin statistics were used to determine the convergence of the posterior distribution (Spiegelhalter et al. 2004; Jiao et al. 2008, 2009). The burn-in iteration and thinning interval were determined based on the convergence criteria following Jiao et al. (2008). The Bayesian inference was generated from samples taken as random draws from the posterior distribution after the three chains reached convergence.

Model selection

Deviance information criterion (DIC) was applied to the Bayesian model selection using MCMC simulation to determine posterior distributions of each model (Spiegelhalter et al. 2002). The DIC was used in this study model selection for the Bayesian models, and formulated as:

$$DIC = 2\overline{D}(\theta) - \widehat{D}(\theta) \; or \; \overline{D}(\theta) + P_D \qquad (5)$$

$$P_D = \overline{D}(\theta) - \widehat{D}(\theta)$$

where D is deviance used to measure predicted goodness-of-fit for all 3 models, P_{D} is the effective number of parameters in the Bayesian model, $\overline{D}$ is the posterior mean of the deviance, and $\widehat{D}$ is the deviance of the posterior mean. Estimated value is denoted by E, y represents all stochastic nodes given the data, and θ represents the stochastic nodes upon which the distribution of y depends, when collapsing over all logical relationships. Models were evaluated using rules of thumb (Burnham & Anderson1998; Spiegelhalter et al. 2002), where a model with the smallest DIC value was defined as the best model, and models within 1-2 DIC units of the 'best' model were also considered the best model, and models within 3-7 DIC units of the 'best' model, were considered deficiently performed.

3. Results

Juveniles of *E. brevidens* were successfully propagated and cultured to tagable sizes for release to the Powell River in accordance with the species recovery plan. A total of 8,310 juvenile mussels was produced and cultured at FMCC. As expected, juveniles grew faster in summer and slower in winter during the culture period at the laboratory, and experienced high mortality from day 1 to day 60, but survival rates stabilized after 2 months (Figure 2). Juveniles had a survival rate of 10% during the first month, 27% during the second month, and 100% thereafter at each sampling occasion.

The increments of individual mussel length (Figure 3-1) and their distribution (Figure 3-2) of the adjacent sampling interval at each observation occasion exhibited fluctuation in the growth curve, with an annual growth cessation or even negative growth starting from about November 1 to May 1, after they were released into the Powell River.

Results from Model 1 showed that the growth rate K, L_∞ and t_0 of released mussels were 0.009 month^{-1}, 99.18 mm and 1.9 months, respectively (Table 2). Model 2 gave four different growth rates of 0.015, 0.031, 0.105 and 0.050 (month^{-1}) respectively, which represented variations in mussel growth at four different stages. Obviously, mussels had the most rapid growth at age 3, followed by age 4 and age 2. Juvenile mussels grew slowly during their culture time in the laboratory and during the non-growth winter season, which lasted about 6 months (5.975 months). Further, L_∞ and t_0 for *E. brevidens* estimated in Model 2 were 53.31 mm and - 0.649, respectively (Table 1). Mean growth rates of mussels at the four stages estimated from the hierarchical Model were 0.015 0.026, 0.110 and 0.050 (month^{-1}), respectively. The parameters of L_∞, GC and t_0 agreed with results from Model 2 as well, with 51.36 mm, 5.98 months and -0.648 months, respectively (Table 1). Model selection can be compared by both the DIC values and the plots of model fit (Table 1 and Figure 4-6). Model 1 (Figure 4) had the lowest fit compared to Model 2 (Figure 5) and Model 3 (Figure 6), indicating that the classic VBGM is less suitable to predicate mussel growth without incorporating non-growth seasons. In this study, juveniles of *E. brevidens* were released on multiple occasions;

hence, cohorts released on the same occasion were grouped together for analyses, indicated by the multiple lines presented in Figure 5 and 6. Juveniles cultured in the laboratory were considered as one cohort, denoted as the blue line in Model 2 (Figure 5). The other parallel lines showed estimated average length of the mussel groups released during multiple time periods throughout the sampling occasions. Model 3 is the hierarchical model that incorporated individual variations. The multiple lines during the juvenile culture period in laboratory indicated variations among the release groups in this model (Figure 6). However, individual variations in length among mussels cultured in the laboratory or released to the river as cohort were not significant.

The results of our study indicated that DIC values of the 3 models were substantially different, with Model 3 having the lowest value of 1683.0, Model 1 the largest DIC value of 3292.8, followed by Model 2 with a DIC value of 2546.0. The difference of DIC units of Models 1 and 2 to Model 3 greatly exceeded 7 DIC units. Hence, Model 3 outperformed the other models and was determined to be the best model. Hierarchical Model 3 that considered the non-growth seasons and incorporated individual variations optimally characterized growth of juvenile *E. brevidens* in the laboratory culture environment and later when released to the river.

Water temperature and organic matter (represented by AFDW) were determined in this study from October 28, 2008 to October 28, 2009 (Table 3). The water temperature dramatically decreased at the end of October and rose at the end of April at the release site. Simultaneously, the AFDW declined about 3 fold during the low temperature period. These two parameters exhibited a strong positive relationship (Pearson's $r = 0.72$). The duration of low temperature and AFDW during a year cycle was in accordance with the period of mussel growth cessations.

4. Discussion

The early life of juvenile mussels aged from day one to 2 months is a critical stage in the life cycle. The mussel culture methods used at FMCC greatly improved survival

and growth of this endangered species (Hua et al. 2013). Rearing larger-sized (>15 mm) juvenile mussels for release is an important first step to restore imperiled populations. Mark-recapture techniques using PIT tags ensured the success of evaluating mussel restoration efforts (Hua et al. in press). Hence, our study from juvenile propagation, culture and release to monitoring process provided a demonstration of conservation and restoration of endangered mussel species.

The quantitative analyses directly influenced results and management decisions. Although the VBGM is perhaps the most commonly used growth model to characterize freshwater mussel length-at-age data (Hastie et al. 2000), this model needs to be evaluated on a case-by-case basis to avoid potential biases and erroneous parameter estimation. The traditional VBGM was applied in our study and resulted in a poor goodness-of-fit. Most of the observations (blue dots) were obviously below the green dotted line (plot of VBGM growth curve) in Figure 4 showing the overestimation. The estimated mean value of L_∞ (99.18 mm) in Model 1 exceeded about 46 mm those of Model 2 and Model 3. Miguel et al. (2004) found that when the VBGM was applied to the youngest age classes (< 6 yr) of freshwater mussels, it underestimated the value of L_∞. Poor fit of a model could be due to applying inappropriate models that are incapable of describing growth variation in individual animals.

In general, juvenile mussels grow rapidly during early life stages, peaking in growth increment, then decreasing in growth increment until achieving a near-constant size. Mussels also exhibit a seasonal growth pattern, with cessation caused by ambient environmental changes of low temperature, food restriction, minimal flow, or inadequate energy storage for reproduction (Shui'man 1974, Adam 1990; Pauly et al. 1992; Downing & Downing 1993; Miguel et al. 2004). Our results indicated that the duration of growth cessation for *E. brevidens* in the Powell River lasted for approximately 6 mo, beginning in late October to early November when water temperature decreased below 15 ℃, and ending in late April to early May when water temperature increased above 15 ℃. During the winter, growth cessation occurred during the laboratory culture period and

after mussels were released to the Powell River. Loss in shell length was detected during their GC periods in the river, although the reduced length of individuals was inconsequential. This result corroborates a previous study reporting negative growth due to resorption in winter (Downing & Downing 1993). Regardless of GC or negative growth of mussels, we found that the duration of mussel GC correlated to the fluctuation of water temperature and food availability. To determine food availability, the ash free dry weight (AFDW) of organic matter in sampled river water, which includes algae, bacteria, zooplanktons and other organic debris as suitable food (Silverman et al., 1997; Hua et al. 2013). The amount of AFDW declined to a low level from the end of October until the following May while water temperature declined to 10-13℃ at the end of October and remained lower until early April, then rose to 20℃ in late April. Environmental changes directly influenced mussel growth, affecting GC or growth loss. However, Model 1 applied with the classic VBGM was not capable of simulating these seasonal growth patterns.

To determine seasonal growth patterns, a modified VBGM were applied by adding a sine wave with two more parameters to allow for seasonal oscillation (Picher and MacDonald 1973):$L_t = L_\infty(1 - e^{-k_s}) + \varepsilon$

$k_s = C \sin[\frac{2\pi(t-s)}{12}] + k\ (t - t_0)$

where k_s is the function that describes seasonal perturbation through a sine wave, C donates the magnitude of the oscillations of sine wave, and s represents the starting point in time relative to t_0. The other parameters, L_t, L_∞, t_0 and ε are defined as in equation (1). The value 12 equals sampling occasions in months.

Although this seasonal growth model was verified and applied to our data, it was incapable of accommodating growth during growth cessation as well as in previous study (Pauly et al. 1992). To solve this problem, Pauly et al. (1992) developed a better seasonal model by incorporating a no-growth time. We initially applied Pauly's improved mathematical function in our study, but it failed to simulate the growth pattern, during the laboratory culture stage and post-release stage with multiple releases. Besides the above

situation, the no-growth time in our study was much longer than the allowance of Pauly's model using the sine wave. Moreover, mussel growth had obvious uneven oscillations that indicated different growth rates at the various stages, which required different growth rates to distinguish growth characteristics for each duration. Hence, two new models (2 and 3) were developed to incorporate GC with a starting point on November 1^{st,} associated with an up bound of 6 months. According to the empirical data, we discarded the sine wave and generated 4 growth rates to fit the uneven oscillation growth pattern of *E. brevidens*. The estimated asymptotic length from Model 2 and Model 3 were 53.31mm and 51.36 mm, respectively, much less the value (99.18 mm) from Model 1. To investigate the reality of these results, we surveyed various museum collections to document the maximum length of *E. brevidens* collected from the Powell River, and found that the largest specimen was 71.9 mm (female) in the North Carolina State Museum of Natural Sciences, 62.4 mm (female) and 64.5 mm (male) in McClung Museum of Natural History and Culture, University of Tennessee, and 75 mm (female) in Museum of Biological Diversity at The Ohio State University. Parmalee and Bogan (1998) reported the average length of mature specimens of *E. brevidens* to be around 50 mm. Our results from Model 2 and 3 were seemingly plausible when compared to the museum records, because L_∞ in this study was decided by laboratory-produced juveniles that experienced environmental changes in the early life stage.

It is know that growth parameters differ from species to species, but they also vary among individuals, and among stocks within the same species due to environmental responses and genetic differences. Our results indicated that growth rates differed at various stages, with the lowest K_1 in the culture period and highest $\overline{K}_3$ at age 3. Newly metamorphosed juvenile mussels experience environmental change after they excysted from host fish that resulted in high mortality and slow growth. The second challenge to released juveniles was adaptation to the ambient environment and available food sources. Hence growth rate (K_2) did not exhibit rapid growth until acclimation and age of 3 yr. Thereafter, mussels became sexually mature and gravid females were observed during the

recapture events at age 4, which was 1 yr earlier than predicted (Jones et al. 2012). The energy investment for reproduction influenced growth in length, resulting a smaller growth rate K_4 at this stage. The other life history trait t_0, estimated from Models 2 and 3, were negative 0.649 and 0.648 month that likely indicated the duration glochidial metamorphosis from parasitic glochidium (larvae of mussel) to juvenile mussel. These t_0 values agreed with empirical data; namely, that glochidia of *E. brevidens* encysted on host fish for 15-20 days before becoming free-living juveniles. However, estimated t_0 value (1.94 mo) from Model 1 was not reasonable. Hence, the life history traits of *E. brevidens* in the Powell River at different stages are best interpreted through Model 2 and Model 3.

To better understand the characteristics of population biology and ecology, individual variation is a important component to be considered in quantitative analyses (Pledger et al. 2003, Jiao et al., 2009, 2010), though it is challenging and requires advanced computing techniques. We developed a hierarchical model (Model 3) with multi-level priors to incorporate individual variation using a Bayesian approach, along with MCMC algorithms. Although the estimated parameters of life history traits of *E. brevidens* were very close to those of Model 2, Model 3 significantly out-performed Model 2 according to the rules of thumb (Spiegelhalter et al. 2002), the criterion of model selection. It was determined to be the best model due to its smallest DIC value (1683.0). Additionally, our results also showed small variances of L_∞, and K values in all models were probably due to the similarity of the same cohorts.

In a previous study, the growth parameters of *E. brevidens* from the Clinch River, Tennessee were estimated using classic VBGM, based on annuli for age at length (Jones and Neves 2011). However, annular growth marks used for delineating annual growth increments to estimate an age has been debated (Downing and Downing, 1993, Strayer et al., 2004). Excluding measurement error and shell erosion, Downing and Downing (1993) reported negative growth in freshwater mussels and indicated that growth rates based on annuli could be inaccurate since growth annuli were not reliable. Indeed, it is

difficult to obtain lengths at defined ages of specimens from field records; hence, parameter estimation through use of annuli and corresponding lengths is still acceptable. We were able to produce juvenile mussels and release them at a precise age, thus resolving the problem of age determination. Information of juvenile growth at these early life stages was invaluable. Our individual growth model allowed for individual variation, duration of growth cessation, and periodic growth rates (K), along with multiple release events (different start points) to provide a flexible fitness to adapted changes to environment and intrinsic factors. For example, it can be applied to growth patterns without growth cessation by setting the GC = 0. The improvement of mark-recapture methodology using PIT technology greatly increased recapture rates and effectively reduced the uncertainties in parameter estimation.

It is important to have complete information for all age classes to determine the growth parameters of a species' lifespan (Haag 2009). Therefore with additional study, we can refine the results of our current estimation for this endangered species by incorporating middle and old age classes in the analytical modeling.

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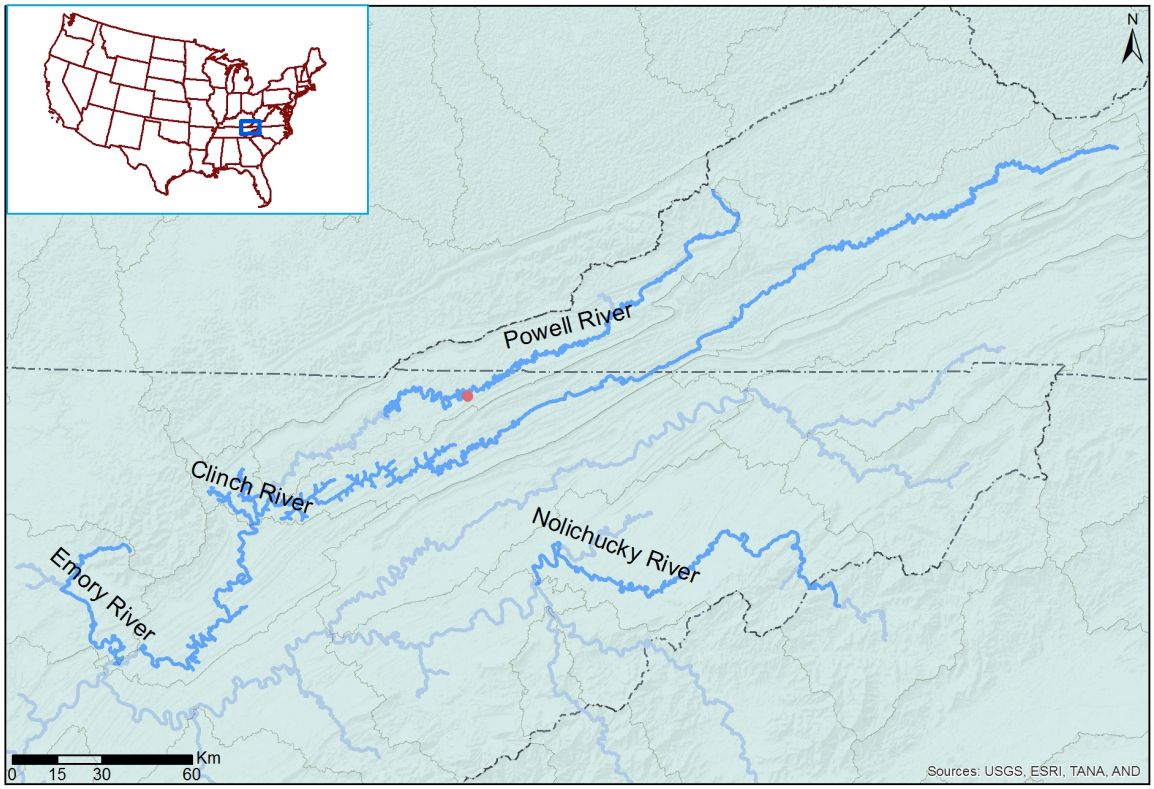

Figure 1. Release of juvenile mussels of *E. brevidens* at Brooks Bridge (36°32'05.5644"N, 83°26'30.3432"W) in the Powell River, Tennessee, USA.

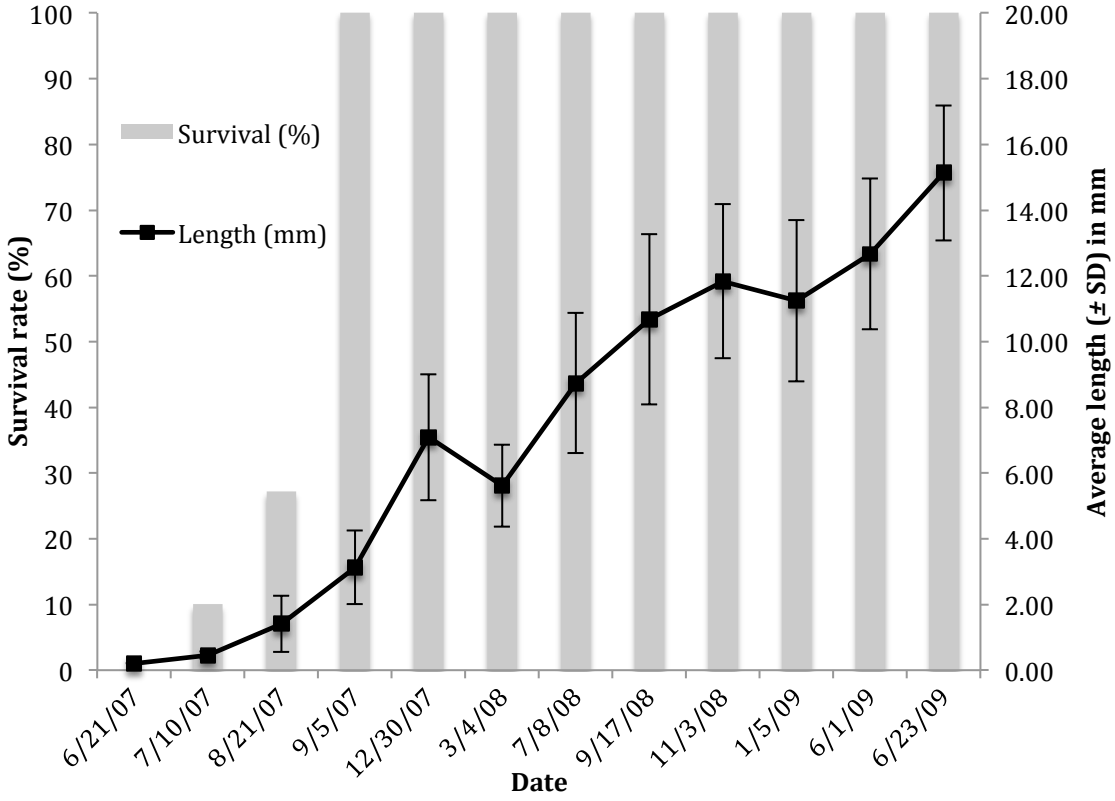

Figure 2. Survival rate and growth of juveniles of E. brevidens from day 1 to tagable size, June 2007 to June 2009.

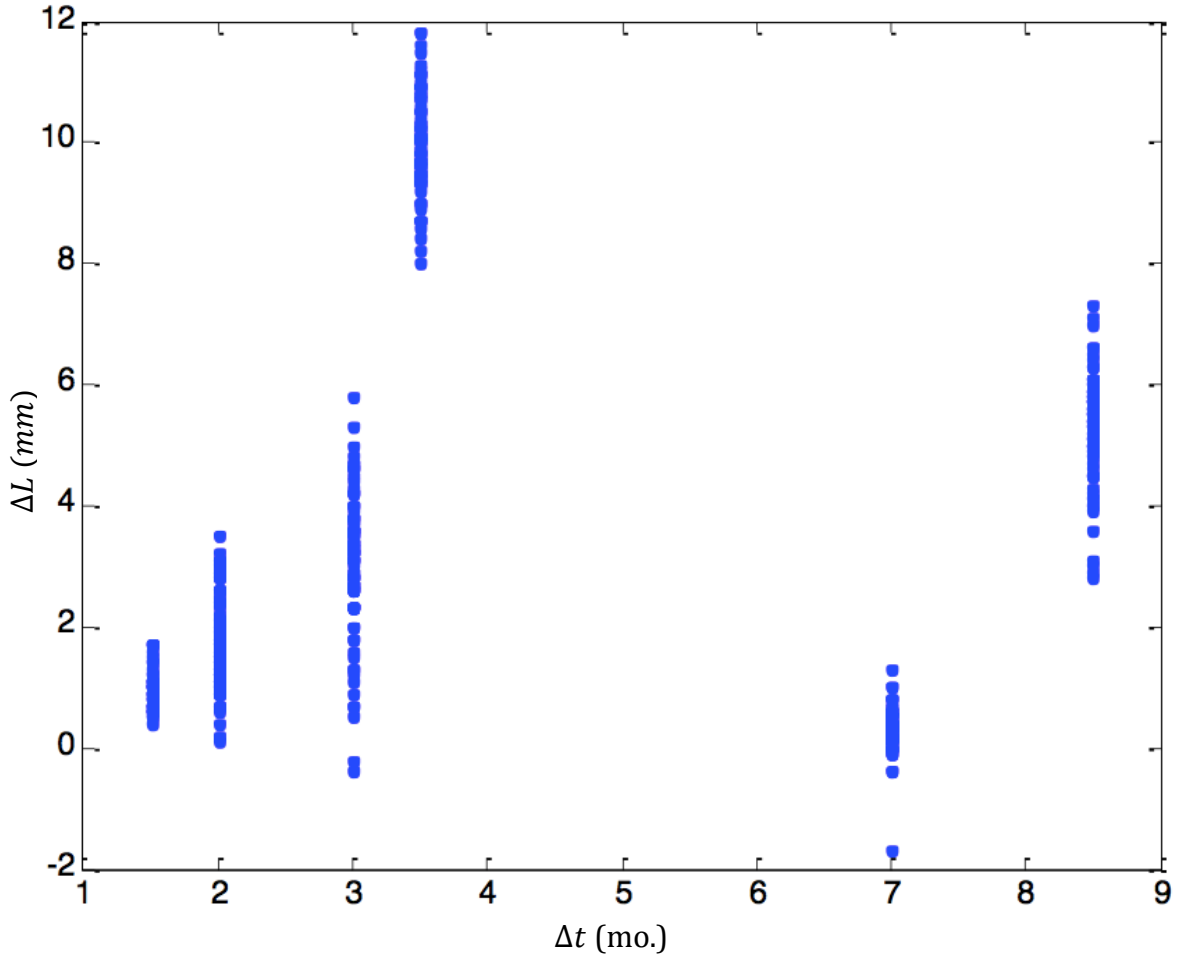

Figure 3-1. Relative growth (incremental length per sample duration $\Delta L/\Delta t$) of individual mussels at each recapture occasion.

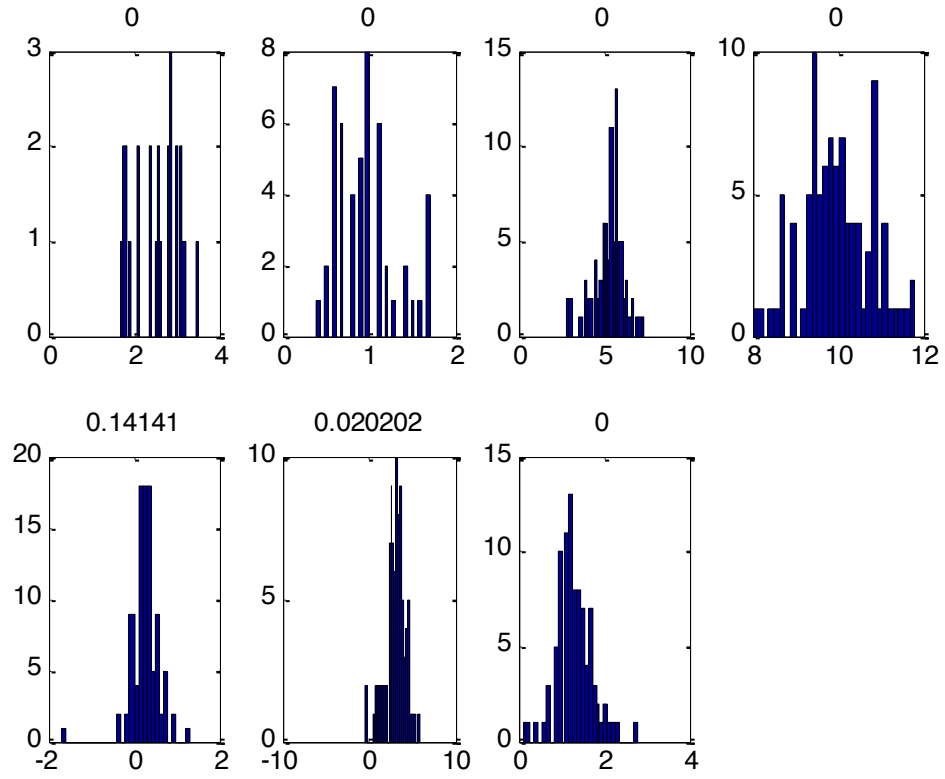

Figure 3-2. Distribution of relative growth of individual mussels at each capture occasion on 8/26/2009, 10/7/2009, 6/25/2010, 10/11/2010, 5/10/2011, 8/17/2011, and 10/12/2011 (from top left to right and bottom left to right). Percentage of negative growth is shown at the top of each histogram.

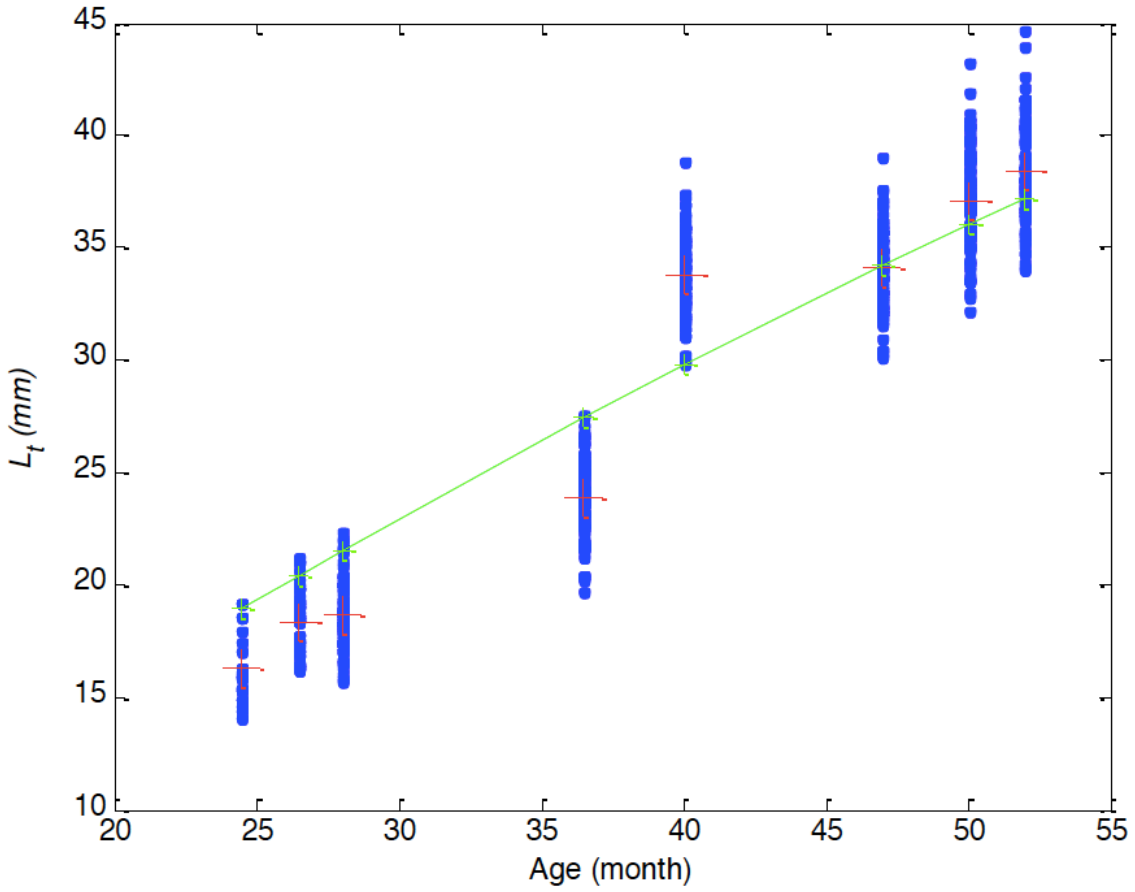

Figure 4: Fits for Model 1 shown as green dotted line with estimated growth in length of released mussels of E. brevidens from 24.5 to 52 mo of age. Blue circles represent observation of each mussel. Red + marker represents mean mussel size at each observation occasion.

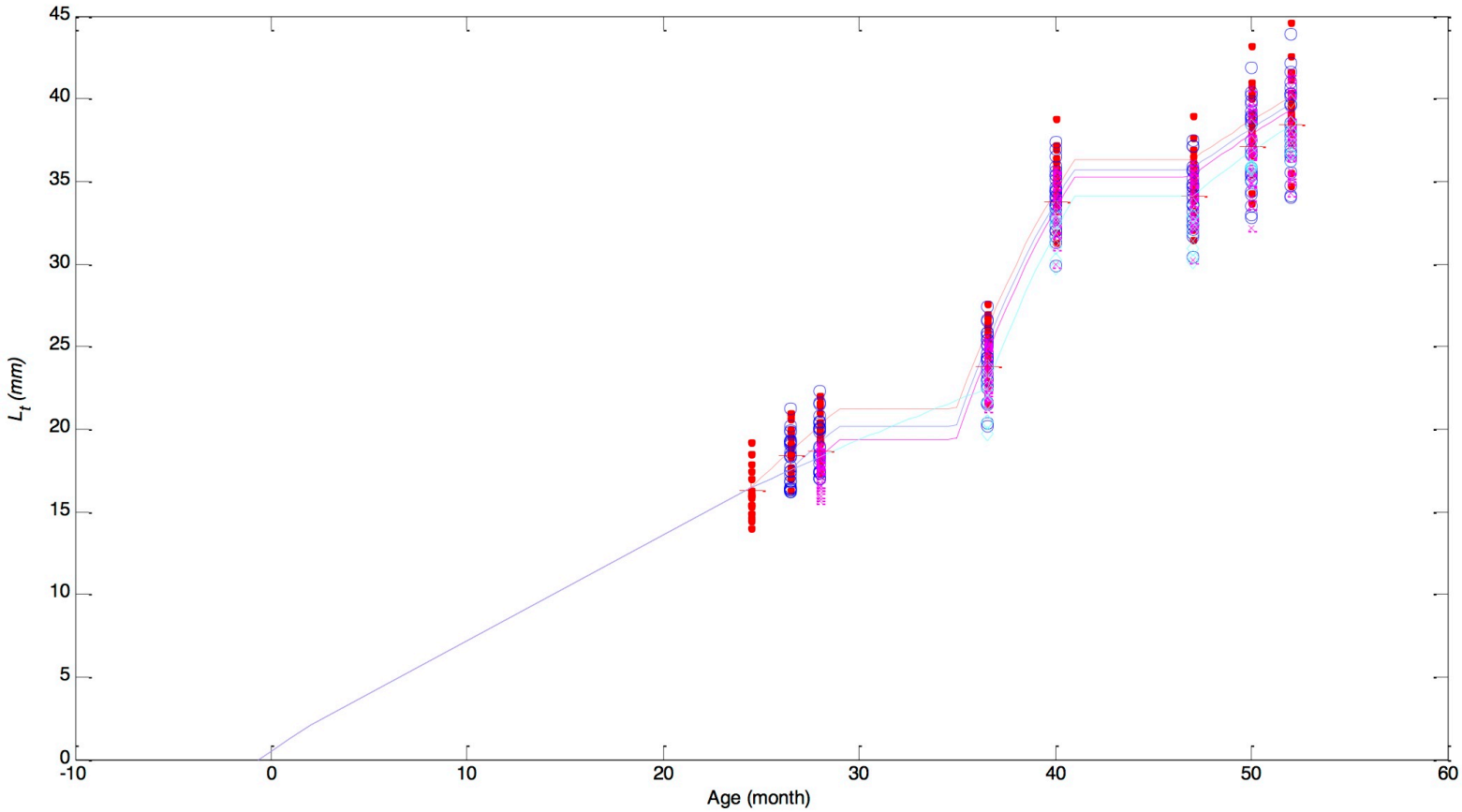

Figure 5. Fits for model 2 with estimated growth in length of juvenile mussels of *E. brevidens* from 0 to 52 mo of age (before and after release). Black line represents mussel growth in the laboratory; red dots (observed mussel sizes) and red line (posterior model fit) represent mussels released in July 2009; blue dots (observed mussel sizes) and blue line represent mussels released in Aug 2009; maroon dots (observed mussel sizes) and line represent mussels released in Oct 2009, and light blue dots (observed mussel sizes) and line represent mussels released in June 2010.

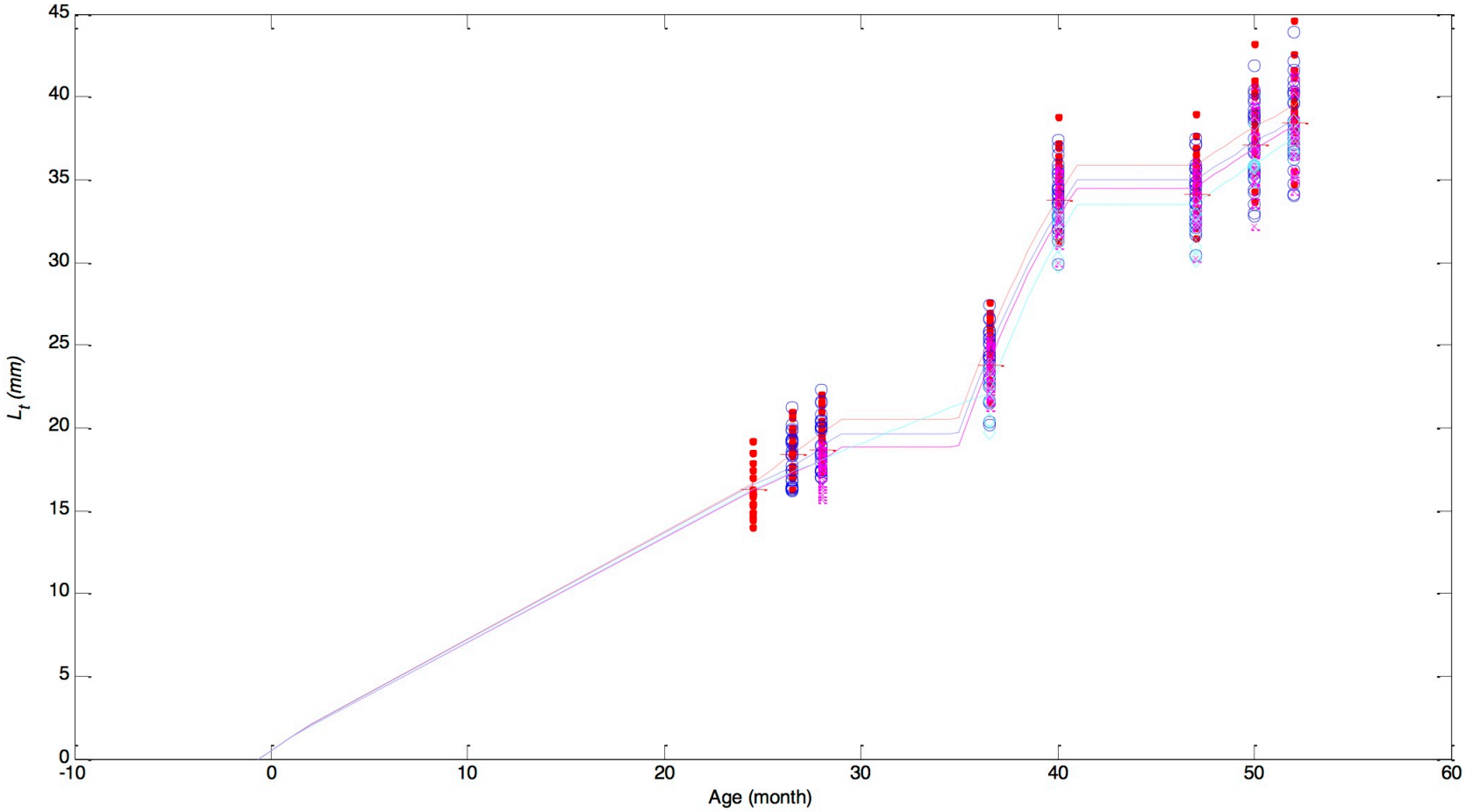

Figure 6. Fits for Model 3 with estimated growth in length of juvenile mussels of *E. brevidens* from 0 to 52 mo of age (before and after release). See Figure 5 for the meaning of markers and lines.

Table 1. Release and recapture of laboratory produced juvenile mussels (*E. brevidens*).

Date	7/1/09	8/26/09	10/7/09	6/25/10	10/11/10	5/10/11	8/17/11	10/12/11
Age (months)	24.5	26.5	28	36.5	40	47	50	52
Number of released mussels	23	28	38	9	1	0	0	0
Number of recaptured mussels		23	50	88	97	96	95	94

Table 2. Estimated parameters of three models and their DIC values (L_∞ denotes the theoretical average maximum body length, *K* represents growth rate coefficient that defines growth rate toward the maximum, and t_0 represents the hypothetical age when mussels were at zero in length. K_g denotes the growth rates of released mussels at various stages (g =1 represents the time in the laboratory; $g = 2, 3, 4$ represents the time in 2009, 2010 and 2011along with the mussel ages at 2, 3 and 4 yr, respectively); denotes the non-growth time (started on November 1). L_∞ and K_g in Model 3 represents the corresponding parameters of mussel length and growth rate coefficient of individuals following normal distributions of mean ($\overline{L}_\infty$) and ($\overline{K}_g$).

Model	DIC	Parameter estimates				
		Parameter	Mean	SD	95% CI	
Model1	3292.8	L_∞ (mm)	99.180	0.806	97.080	99.980
		K(month^{-1})	0.009	1.068E-4	0.009	0.010
		t_0 (month)	1.939	0.061	1.773	1.998
Model2	2546.0	L_∞(mm)	53.310	6.262	45.940	68.380
		K_1(month^{-1})	0.015	0.002	0.010	0.018
		K_2(month^{-1})	0.031	0.005	0.021	0.039
		K_3(month^{-1})	0.105	0.023	0.062	0.142
		K_4(month^{-1})	0.050	0.017	0.022	0.083
		GC (month)	5.975	0.025	5.908	5.999
		t_0(month)	-0.649	0.977	-1.949	1.566
Model3	1683.0	$\overline{L}_\infty$(mm)	51.360	0.993	49.740	53.220
		$\overline{K}_1$(month^{-1})	0.015	5.758E-4	0.014	0.017
		$\overline{K}_2$(month^{-1})	0.026	0.002	0.022	0.029
		$\overline{K}_3$(month^{-1})	0.110	0.005	0.102	0.119
		$\overline{K}_4$(month^{-1})	0.050	0.004	0.044	0.057
		$\overline{GC}$(month)	5.980	0.013	5.950	5.998
		t_0(month)	-0.648	0.961	-1.948	1.525

Table 3. Values for water temperature and organic matter near the release site in Powell River, from October 2008 to October 2009. Organic matter content is indicated by ash free dry weight (AFDW).

Date	Water temperature (℃)	AFDW (μg/ml)
10/28/08	10.00	-
01/22/09	1.20	-
02/24/09	5.40	-
03/25/09	12.70	-
04/29/09	19.90	0.20
05/28/09	21.40	0.73
06/25/09	23.80	0.63
07/17/09	24.00	0.93
08/27/09	21.70	0.95
10/28/09	13.30	0.23

CHAPTER 5: Summary and Management implications

A split-plot design was successfully used to test the effects of algal density, water flow, and substrate type on the culture of juveniles of *V. iris* in a recirculating system. Substrate was the most significant factor in successfully culturing newly metamorphosed mussels. Juvenile mussels reared in fine sediment exhibited significantly greater growth and survival, while those reared in culture units without substrate had the lowest growth and survival. Algae at three concentrations and water flow at two levels did not result in significant differences in growth and survival. Juveniles of *L. fasciola* fed on natural food in pond water had significantly greater survival and growth rates than those fed on diets of commercial algae. In addition to common species, juvenile mussels of the endangered *E. capsaeformis* and *E. brevidens* were successfully produced and grown in systems with fine sediment and pond water. The negative effects of flatworm predation and filamentous algae to survival and growth of juveniles were controlled through host fish quarantine, pond-water filtration, and appropriate manipulation to prevent filamentous algal growth. Juvenile escapement during the culture period was prevented by using sieve bags with a proper mesh size at the culture unit outlets. An initial protocol for juvenile mussel propagation and culture was developed on the basis of these study results.

I recommend a substrate of fine sediment, an algae feeding density of about 35,000 cells ml^{-1}, and a flow rate from 3.75 to 7.5 ml s^{-1} for rearing juvenile mussels of these and similar species. Continuous feeding of algae is essential to maintain algae density in recirculating culture systems. Ammonia and nitrite are critical parameters for juvenile mussel culture, and I recommend a maximum concentration of NH_3-N 0.02 mg L^{-1} and $NO2^{-1}$- N of 0.01 mg L^{-1} during the culture of juveniles. A source of natural food in pond water or stream water is recommended to supplement any commercial diet of algae for juvenile mussels or to directly feed mussels in the laboratory. Growing mussels to suitable stocking sizes is a critical step toward effective augmentation and reintroduction of mussel populations.

However, essential nutritional requirements at various life stages of mussels needs

to be qualitatively and quantitatively assessed, such that balanced nutrition becomes known. With suitable diets, the use of laboratory methods for propagation can achieve consistent results and eventually increase the survival and growth of juvenile mussels to aid recovery of endangered species.

Release of propagated juvenile mussels and periodic monitoring efforts greatly facilitated the evaluation of environmental suitability for these animals. Results in my study indicated that growth rates of released mussels were significantly different among the 3 sites. Mussels released at Horton Ford, Clinch River, exhibited significantly faster growth than those released at Fugate Ford, Powell River. Mussels released at Fugate Ford had slower growth than those at the other two sites; however, mussels kept in cages and deployed at Fugate Ford and Payne Property, Clinch River, exhibited slightly different trends in growth rates. Body burdens of Al, Cu and Zn were significantly different in mussels collected from the 3 sites but no detrimental effects of such levels are suspected. Estimates of natural mortality for *L. fasciola* were much less in caged mussels than those for tagged and free-released mussels because of the protected environment. Evaluation of site suitability from in-situ survival and growth of juveniles indicated that Horton Ford is the most suitable site for mussel restoration, while environmental conditions at Fugate Ford are deemed unsuitable for mussel restoration and recovery. A longer-term study of site suitability for mussel restoration at Payne Property is needed. This study demonstrated a protocol for evaluating site suitability in mussel restoration through a mark-recapture study that can be applied to an array of species that prefer environmental conditions similar to that of *V. iris* and *L. fasciola*. Use of cages was the most effective method to determine site suitability because the free-release of mussels (untagged, tagged) had low catchability. This methodology can be applied to effectively monitor the growth and survival of released mussels at sites in rivers. Continued release of propagated mussels to suitable augmentation and reintroduction sites will begin the recovery process for many endangered species.

However, the commonly used mark-recapture method using Hallprint tags needs

to be refined to increase catchability.

Methodology using PIT tags on endangered mussels of *E. brevidens* released near Brooks Bridge, Powell River, greatly increased mean detection probability to about 98%. Hierarchical Bayesian models incorporating individual variations, periodic growth and growth cessations, along with multiple release occasions, gave the best estimates of model parameters and yielded the lowest deviance information criterion value. Survival rate of released individuals exceeded 99% per month during 9 successive recapture occasions in the 2-yr study period, regardless of seasonality. Mussels exhibited different growth increments at various rates of K (0.015, 0.026, 0.110 and 0.050 $year^{-1}$), corresponding to the duration of laboratory culture, ages 2, 3 and 4 yr old, respectively; and a growth cessation (GC) for 5.98 mo. The other parameters of asymptotic length L_∞ and age at the length of zero (t_0) were 51.36 mm and -0.648 mo, respectively. The flexible structure of Bayesian hierarchical models allowed me to examine growth characteristics of *E. brevidens* in a changing environment, to better understand details of its growth and lifespan, and thus, provide useful data for conservation management. Additionally, the methodological implication of this model is that its use and modification can be extended to other growth models and used under various environmental circumstances. Laboratory-reared mussels can exhibit high survival rates when released into suitable habitats. Results indicated that mussel release site in the lower Powell River of Tennessee appear suitable for conducting large-scale population augmentations of these and other endangered species to implement recovery. Mark-recapture method using PIT tags can be applied to other faunal groups that are difficult to collect and monitor for conservation and restoration.

Because recruitment was not observed, even though gravid individuals of released mussels were observed during sampling occasions, a long-term monitoring project is needed to investigate population recruitment for risk assessment, and to evaluate the rate of success of restoration efforts using laboratory-produced mussels.