# A suitable diet and culture system for rearing juvenile freshwater mussels at White Sulphur Springs National Fish Hatchery, West Virginia

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ABSTRACT

Propagation and culture has been accepted as an approved aquaculture method for resource managers to enhance and recover freshwater mussel populations. Although juveniles can be produced readily for many mussel species, achieving high growth and survival in the laboratory remains difficult. The goal of my project was to improve growth and survival of juvenile mussels by comparing diets, algae concentration, and culture systems.

The first objective determined a suitable diet for feeding juvenile northern riffleshell, *Epioblasma torulosa rangiana*, a species listed as federally endangered. Three algal diets were evaluated to determine differences in growth and survival of juveniles of *E. t. rangiana.* After 60 d, mean survival on Phytofeast, Shellfish Diet, and WSSNFH mix were 75.1 (95% CI: 72.2 to 78.0), 78.9 (95% CI: 74.5 to 83.2), and 85.0 (95% CI: 80.6 to 89.3) percent, respectively. WSSNFH mix had the highest survival which was significantly different from Phytofeast ($p$=0.01). Mean shell lengths were 2.37 mm (95% CI: 2.27 to 2.47), 2.62 mm (95% CI: 2.52 to 2.72), and 3.11 mm (95% CI: 3.01 to 3.22), respectively. Juvenile length on all three diet treatments was significantly different from each other ($p$<0.0001), with the WSSNFH mix exhibiting the highest growth, and Phytofeast with the lowest growth.

My second objective evaluated the effect of algal concentration (cells $mL^{-1}$) on growth and survival of juveniles of *E. t. rangiana* and mucket,

*Actinonaias ligamentina*. After 60 d, mean survival of *E. t. rangiana* for the low (30,000 cells $mL^{-1}$), medium (80,000 cells $mL^{-1}$), and high (140,000 cells $mL^{-1}$) algal concentrations were 39.1 (95% CI: 30.7 to 47.4), 20.7 (95% CI: 12.8 to 28.6), and 12.7 (95% CI: 4.82 to 20.5) percent, respectively ($p<0.01$). Mean shell lengths were 1.58 mm (95% CI: 1.49 to 1.66), 1.30 mm (95% CI: 1.19 to 1.40), and 1.01 mm (95% CI: 0.936 to 1.08), respectively ($p<0.0001$). Mean survival of *A. ligamentina* for the low, medium, and high algae concentrations were 46.8 (95% CI: 35.2 to 58.4), 24.6 (95% CI: 15.1 to 34.0), and 10.7 (95% CI: 5.45 to 15.9) percent, respectively ($p<0.01$). Significant differences were observed between the low feed concentration versus the medium and high feed concentrations. Mean shell lengths for the low, medium, and high concentrations were 1.15 mm (95% CI: 1.08 to 1.22), 0.994 mm (95% CI: 0.930 to 1.06), and 0.833 mm (95% CI: 0.770 to 0.896), respectively. All concentrations were significantly different, and the low concentration had the highest mean shell length ($p<0.0001$).

The third objective compared the performance of three recirculating aquaculture systems for rearing juvenile mussels >5 mm. Mean incremental length of juveniles of *E. t. rangiana* at 60 d in Pans, Buckets, and Upwellers was 1.19 mm (95% CI: 0.746 to 1.62), 1.05 mm (95% CI: 0.608 to 1.49), and 2.07 mm (95% CI: 1.63 to 2.51), respectively. Incremental lengths were significantly higher in the Upwellers ($p=0.03$). The mean lengths for Bucket and Pan systems were not significantly different from each other ($p=0.54$). Percent survival of juveniles for the Pans, Buckets, and Upwellers were 91.7 (95% CI: 87.4 to 96.0), 90.0 (95% CI: 80.6 to 99.4), and 100 (95% CI: 100 to 100), respectively. Survival in the Upwellers was significantly higher than in the Buckets

($p$=0.018). Survival of juveniles in the Pan system and Upwellers were not significantly different from each other ($p$=0.05). Mean growth for *A. ligamentina* was 1.96 mm (95% CI: 1.03 to 2.9), 0.88 mm (95% CI: 0.048 to 1.80), and 2.46 mm (95% CI: 1.537 to 3.38), respectively ($p$=0.07). Mean percent survival of juveniles of *Actinonaias ligamentina* in the Pans, Buckets, and Upwellers were 100 (95% CI: 100 to 100), 86.7 (95% CI: 74.0 to 99.4), and 100 (95% CI: 100 to 100), respectively. Survival of *A. ligamentina* in the Upwellers was significantly higher than in Buckets ($p$<0.0001). Juvenile survival in the Pan system and Upwellers was not significantly different ($p$=0.998). Results indicate that the Upweller culture system supported the highest growth and survival in culturing *E. t. rangiana* and *A. ligamentina*.

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## Chapter 1. General Introduction

Freshwater mussels are the most imperiled animals in North America, with over 72% listed as endangered, threatened, or special concern (Neves 1999; Strayer 2004). Causes for the declines include destruction of habitat due to water pollution, impoundments, and sedimentation (Neves 1999). Perhaps the most current threat is water pollution, and data show mussels are more sensitive to a number of toxicants than other animals (Valenti et al. 2006; March et al. 2007; Wang et al. 2007). In addition, our freshwater ecosystems are becoming increasingly impaired by sedimentation, nutrient enrichment, resource extraction, and municipal discharges. In 2009, over 55% of our nation's assessed river and stream miles were in poor condition (USEPA 2013).

The introduction of the Endangered Species Act in 1973 supported the eventual listing of over 68 freshwater mussel species as endangered, which prompted conservation action by state and federal agencies. Actions included writing recovery plans for these species, which identified the need for propagation and culture of mussels as a restoration method. This prompted research facilities to identify life history needs and develop propagation techniques for restoration.

Unlike most recreational fish species, such as rainbow trout (*Oncorhynchus mykiss*) and channel catfish (*Ictalurus punctatus),* the life history and propagation techniques for mussels have large data gaps, with limited biological information for many species. Additionally, the complicated reproductive cycle and large number of imperiled mussels in the United States hindered the ability of aquatic biologists to produce and culture juvenile mussels for large-scale release.

During the past 20 y, technological advances for propagation of freshwater mussels have been made. Basic life history information including the identification of host fishes for many mussel species has been determined. Also, reproductive timing and locations to collect mussel broodstock have enabled us to produce juveniles of numerous mussel species. We also have learned which fish species are abundant, collection locations, and how to hold fish in captivity which further increased our production capabilities. Limited information was available on diet, including appropriate algae species and cell size which increased growth and survival in the laboratory. Furthermore, the invention of the bucket culture systems (Missouri State University, Springfield, Missouri) also increased growth and survival among juvenile mussels. This compact rearing system simplified replication for culture experiments and enabled facilities to produce and release a wide variety of species to stream restoration areas. However, it was not until the last 5 y that facilities and researchers have been able to grow juvenile mussels to sufficient size (20-30 mm) to tag (hallprint or pit tag) an individual for monitoring at release sites.

Diet requirements are extremely important to hold adult mussels for propagation, translocation, or relocation efforts. It is imperative that captive-held adults are kept healthy until they are returned to source rivers. When mussels are relocated to restoration areas, there is a risk of spreading diseases and invasive species unless they are quarantined in isolated systems. Isolated systems rely on non-natural food sources such as commercial diets or hatchery-grown algae, making the dietary requirements of juvenile and adult mussels a research need for program success.

Hatchery-reared juvenile mussels provide resource managers with an opportunity to increase mussel populations and establish new populations where suitable habitat exists. In order to grow freshwater mussels to a size (>10 mm) suitable to be tagged, released, and monitored, it is crucial to know the dietary needs and appropriate culture system for successful rearing in a hatchery.

Mussel species have different diet preferences (Bisbee 1984), therefore I chose to test algae concentration and type of culture system on two species of juvenile mussels. The northern riffleshell (*Epioblasma t. rangiana*) was chosen for this study to improve current propagation technology for this species and others in the genus *Epioblasma.* The mussel genus *Epioblasma* is considered the most imperiled taxonomic group in North America. This genus historically had 25 species within it, although 72% of these species are now extinct. The other 7 species are listed as federally endangered (Jones and Neves 2010). All 7 species are either extinct or endangered, with possible extinctions of some remaining species expected within the next 10-20 y if recovery goals are not met. The *E. t. rangiana* was listed as a federally endangered species in 1993 and has been extirpated from most of its historic range (USFWS 1994).

Within the United States, *E. t. rangiana* currently resides in Kentucky, Illinois, Ohio, Pennsylvania, and West Virginia. It is considered extant in only 7 streams from these states, with the largest population occurring in the Allegheny River, Pennsylvania. In Canada, *E. t. rangiana* occurs in the Sydenham and Ausable rivers and was ranked as Endangered by the Committee on the Status of Endangered Wildlife in Canada in 2010. Individuals are relatively short-lived (max. 10-15 y), small in size (max. 65 mm), thin-shelled, sexually dimorphic, and primarily use darters as host fishes. Reported host fishes

include banded sculpin (*Cottus carolinae*), bluebreast darter (*Etheostoma camurum*), banded darter (*Etheostoma zonale*), and brown trout (*Salmo trutta*) (Watters, 1996).

The other species I chose was the mucket, *Actinonaias ligamentina.* In contrast, *A. ligamentina* is a common mussel species found throughout most of its historic range in the Ohio, Tennessee and Mississippi River systems, and has reproducing populations in 20 states, from Minnesota east to New York and south to Louisiana. It also can be found in the St. Lawrence River basin and tributaries of lakes Erie, Michigan, and Ontario (Burch 1975; Parmalee and Bogan 1998). The species is long-lived (>20 y), large in size (>120 mm), thick shelled, does not show sexual dimorphism, and uses a variety of hosts, including largemouth bass (*Micropterus salmoides*) and smallmouth bass (*Micropterus dolomieu*) as primary host fishes (Parmalee and Bogan 1998).

The goal of my project was to improve growth and survival of juvenile mussels by completing three objectives. The first objective was to compare growth and survival of juvenile mussels fed a live algal diet and two commercially available diets. The second, was to determine a suitable algae concentration to feed mussels. The last objective was to use larger juveniles (>5 mm) to evaluate three culture systems for long-term rearing.

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## Chapter 2. A suitable diet for rearing endangered northern riffleshell, *Epioblasma torulosa rangiana*, in recirculating aquaculture systems at White Sulphur Springs National Fish Hatchery, West Virginia.

ABSTRACT

Three algal diets were evaluated for differences in growth and survival of *E. t. rangiana* juveniles; Shellfish Diet and Phytofeast which are produced by Reed Mariculture (Reed Mariculture, Inc., Campbell, California) and a live hatchery-grown WSSNFH mix. Juveniles were reared on one of the three diets at a concentration of 30,000 cells $mL^{-1}$. After the 60 d experiment, mean percent survival of *E. t. rangiana* of Phytofeast, Shellfish Diet, and WSSNFH mix were 75.1 (95% CI: 72.2 to 78.0), 78.9 (95% CI: 74.5 to 83.2), and 85.0 (95% CI: 80.6 to 89.3), respectively. Significant differences ($p$=0.01) were observed between WSSNFH mix when compared to Phytofeast. Mean shell lengths of juveniles at 60 d for the Phytofeast, Shellfish Diet, and WSSNFH mix were 2.37 mm (95% CI: 2.27 to 2.47), 2.62 mm (95% CI: 2.52 to 2.72), and 3.12 mm (95% CI: 3.01 to 3.22), respectively. Significant differences also were observed for mean shell lengths, and all diet treatments were significantly different from each other ($p$<0.0001). The WSSNFH mix had the highest growth, and Phytofeast had the lowest growth. Results from this experiment indicate that until better commercial diets can be developed, a live algal mix is preferred for growing juveniles of *E. t. rangiana*.

### Introduction

Quantity and quality of food is recognized by marine bivalve specialists as the limiting factor in successful aquaculture efforts (Winter 1978). Specific nutritional

requirements for juvenile freshwater mussels are not known, however successful rearing of juvenile mussels in the laboratory is dependent on feeding a balanced algal diet.

Proteins, carbohydrates, and lipids are known to be the most important biochemical constituents of an acceptable bivalve diet. Proteins, for example, are responsible for tissue production and are essential in growth of juveniles; protein-limiting diets have been shown to decrease growth in mussels (*Mytilus trossulus*) (Kreeger and Langdon 1993). Carbohydrates, mainly glycogen, are the primary energy source for bivalves. Dietary carbohydrates also may be important in balancing proteins and lipids for energy production (Whyte et al. 1989). Enright et al. (1986) showed that a diet with high levels of carbohydrates increased growth of oyster larve (*Ostrea edulis*), if adequate levels of proteins and lipids were available. Lipids also are important in reproductive development and likely a major energy source for developing larvae (Berenberg and Patterson 1981). It is unlikely that lipids are synthesized by mussels and therefore must be available in the diet. Successful propagation and culture of mussels must include a balanced diet with adequate levels of proteins, carbohydrates, and lipids. Algae can be high in lipids (i. e., plant sterols) and are therefore important components in any bivalve diet. Successfully reared juveniles have been fed algal diets containing high levels of polyunsaturated fatty acids (Gatenby 1994; Gatenby et al. 1997).

Size of food particles is also a consideration when developing a suitable diet to feed mussels and increase growth and survival of juveniles. In nature, mussels consume small particles less than 28 μm in diameter, which include algae, detritus, and bacteria (Nichols and Garling 2000). Lasee (1991) determined a two-d old juvenile has a mouth

size of 16 µm and an esophagus of 6 µm. Beck and Neves (2003) determined that smaller cells (2.8-8.5 µm) are preferred to larger cells (22.8-44.5 µm) which are passed over.

Microalgae species differ in their biochemical components and by providing a multi-species diet, a more balanced diet can be provided. A combination of several microalgae including diatoms has been shown to produce greater growth and survival for bivalves (Brown et al. 1997; Gatenby et al. 2003; Helm and Bourne 2004). Additionally live algae and algae mixes have been shown to be more successful in rearing bivalves (Gatenby 1994, Heasman et al. 2001).

However, to culture a combination of algae and diatoms, a controlled indoor environment is required, which contributes significantly to operational costs of hatcheries (Southgate et al. 1992; Heasman 2001). Similarly, production costs for algae culture in the United States range from \$160-400 $kg^{-1}$, which accounts for almost 30% of marine production costs (Southgate et al. 1992; Knauer and Southgate 1999; Heasman et al. 2001). Algae culture costs are similarly high for freshwater mussel production. As with marine algae culture, freshwater algae culture can have unpredictable failures or crashes that can occur at crucial production time periods. To reduce operational costs and potentially devastating results from algae crashes associated with production, artificial diets are being produced and tested for their commercial value in the marine bivalve industry (Knauer and Southgate 1999; Heasman et al. 2001). For freshwater mussels, however, information on nutritional requirements is limited, and recent successes in propagation and culture of unionids by some facilities mainly incorporate a natural food source (i.e., pond or river water) for the diet by rearing mussels in outdoor cages. Other facilities use river or pond water and supplement the diet with either cultured algae or

commercial diets which are now readily available. To culture mussels indoors throughout the year, microalgae are required but at a high operational cost.

The goal of this study was to compare juvenile growth and survival of freshwater mussels fed commercially available shellfish diets, Phytofeast and Shellfish Diet, with a hatchery-grown (WSSNFH) live algae mix. These algae diets were evaluated for their effects upon growth and survival of juveniles of northern riffleshell, *Epioblasma torulosa rangiana*.

## **Methods**

### *Adult Mussel Collection*

Gravid female mussels of *E. t. rangiana* were collected from the Alleghany River (River km 111.7) at East Brady, Clarion County, Pennsylvania during a Pennslyvania Department of Transportation bridge relocation project in June 2008. Divers from the Ohio River Islands National Wildlife Refuge (ORINWR, USFWS), West Virginia Department of Natural Resources (WVDNR), and White Sulphur Springs National Fish Hatchery (WSSNFH) collected the mussels during relocation efforts to other sites in West Virginia and Ohio. Mussels were collected and checked for gravidity by carefully opening the valves 5-10 mm to look for inflated gills containing glochidia. After gravidity was assessed, 10 gravid females of *E. t. rangiana* were transported back to WSSNFH. Mussels were transported in a 94.6 L cooler with a 12-volt Super Fish Saver aerator from (Marine Metal Products, Inc., Clearwater, Florida). In addition, extra river water was transported in a cooler to perform a 50% water change during transport since the travel time was over 6 h in duration.

Upon arrival at WSSNFH, mussels were slowly acclimated to the facility water temperatures. Mussels were temporarily held in recirculating water in troughs containing a 15 cm layer of mixed gravel and sand substrate. Substrate consisted of 40% small pebble (<2 cm) and 60% rinsed sand. Holding troughs were equipped with a three-quarter hp chiller (Aqua Logic, Inc., San Diego, California) and 1 kW submersible heaters to maintain water temperature at 20 ± 1 degree C.

*Automatic Feeding System*

The WSSNFH developed an automatic feeding system that provides continuous feeding of juvenile and adult mussels using a series of solenoid valves and timers (Figure 1). For this experiment, with three diets under evaluation, the automatic feeding system was modified. In the sump of the bucket culture system, a 18.9 L bucket was filled with one of the diets being tested. Each bucket had a pump to transport the diet into its own 1.9 cm pvc feeding manifold, forming a loop to return the unconsumed algae to the 18.9 L bucket. For each diet, the manifold had three solenoid valves which corresponded to the three timers. Each solenoid would feed one bucket, with three buckets per diet treatment. Each solenoid valve was electronically connected to a timer that controlled when the solenoid opened and distributed algae. The length of time the solenoid valve was opened controlled the amount of algae added to the culture buckets.

Prior to the experiment, the amount of algae to be dispensed and its frequency was determined. Cell counts of each treatment were conducted every 5 min for 2 h. After 30 min, treatment concentrations dropped below 50%; hence, the timers were set to dispense algae at 30-min intervals. To achieve the desired culture concentration of 30,000

cells mL$^{-1}$, each diet was diluted to approximately 4.4 X $10^8$ cells mL$^{-1}$. Algae from each treatment was dispensed at a rate of 14 mL every 30 min (2 mg L$^{-1}$ dry wt.).

*Culture of Live Algal Diet*

Methods to culture algae used in the following experiments were a modification of Hoff and Snell (1999). Once cultures were concentrated sufficiently, a larger continuous algae production system, called a Biofence (http://www.variconaqua.com/, Varicon Solutions Ltd., Malvern, United Kingdom) was used.

*Host Fish Collection*

Sculpins (*Cottus sp.*) were used to metamorphose glochidia of *E. t. rangiana* to the juvenile stage. A total of 120 sculpins was collected by staff at WSSNFH from Howards Creek, Greenbrier County, WV, in June 2008. Sculpins were collected using a Smith-Root LR-21 backpack electrofisher (Smith-Root, Inc., Vancouver, Washington) and an 2.4 m seine net (Aquatic Ecosystems, Inc., Apopka, Florida). The seine net was set 3 to 5 m below the electroshocker. Stunned fish floated downstream into the seine and were immediately placed into a 18.9 L bucket with river water. After 25-40 fish were collected in the bucket, the fish were transferred to a cooler filled with river water and a dilute concentration of salt (NaCl) to reduce stress on the fish during transport. Sculpins were transported in a 94.6 L cooler using a 12-volt Super Fish Saver aerator (Marine Metal Products, Inc., Clearwater, Florida).

*Production of Juvenile Mussels*

Glochidia were extracted from gravid females using a 21 gauge 3.8 cm hypodermic needle and syringe filled with water. The needle was inserted into the posterior end of each outer gill to flush glochidia from the gill into a Petri dish. Viability

was assessed by applying a dilute NaCl solution to a sample of glochidia and visually inspecting the speed at which glochidial valves closed shut.

To infest the sculpins with glochidia of *E. t. rangiana,* 60 fish were placed in two well-aerated 6-L plastic containers with approximately 1.0 L of water. Glochidia extracted from three female mussels were combined and added to both containers of host fish, which were aerated vigorously for approximately 1 h. Fish and glochidia were stirred after about 30 min during the infestation process in order to keep glochidia in suspension. Once the infestations were completed, sculpins were separated by size to prevent cannibalism and placed into an AHAB system (Aquatic Ecosystems, Inc., Apopka, Florida). No more than 15 sculpins were put in each 3-L AHAB tank. All fish-holding systems were set at 20º C. Sculpins were held in stand-alone AHAB recirculating systems, with 15 fish in each 9-L acrylic tank.

*Culture of Juvenile Mussels*

Juvenile mussels excysted from host fish within 2 wk and continued through to 4 wk post-infestation. Once juvenile mussels began excysting from the host fish, sieves from the self-siphoning AHAB aquaria were checked every 1-2 d to recover juveniles. Once excysted, juvenile mussels were collected and held in a 'bucket of muckets' (Barnhart Buckets) juvenile culture system (Barnhart 2006) for 3 wk prior to initiating the experiment, to allow premature or poor-condition juveniles to die and avoid initial mortality during the experiment. Each bucket culture system consisted of two nested buckets as described in Barnhart (2006). In this experiment, since juveniles had an initial mean length of 1.2-1.3 mm, mesh size on the juvenile cups was 200 µm. A total of 18L of water was recirculated between the two buckets by pumping water through the mesh

containers using a small submersible pump (Marineland, Inc., Blacksburg, Virginia) to create a down-welling effect.

Juvenile mussels were fed one of three treatments; Phytofeast, Shellfish Diet, or WSSNFH mix (Figure 1). Algae concentrations for all treatments were maintained at 30,000 cells $mL^{-1}$. Algal density in the culture systems was checked and counted daily using a Beckman Coulter Counter (Beckman Coulter, Inc., Brea, California) and recorded as cells $mL^{-1}$ and $\mu m^3$ $mL^{-1}$ for the 60-d duration of the experiment. In addition to algal density estimates, the culture water was filtered, dried, and ashed to calculate ash-free-dry weight and organic content.

The experimental design consisted of three buckets per treatment, and three chambers with juveniles per bucket (Figure 2). Therefore, 3 chambers per bucket were occupied with juveniles, and 3 chambers had 150 µm place holders or empty chambers. In total, 9 buckets and 27 containers of juveniles were used in the experiment (Figure 2). During experimental set-up, 150 juveniles were counted and placed in labeled juvenile containers, and lengths of 10 juveniles per chamber were measured using a microscope and ocular micrometer.

The mesh screens on the juvenile containers were sprayed and cleaned daily with a household garden sprayer to avoid clogging of the holding chambers, especially given the high juvenile densities per bucket system. Weekly, each bucket received a water exchange, replacing the culture water with clean, filtered (<30 µm) spring water. Juveniles were sampled every 15 d, and growth and survival were recorded during each sample event. Sample events consisted of removal of the containers from the bucket and inspection and measurement of juveniles with the microscope. Growth (length in mm)

and percent survival were evaluated for all containers and treatments. In addition, every 15 d, the nylon mesh screens were changed in all of the juvenile chambers to prevent clogging. Juveniles were cultured throughout the experiment at 20-21° C, and temperature was maintained for each bucket by a water bath. A recirculating trough system that included a heater and chiller controlled temperature for all buckets (Figure 1). Other water quality data including ammonia were not taken during the experiment because 100% water exchanges occurred weekly, and the diet ration was of low concentration.

*Juvenile Mussel Diets*

Juveniles were fed live algae cultured at WSSNFH containing the following mix of 4 species: *Bracteacoccus grandis, Neochloris oleoabundans, Phaeodactylum tricornutum,* and *Oosystis polymorpha* (Table 1). Algae were grown in photo-reactors known as Biofences (http://www.variconaqua.com/, Varicon Aqua Solutions, Ltd., Malvern, United Kingdom). Algae was harvested and centrifuged into a thick paste, vacuum sealed, and refrigerated. Algae paste was refrigerated for a maximum of 4 wk to use in these experiments. Phytofeast and Shellfish Diet are produced by Reed Mariculture, (Campbell, California). Phytofeast is a blended, preserved algae mix used for marine bivalves and has seven genera in its formulation; *Tetraselmis, Isochrysis, Pavlova, Nannochloropsis, Thalassiosira, Amphora, and Synechococcus*. Phytofeast is preserved using food grade additives including citric acid, ascorbic acid, and larixinic acid. Shellfish Diet is not live algae, consisting of four species of marine microalgae; *Isochrysis, Pavlova, Tetraselmis,* and *Thalassiosira weissflogii*.

*Dry Weight and Ash-free Dry Weight*

The Phytofeast, Shellfish Diet, and WSSNFH mix were each analyzed twice (n=2) for dry and ash-free dry weight to evaluate organic content of the respective juvenile diets. A water sample from each juvenile bucket was analyzed for dry and ash-free dry weight twice (n=2). Samples were collected on filters (GF/F 25 mm) and first ashed in a muffle furnace at 450º F for 24 h and weighed. Coulter counts (Beckman Coulter, Inc., Brea, California) recorded mean cell size in µm, cells $mL^{-1}$, and $\mu m^3$ $mL^{-1}$ for each sample filter. Feed mix and juvenile buckets were filtered using a vacuum pump with previously ashed filters. Volume of sample water filtered was recorded in mL. Filters then were dried in a muffle furnace for 24 h at 60º F and then weighed to obtain dry weight (g). Filters then were ashed once again at 450º F for another 24 h to record ash-free dry weight for each sample.

The diets were evaluated prior to the start of the experiment and after 30 d, with three samples filtered and processed for dry and ash-free dry weight. Additionally, three samples were taken from each bucket, and dry and ash-free dry weight were evaluated twice during the experiment (n=2) (Table 2).

*Statistical Analysis*

All statistical analyses were conducted in JMP version 10.0, (SAS Institute 2010). I compared percent survival among the three diet treatments over time, sampling juvenile mussels every 15 d. Survival of juveniles is reported as a percentage of survival at 60 d. Differences in mean shell lengths over 60 d also were compared among the three diet treatments. One-way analysis of variance (ANOVA) and student *t*-tests were used to

analyze growth and survival within treatments during the experiment. All statistical tests conducted in this study were considered significant at an alpha level of 0.05 or less.

**Results**

*Juvenile Growth and Survival*

Mean percent survival of juveniles of *E. t. rangiana* at 60 d for the Phytofeast, Shellfish Diet, and WSSNFH mix treatments were 75.1 (95% CI: 72.2 to 78.0), 78.9 (95% CI: 74.5 to 83.2), and 85.0 (95% CI: 80.6 to 89.3), respectively (Table 2; Figure 3). Significant differences ($p$=0.01) were observed among the three diets. WSSNFH mix had higher percent survival than Phytofeast ($p$=0.01) (Figure 3). A significant difference also was observed between the WSSNFH mix and Shellfish Diet ($p$=0.05). No difference occurred between Shellfish and Phytofeast (p=0.22).

Mean shell lengths of juveniles of *E. t. rangiana* at 60 d for the Phytofeast, Shellfish Diet, and WSSNFH mix treatments were 2.37 mm (95% CI: 2.27 to 2.47), 2.62 mm (95% CI: 2.52 to 2.72), and 3.12 mm (95% CI: 3.01 to 3.22), respectively, and all values were significantly different from each other ($p$<0.0001)(Table 2; Figure 4). The WSSNFH mix had the greatest growth, and Phytofeast had the lowest growth (Figure 4).

*Cell Counts*

Mean cell counts ($mL^{-1}$) for the Phytofeast, Shellfish Diet, and WSSNFH mix treatments were 38,560 (95% CI: 23,548 to 53,573), 50,609 (95% CI: 35,493 to 65,726), and 24,100 (95% CI: 8,984 to 39,216), respectively (Table 2; Figure 5), and were not significantly different from each other ($p$=0.05). The Shellfish Diet and WSSNFH mix means significantly different. Mean cell volumes for the Phytofeast, Shellfish Diet, and WSSNFH mix were $6.70 \times 10^5$ $\mu m^3$ $mL^{-1}$ (95% CI: $4.06 \times 10^5$ to $9.25 \times 10^5$), $1.01 \times 10^6$

μm$^3$ mL$^{-1}$ (95% CI: 7.43 x $10^5$ to 1.28 x $10^6$), and 1.34 x $10^6$ μm$^3$ mL$^{-1}$ (95% CI: 1.08 x $10^6$ to 1.71 x $10^6$) (Table 2; Figure 6). The WSSNFH mix and Shellfish Diet were significantly different from Phytofeast ($p$<0.01); however, Shellfish Diet and Phytofeast were not significantly different from each other ($p$=0.08). Mean cell size (μm) of diet treatments also was compared, with the mean cell size for Phytofeast at 4.15 μm (95% CI: 3.2 to 5.06), Shellfish at 4.76 μm (95% CI: 3.84 to 5.67), and WSSNFH was 6.21 μm (95% CI: 5.30 to 7.12) (Table 2; Figure 7). One-way ANOVA showed a significant difference ($p$<0.01) between the mean cell size of the WSSNFH mix and both Phytofeast and Shellfish Diet; however, Shellfish Diet and Phytofeast were not significantly different from each other ($p$=0.35).

*Dry and Ash-Free Dry Weights of Diets*

Mean dry weights of diets were not significantly different ($p$=0.79); Phytofeast, Shellfish Diet, and WSSNFH mix were 3.60 mg L$^{-1}$ (95% CI: 2.72 to 4.49), 4.01 mg L$^{-1}$ (95% CI: 3.12 to 4.89), and 3.73 mg L$^{-1}$ (95% CI: 2.84 to 4.61), respectively (Table 2). Dry weights of culture bucket treatments also were not significantly different ($p$=0.08). The Phytofeast diet had a mean dry weight of 2.23 mg L$^{-1}$ (95% CI: 1.90 to 2.54), whereas the Shellfish Diet was 1.96 mg L$^{-1}$ (95% CI: 1.62 to 2.62), and the WSSNFH mix was 2.14 mg L$^{-1}$ (95% CI: 1.79 to 2.49) (Table 2; Figure 8).

Mean ash-free dry weight (AFDW) for Phytofeast, Shellfish Diet, and WSSNFH mix were 2.05 mg L$^{-1}$ (95% CI: 1.93 to 2.17), 1.98 mg L$^{-1}$ (95% CI: 1.87 to 2.09), and 2.04 mg L$^{-1}$ (95% CI: 1.90 to 2.18), respectively, and were not significantly different ($p$=0.58) (Table 2). Mean AFDW of each treatment, taken from the bucket culture system, was 0.698 mg L$^{-1}$ (95% CI: 0.49 to 0.90) for Phytofeast, 0.826 mg L$^{-1}$ (95% CI:

0.67 to 0.98) for Shellfish Diet, and 0.781 mg $L^{-1}$ (95% CI: 0.61 to 0.95) for WSSNFH, with no significant differences observed ($p$=0.50) (Table 2; Figure 9).

**Discussion**

Study results indicated that juveniles of *E. t. rangiana* had higher mean length (mm) and survival (%) with the live WSSNFH mix than with the two commercially available diets when cultured in the bucket systems. Although few published diet studies are available, Vincie (2008) compared growth and survival of oyster mussel juveniles, *Epioblasma capsaeformis*, when fed combinations of Reed Mariculture's Shellfish Diet 1800 and *Nannochloropsis* 3600, with additions of bacteria flocs or probiotics. Although no live algae were tested in Vincie (2008), results showed that juveniles grew slightly better in a 3X higher concentration of the two commercial diets. Bacterial floc and probiotics did not seem to improve either growth or survival. However, at the end of her study, mean lengths ranged from 0.720 to 0.813 mm, and survival ranged from 3.17 to 9.92 %, which are both considerably lower than results in my study. Each mussel chamber had a lower number of juveniles in her study compared to my study, which should have increased growth (n=42-50) (Vincie 2008). Bacteria also were not effective at increasing growth and survival of juveniles of rainbow mussels, *Villosa iris* (Gatenby et al. 1996).

Another study with *E. capsaeformis* compared two live algal species, *Neochloris oleoabundans* and *Nannochloropsis occulata*, and found no difference in growth or survival between them; survival for that study was >29% (Jones et al. 2005). It is known that multi-species algal mixes support better growth and survival of juvenile mussels than a single-species diet (Heasman 2001). Some algal species are too large for juvenile

mussels to consume. For example, Beck and Neves (2003) showed that juveniles can selectively feed on algal species based on cell size, where larger cells (22.8-44.5 µm) are passed over for smaller cells (2.8-8.5 µm). In their study, the smaller particle algae were selected for and consumed by the mussels; *Nannochloropsis oculata* and *Selenastrum capricornutum* were selected over *Scenedesmus quadricauda*. Mean cell sizes for the three diets ranged from 4.15 to 6.21 µm, which occur in the range of preferred cell sizes observed by Beck and Neves (2003). Other freshwater mussel studies have identified a fine (<200 µm) organic sediment in combination with live algae to enhance growth and survival (Gatenby et al. 1997; Jones et al. 2005); however, mussels in my study were not grown in sediment and yet experienced high survival at the time of the experiment.

The mariculture industry has been assessing the effects of diets on various bivalves since the mid-1900s to reduce hatchery operating costs and maintain or improve condition of broodstock, larvae and juveniles. Diet experiments conducted with Sydney rock oysters (*Saccostrea glomerata*) fed fresh algae had twice the growth of oysters fed algae with additives (Heasman 2001). Another study also showed a decrease in growth for juvenile oysters when fed concentrated diets (Ponis et al. 2003). Survival during these experiments was not significantly different when oysters were fed live algae instead of centrifuged or preserved algae. Algae that were frozen (-15 ºC) or low speed centrifuged slurries with or without additives seemed to adversely affect survival relative to live or stored algae. Some of the additives used in preserved algae include citric acid and ascorbic acid, the same additives used in the production of Phytofeast, but did not seem to have an adverse effect on oyster larvae. Other studies compared growth and survival among microalgae concentrates and reported that generally as long as the concentrates

were not old (<4 wk), at least partial substitution of concentrates for live algae did not yield detrimental results (Aji 2011). Results from experiments with dried microalgae or yeast diets were not as favorable for replacing or supplementing a live algal diet (Aji 2011).

Although biochemical analyses was not completed as part of this study, some analyses by Gatenby et al. (2003) were conducted on three of the four species in the WSSNFH mix; *Neochloris oleoabundans*, *Bracteocauccus grandis*, and *Phaeodactylum tricornutum*. Basic comparison between three of the four species in the WSSNFH mix and Shellfish Diet showed that lipids and carbohydrates were higher in the WSSNFH mix than in the Shellfish Diet. Although Reed Mariculture was contacted concerning the nutritional profile of Phytofeast, only limited information was provided. The supplied data (Table 1) reported that Phytofeast is low in protein, carbohydrates, and lipids because it is mostly water and meant for the hobby aquarist. Phytofeast does, however, have a diverse selection of algae species in its mix, which could provide a diversity of amino acids and essential nutrients to juvenile mussels.

Cell counts and cell volumes were highly variable during the experiment, due to the settling rate of the algae (Table 2). The cell counts for the treatments were significantly different between Shellfish Diet and WSSNFH mix and the cell volumes were significantly different between the Phytofeast and WSSNFH (Table 2). This discrepancy is likely due to the high variation among samples and the larger cell sizes of WSSNFH mix. No significant differences were observed between the treatment dry weights or AFDW, which indicates little difference in the amount of each diet being fed to the mussels. During the experiment, it was more difficult to keep Phytofeast and

Shellfish Diet in suspension, especially Phytofeast. To maintain the desired concentration of 30,000 cells $mL^{-1}$ in each culture system, algae needed to be added more often. Both of the commercial diets exhibited clumping, which seemed to foul the culture water faster than did the WSSNFH mix.

**Conclusions**

Comparing diets and learning what to feed mussels is an important step in improving laboratory rearing techniques. In my study, growth and survival of juveniles of *E. t. rangiana* were higher when fed the live WSSNFH mix over the Shellfish and Phytofeast commercial diets. These results indicate that incorporating live algae that have adequate levels of proteins, carbohydrates, and lipids can provide a suitable diet for mussels.

Growing multiple species of live algae to supply food to a large mussel facility is expensive and time consuming. Results from my experiment show that the production of live algae can significantly increase growth and survival of juvenile mussels and that the expense to grow algae is justified. A live algal diet has been shown to be highly successful in rearing bivalves (Gatenby 1994; Heasman et al. 2001). Juveniles fed mixed diets containing multiple species have been shown to produce higher growth (Napolitano et al. 1990; Helm and Bourne 2004). Future research is needed to identify other commercially available diets or live algal species suitable for mussel culture.

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Table 1. Basic nutritional properties of each algal diet.

| Diet | Protein (% dry wt.) | Carbohydrate (% dry wt.) | Lipid (% dry wt.) |
|---|---|---|---|
| WSSNFH mix[1] | 45.2-54.1 | 8-56 | 10.0-28.5 |
| Shellfish Diet[2] | 46-54 | 18-24 | 14.3-19.6 |
| Phytofeast[2] | 1.3 | Not known | 0.5% |

1. Data from Gatenby et al. 2003 for *Neochloris oleoabundans*, *Bracteocauccus grandis*, and *Phaeodactylum tricornutum*.
2. Data provided by Reed Mariculture (Campbell, California)

Table 2. Summary of mean values with 95% Confidence Intervals.

| Mean measurement (unit) | WSSNFH Mix (95% CI) | Shellfish Diet (95% CI) | Phytofeast (95% CI) | *p* value |
|---|---|---|---|---|
| Cell count (cells $mL^{-1}$) | 24,000 (8,984 to 39,216) | 50,609 (35,493 to 65,726) | 38,560 (23,548 to 53,573) | $p$=0.0521 |
| Cell volume (µm3 $mL^{-1}$) | 1,340,000 ($1.08 \times 10^6$ to $1.71 \times 10^6$) | 101,0000 ($7.43 \times 10^5$ to $1.28 \times 10^6$) | 670,000 ($4.06 \times 10^5$ to $9.25 \times 10^5$) | $p$=0.0024 |
| Cell size (µm) | 6.21 (5.30 to 7.12) | 4.76 (3.84 to 5.67) | 4.15 (3.2 to 5.06) | $p$=0.0077 |
| Dry wt. diet (mg $L^{-1}$) | 3.73 (2.84 to 4.61) | 4.01 (3.12 to 4.89) | 3.60 (2.72 to 4.49) | $p$=0.7934 |
| AFDW diet (mg $L^{-1}$) | 2.04 (1.90 to 2.18) | 1.98 (1.87 to 2.09) | 2.05 (1.93 to 2.17) | $p$=0.5792 |
| Dry wt. bucket systems (mg $L^{-1}$) | 2.14 (1.79 to 2.49) | 1.96 (1.62 to 2.62) | 2.23 (1.90 to 2.54) | $p$=0.0750 |
| AFDW bucket systems (mg $L^{-1}$) | 0.781 (0.61 to 0.95) | 0.826 (0.67 to 0.98) | 0.698 (0.49 to 0.90) | $p$=0.5003 |
| 60-d survival (%) *E. t. rangiana* | 85.0 (80.6 to 89.3) | 78.9 (74.5 to 83.2) | 75.1 (72.2 to 78.0) | $p$=0.0098 |
| 60-d growth (mm) *E. t. rangiana* | 3.12 (3.01 to 3.22) | 2.62 (2.52 to 2.72) | 2.39 (2.27 to 2.47) | $p$<0.0001 |

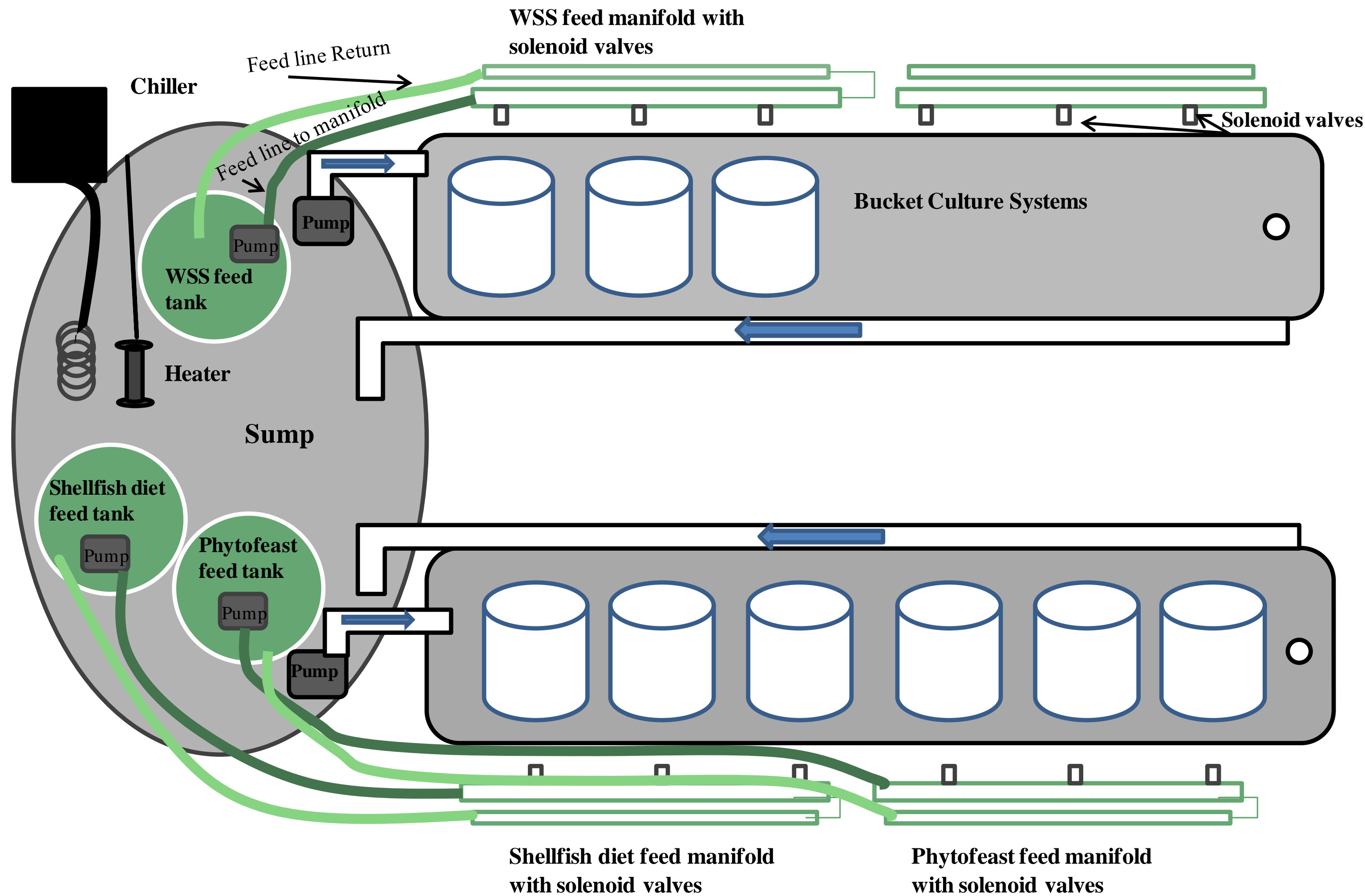


Figure 1. Top view of culture system. A total of nine independent bucket culture systems with three for each diet treatment were placed in a temperature-controlled water bath. Water temperature was controlled within 1 degree C by a heater and chilling unit. There were two pumps inside the sump which moved water into each trough. Each trough had a return line indicated by blue arrow's which returns water to the sump for even temperature control of the water bath. For each diet treatment there was a 18.9 L bucket which had a pump inside that moved the diet into a recirculating PVC manifold (in dark green) and then a return (light green) back into the bucket. Each manifold had three solenoids which controlled the amount of diet for the three replicates within that treatment. Each solenoid was controlled by its own timer (not shown).

## Experimental Design

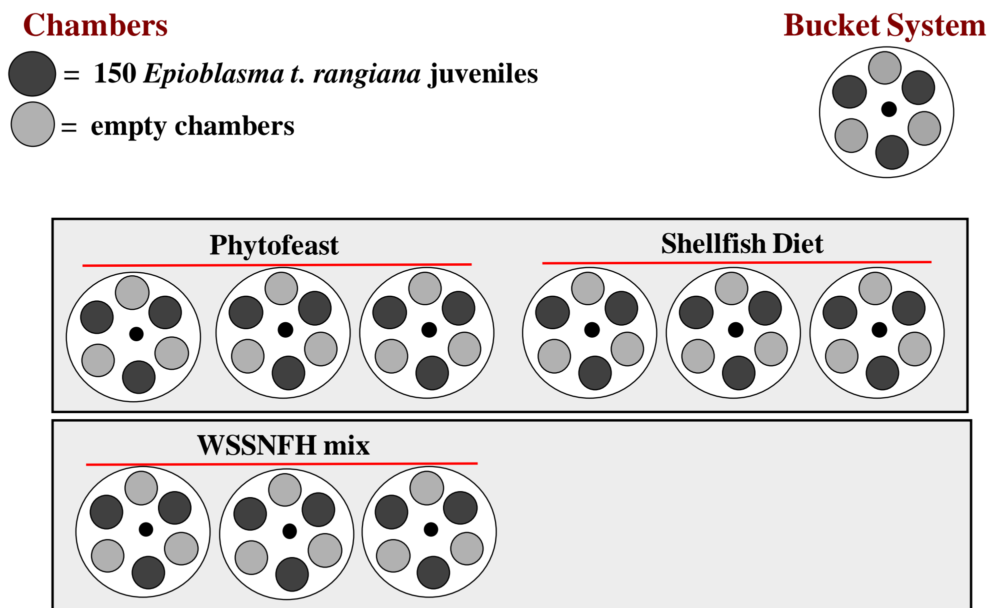


Figure 2. Top view of experimental design for evaluating three diets; Phytofeast, Shellfish Diet, and WSSNFH mix. Three buckets were used for each diet treatment; each bucket had three chambers of juveniles and three empty chambers with 150-µm mesh.

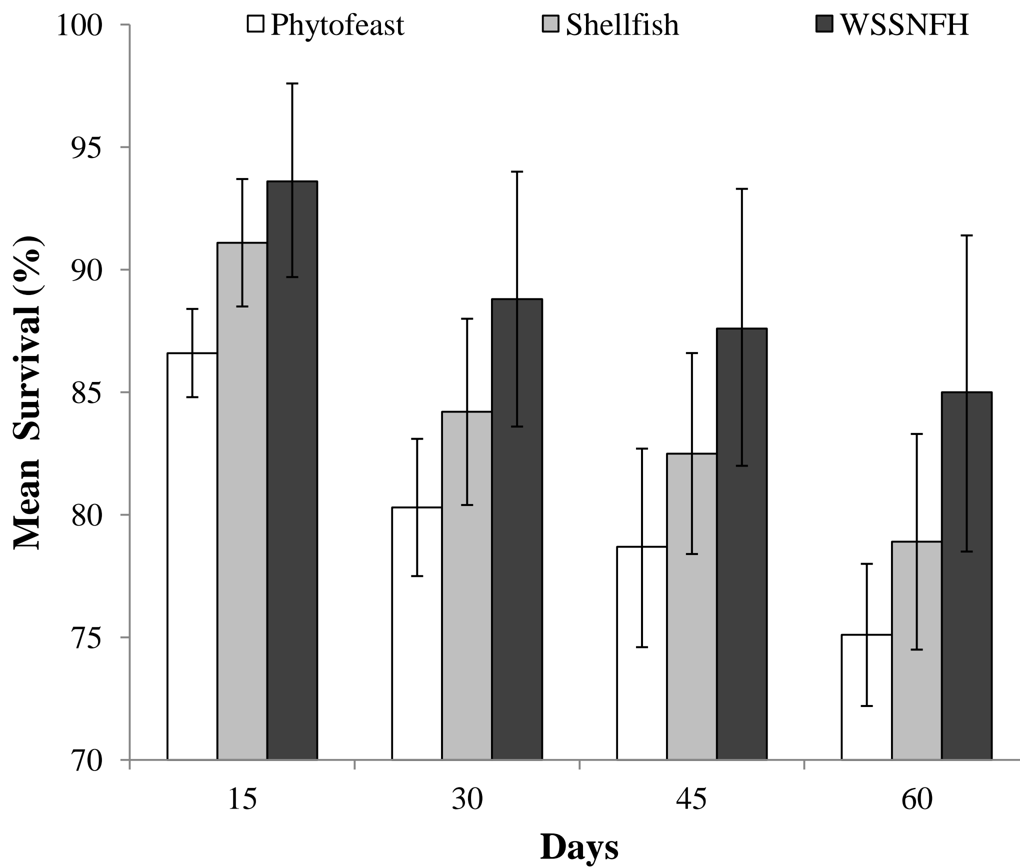


Figure 3. Percent survival (%) of juveniles of *Epioblasma t. rangiana* at 15-d intervals for the three diets; Phytofeast, Shellfish Diet, and WSSNFH mix. WSSNFH mix had significantly higher survival than Phytofeast (Mean ± 95% CI; $p$=0.01).

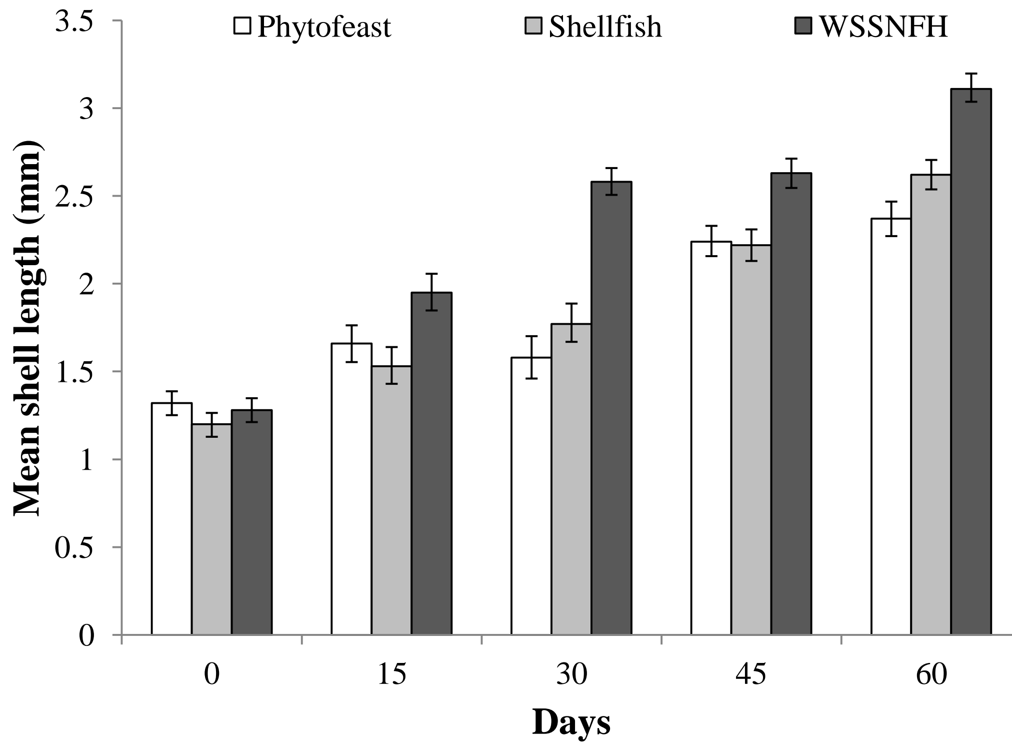


Figure 4. Shell length (mm) of juveniles of *Epioblasma t. rangiana* at 15 d intervals during the 60-d experiment for the three diet treatments. All three diets were significantly different, and WSSNFH mix produced the largest shell length (Mean ± 95% CI; $p<0.0001$).

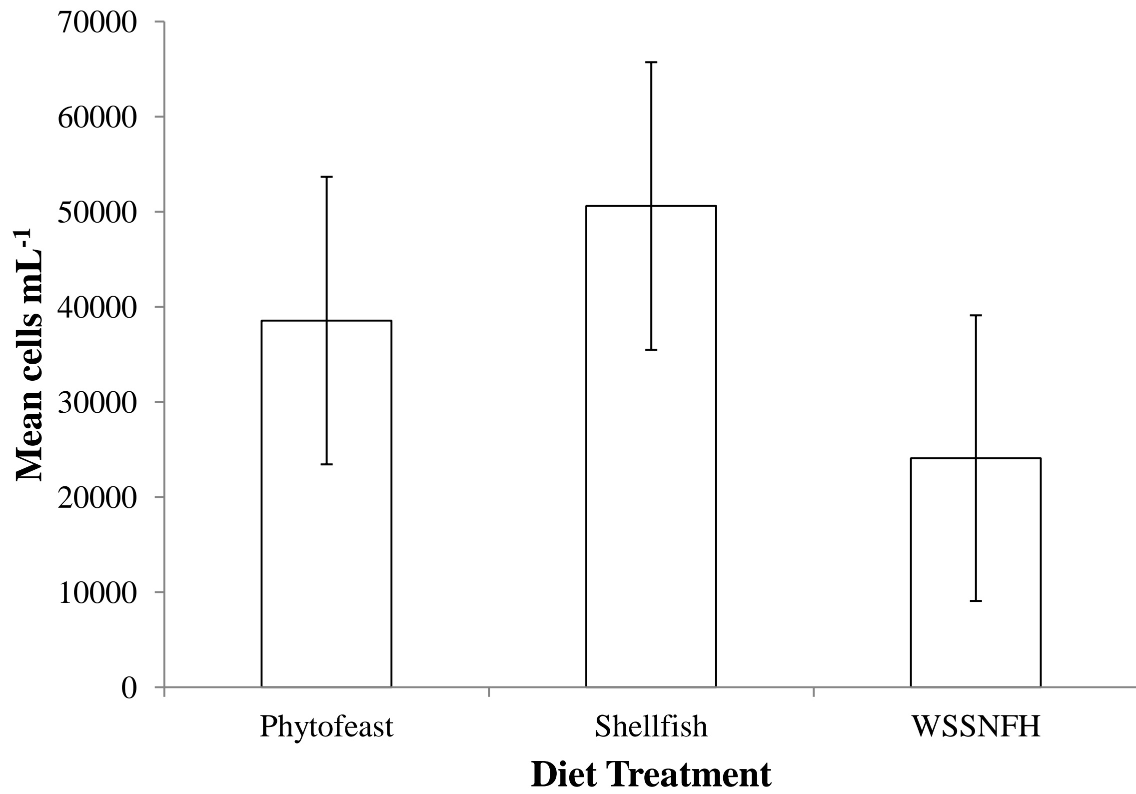


Figure 5. Cell counts (cells $mL^{-1}$) for the three diets during the 60-d experiment. No significant difference was observed among the three diets (Mean ± 95% CI; $p=0.0521$). Cell counts were recorded daily.

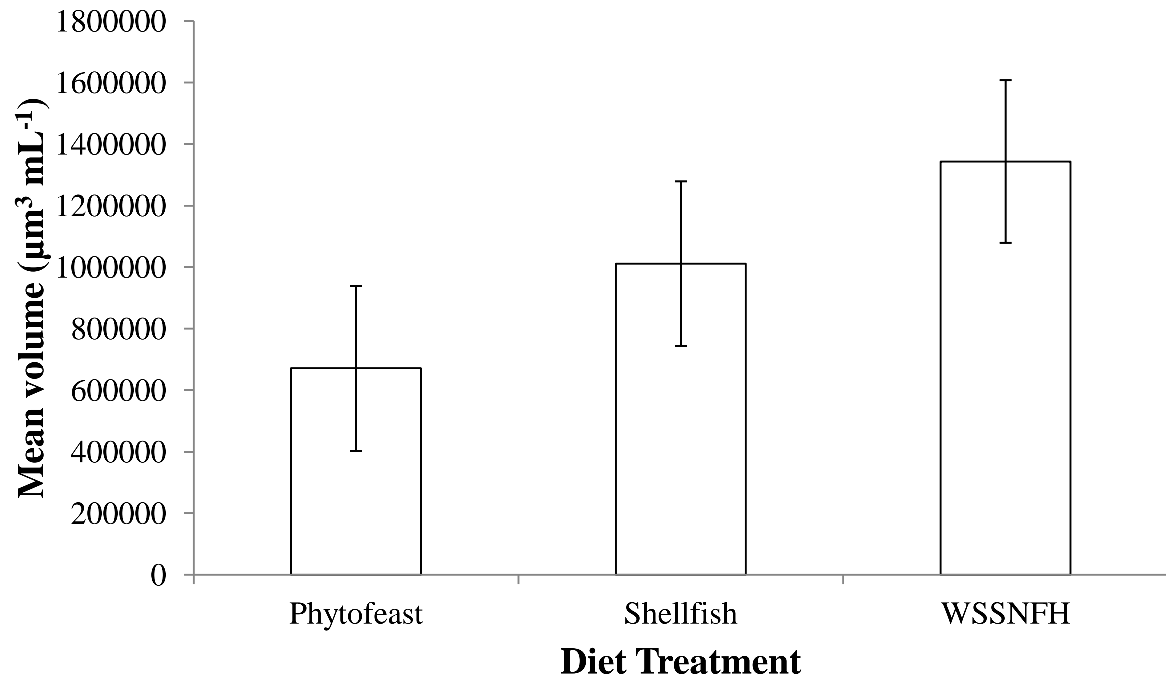


Figure 6. Cell volumes (cells $\mu m^3$ $mL^{-1}$) for the three diets during the 60-d experiment. WSSNFH mix and Phytofeast were significantly different (Mean ± 95% CI; $p<0.01$). Cell volumes were recorded daily.

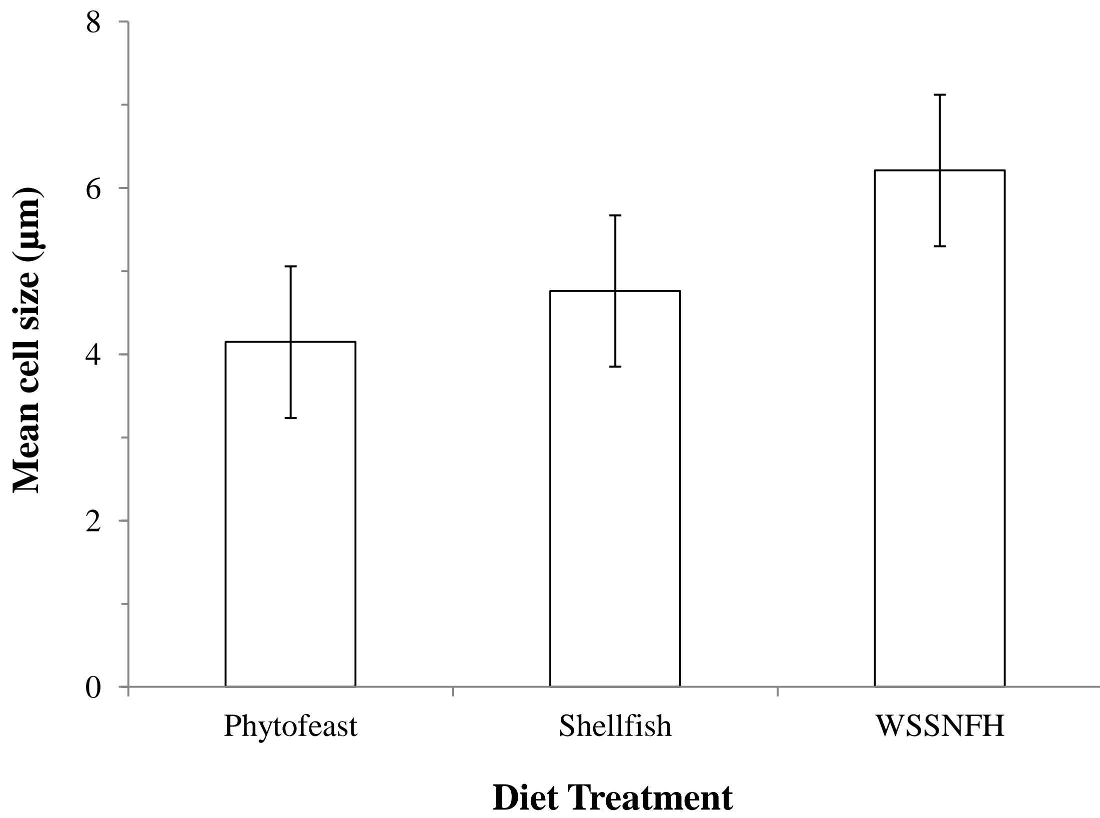


Figure 7. Cell size (µm) for the three diets during the 60-d experiment. WSSNFH mix had significantly larger cells than Phytofeast (Mean ± 95% CI; $p$=0.01). Cell sizes were recorded daily.

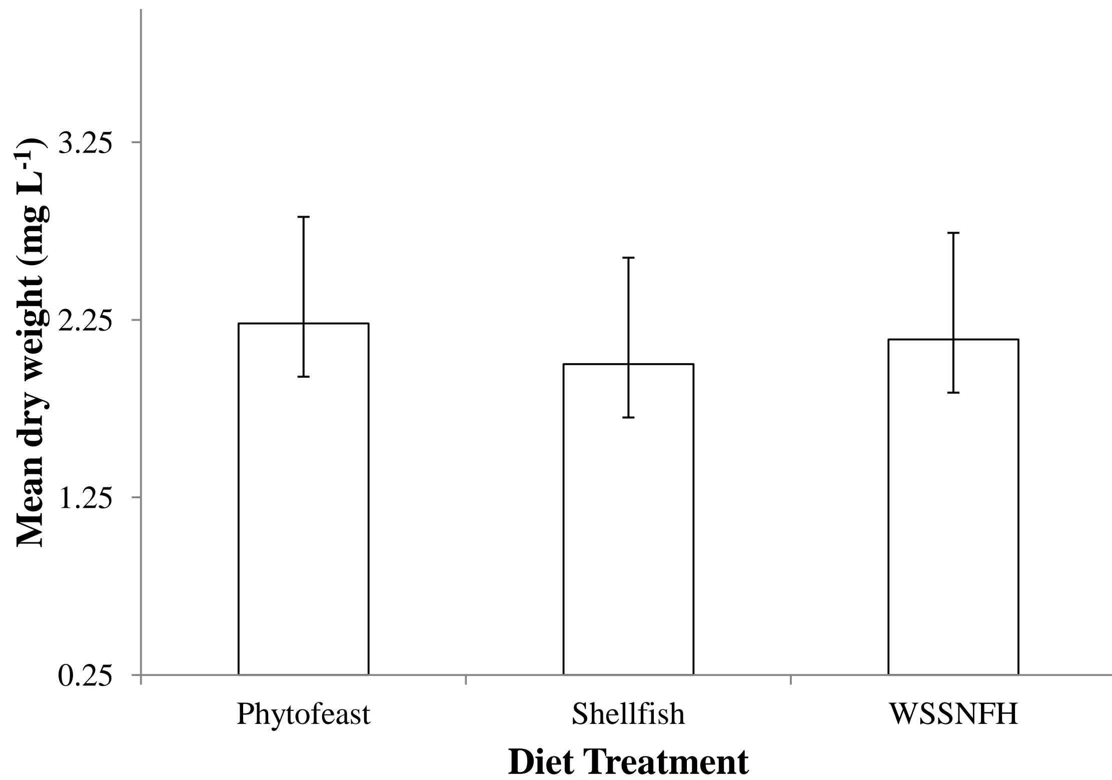


Figure 8. Dry weight (mg $L^{-1}$) of algae in culture buckets for the three diets during the 60-d experiment, with no significant difference among treatments (Mean ± 95% CI; $p$=0.08). Dry weight analysis was performed twice on each treatment.

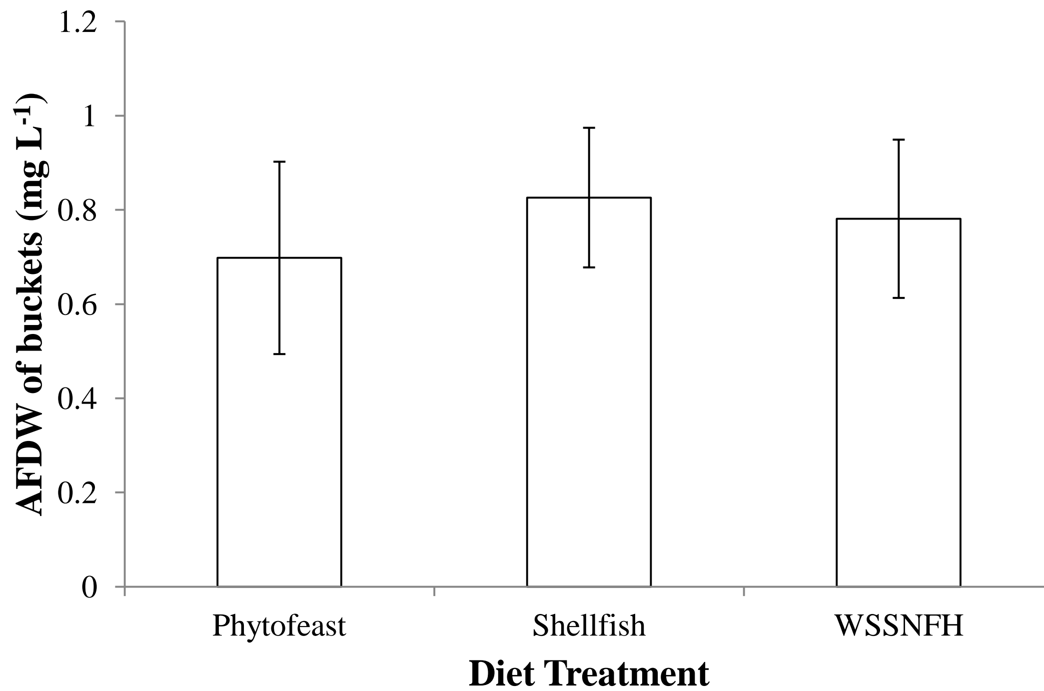


Figure 9. AFDW (mg $L^{-1}$) of algae in culture buckets for the three diets during the 60-d experiment, with no significant differences observed among treatments (Mean ± 95% CI; $p$ =0.50). Dry weight analysis was performed twice on each treatment.

## Chapter 3. A suitable density of algae for feeding juvenile mussels reared in recirculating aquaculture systems.

ABSTRACT

In recent years, propagation and culture has been accepted as a method for resource managers to enhance current mussel populations, establish new populations, and revitalize historic populations. This experiment evaluated the effect of food concentration in cells $mL^{-1}$ on growth (shell length) and survival of juveniles of *E. t. rangiana* and *A. ligamentina*. Juveniles were fed a live algae diet at three concentrations: 30,000, 80,000, and 140,000 cells $mL^{-1}$. Mean percent survival of juveniles of *E. t. rangiana* for the low (30,000 cells $mL^{-1}$), medium (80,000 cells $mL^{-1}$), and high (140,000 cells $mL^{-1}$) feed concentrations were 39.1 (95% CI: 30.7 to 47.4), 20.7 (95% CI: 12.8 to 28.6), and 12.7 (95% CI: 4.82 to 20.5), respectively. Significant differences ($p<0.01$) were observed between the low concentration when compared to the medium and high feed concentrations. Mean shell lengths of juveniles of *E. t. rangiana* at 60 d for the low, medium, and high feed concentrations were 1.58 mm (95% CI: 1.49 to 1.66), 1.30 mm (95% CI: 1.19 to 1.40), and 1.01 mm (95% CI: 0.936 to 1.08), respectively. Mean shell length of juveniles for all three feed concentrations were significantly different from each other ($p<0.0001$), with the lowest concentration having the highest growth and the highest feed concentration having the lowest growth.

Mean percent survival for juveniles of *Actinonaias ligamentina* for the low, medium, and high feed concentrations were 46.8 (95% CI: 35.2 to 58.4), 24.6 (95% CI: 15.1 to 34.0), and 10.7 (95% CI: 5.45 to 15.9), respectively. A significant difference was observed between the low feed concentration and the medium and high feed concentrations ($p<0.01$). Mean shell lengths for the low, medium, and high

concentrations were 1.15 mm (95% CI: 1.08 to 1.22), 0.994 mm (95% CI: 0.930 to 1.06), and 0.833 mm (95% CI: 0.770 to 0.896), respectively. Significant differences were observed ($p<0.0001$) among all treatments, with the largest growth again occurring in the low concentration treatment. Results showed that juveniles of *E. t. rangiana* and *A. ligamentina* have higher growth and survival when fed the low concentration of algae.

## Introduction

Algae are perhaps the most commonly utilized food source for rearing juvenile mussels in the laboratory. Despite this importance for mussel culture, few studies have investigated dietary requirements, including the quantity to feed. Most studies have investigated particle-size selection of juvenile and adult mussels, revealing that they are able to select and sort algal based on size prior to ingestion (Vanderploeg 1995; Paterson 1984; Beck 2003). Lasee (1991) investigated the digestive tract of 2 d-old *Lampsilis ventricosa* and discovered that the mouth of this species is only 16 µm wide and the esophagus 6 µm wide. Beck (2003) determined the size of algal cells suitable for ingestion by young juveniles to be 2.8 to 8.5 µm. Vanderploeg et al. (1995) determined that adult *Lampsilis radiata siliquoidea* filter particles from 1.3 to 3.0 µm with 100% efficiency, and similarly Patterson (1984) reported that *Elliptio complanata* selected particles in the 4-5 µm range. Yeager (1994) suggested that the primary food source for juvenile mussels changes as they transition from pedal-feeding to suspension feeding, with younger (3-5 d-old) juveniles feeding on flagellated bacteria, diatoms, algae, and detritus 2-5 µm in size. Older juveniles (10-14 d-old) ingest inorganic sediments and algae, with bacteria of lesser importance. Nutritional value of algae is important, with

newly metamorphosed juveniles requiring a diet high in lipids and polyunsaturated fatty acids (Gatenby et al. 1997).

Another important factor for determining a suitable diet for juveniles is the concentration of algae in the culture system. Early studies indicated that a ration of 20,000-30,000 cells $mL^{-1}$ was sufficient for juveniles; however, some studies have shown excellent survival at much higher densities, 300,000-500,000 cells $mL^{-1}$ (Gatenby et al. 1997; Rodgers 1999; Henley et al. 2001; Barnhart unpublished data). It is important to determine a suitable algal density in recirculating aquaculture systems to feed mussels to promote adequate growth and survival without fouling the culture water and wasting resources.

Two mussel species were chosen for this study, the northern riffleshell (*Epioblasma torulosa rangiana*) and the mucket (*Actinonaias ligamentina*). Each species was chosen because of differences in life history, distribution, status, and high variability in juvenile growth and survival among species in hatchery settings (Jones et al. 2005; Liberty 2007; Vincie 2008). For example, *E. t. rangiana* was listed as federally endangered in 1993 and has been extirpated from most of its historic range in the United States (USFWS 1994); current range includes Kentucky, Illinois, Ohio, Pennsylvania, and West Virginia (WV). However, the species is generally considered extant in only seven streams from these states, with the largest population occurring in the Allegheny River, Pennsylvania.

In Canada, *E. t. rangiana* occurs in the Sydenham and Ausable rivers and was ranked as Endangered by the Committee on the Status of Endangered Wildlife in Canada in 2010. Individuals are relatively short-lived (max. 10-15 y), small in size (max. 65

mm), thin-shelled, sexually dimorphic, and primarily use darters as host fishes. In particular, documented host fish include banded sculpin (*Cottus carolinae*), bluebreast darter (*Etheostoma camurum*), banded darter (*Etheostoma zonale*), and brown trout (*Salmo trutta*) (Watters, 1996). The genus *Epioblasma* is considered the most imperiled taxon of mussels in North America. Because results from my experiment may be used to develop culture methods for other species of *Epioblasma,* I included a representative of this group in my study.

The mucket *A. ligamentina* is a common mussel species found throughout most of its historic range in the Ohio, Tennessee and Mississippi River systems, with reproducing populations in 20 states, from Minnesota east to New York and south to Louisiana. It also can be found in the St. Lawrence River basin and tributaries of lakes Erie, Michigan, and Ontario (Burch 1975; Parmalee and Bogan 1998). The species is long-lived (>20 y), large in size (>120 mm), thick shelled, does not show sexual dimorphism, and uses a variety of host fishes, including largemouth bass (*Micropterus salmoides*) and smallmouth bass (*Micropterus dolomieu*) as primary host fishes (Parmalee and Bogan 1998).

This experiment compared the effects of three algal concentrations on growth and survival of two captively-reared species, *Epioblasma torulosa rangiana* and *Actinonaias ligamentina,* to identify an adequate algal feed concentration for rearing juvenile mussels. A suitable algal density should increase growth and survival and allow more mussels to be produced and released into restoration areas.

## Methods

*Adult Mussel Collection*

Gravid females of *A. ligamentina* and *E. t. rangiana* were collected from the Alleghany River (River Km 111.7) at East Brady, Clarion County, Pennsylvania during a bridge relocation project in May 2007. Divers from the Ohio River Islands National Wildlife Refuge (ORINWR, USFWS), West Virginia Department of Natural Resources (WVDNR), and White Sulphur Springs National Fish Hatchery (WSSNFH) collected the mussels during relocation efforts at other sites in West Virginia and Ohio. Both species were collected and checked for gravidity by carefully opening the valves 5-10 mm and looking inside for gills inflated with glochidia. After gravidity was assessed, I transported 12 gravid *E. t. rangiana* and five gravid *A. ligamentina* to WSSNFH. Mussels were transported in a 94.6 L cooler with a 12-volt Super Fish Saver aerator (Marine Metal Products, Inc., Clearwater, Florida). In addition, extra river water was transported in a cooler to perform a 50% water change during transport since travel time was over 6 h in duration.

Upon arrival at WSSNFH, mussels were slowly acclimated to the facility's holding system temperatures. Mussels were held in recirculating troughs containing approximately 15 cm of mixed gravel and sand substrate. The substrate consisted of 40% small pebble (<2 cm) and 60% rinsed sand. Holding troughs were equipped with a three-quarter hp chiller (Aqua Logic, Inc., San Diego, California) and 1 kW submersible heaters (Innovative Heat Concepts, LLC, Homestead, Florida) to maintain water temperature at 20± 1º C.

*Automatic Feeding System*

The WSSNFH developed an automatic feeding system that allows for continuous feeding of juvenile and adult mussels using a series of solenoid valves and timers. A 120 L feed tank with two pumps delivered concentrated, live, cultured algae. One submersible pump kept algae in suspension within the tank. The other external pump recirculated the algae through a 1.9 cm pvc manifold that extended the length of the laboratory and returned to the feed tank. The manifold had solenoid valves attached that opened to release algae into the rearing systems. Each solenoid was connected to a timer that controlled the opening time and frequency of the solenoid valve and therefore controlled how much algae was delivered to juvenile rearing systems distributed throughout the laboratory.

*Culture of Live Algal Diet*

Methods to culture algae for use in the experiment were a modification of those used by Hoff and Snell (1999). Once cultures were sufficiently concentrated, a larger continuous algae production system, called a Biofence (http://www.variconaqua.com/, Varicon Solutions Ltd., Malvern, United Kingdom) was used.

The Biofence is a 350-L photo-reactor where algae is cultured inside clear plastic tubes which recirculate into a head tank. Carbon dioxide and nutrients were automatically adjusted according to pH levels and water flow into the system. Once sufficient water and nutrients were added, the excess algae was pumped into a centrifuge and spun down into a paste. The algae mix paste was vacuum-sealed and refrigerated for later use.

*Host Fish Collection*

Largemouth bass (*Micropterous salmoides*) were used to metamorphose glochidia of *A. ligamentina* to juvenile mussels, and sculpins (*Cottus sp.*) were used to metamorphose glochidia of *E. t. rangiana.* A total of 100, 13 to 18 cm largemouth bass were purchased from Rainbow Head Farms, Clarksburg, West Virginia, in May 2007. Fish were transported in a 946 L fish hauling tank with aeration back to WSSNFH. A total of 100 sculpins were collected by staff at WSSNFH from Howards Creek, Greenbrier County, West Virginia, also in May 2007. Sculpins were collected using a Smith-Root LR-21 series backpack electrofisher (Smith-Root, Inc., Vancouver, Washington) and an 2.4 m seine net (Aquatic Ecosystems, Inc., Apopka, Florida). The seine net was set 3 to 5 m below the electroshocker. Stunned fish floated downstream into the seine and were immediately placed into a 18.9 L bucket with river water. After 25 to 40 fish were collected in the bucket, the fish were transferred to a cooler filled with river water and a dilute concentration of salt (NaCl) to reduce stress during transport. Sculpins were transported in a 94.6 L cooler using a 12-volt Super Fish Saver aerator (Marine Metal Products, Inc., Clearwater, Florida) to provide aeration during transport.

*Production of Juvenile Mussels*

Glochidia were extracted from gravid females using a 21-gauge 3.8-cm hypodermic needle and syringe filled with water (Zale and Neves 1982). The needle was inserted into the posterior end of each gill to flush the glochidia from the gill into a Petri dish. Viability was assessed by using a dilute NaCl solution to a sample of glochidia to assess response time. This viability test showed prompt response by valve closure to the NaCL solution. To infest sculpins with *E. t. rangiana,* 60 fish were placed in a well-

aerated 6L plastic container with 1.0 L of water. Glochidia extracted from three female mussels were combined and added to the container holding the host fish and aerated vigorously for approximately 1 h. Fish and glochidia were stirred about 30 min during the infestation process to keep glochidia in suspension.

Once the infestation was complete, sculpins were separated by size and placed into an AHAB system (Aquatic Ecosystems, Inc., Apopka, Florida). In order to prevent cannibalism among individuals, fish were graded into comparable size groups. No more than 15 sculpins were put in each 3-L AHAB tank. To infest *A. ligamentina,* glochidia of all five mussels were extracted using the same technique described above. One hundred largemouth bass were split into two 18.9 L buckets, each filled one-third full of water. The extracted glochidia were split into two Petri dishes. Immediately following placement of the fish into buckets, the glochidia were added and the fish gently stirred. The fish were heavily aerated with two 7.6 cm airstones. After about 5 min, the gills of the fish were carefully examined for larval attachment and visually checked every 3-4 min until larvae were visually conspicuous on the gills. After about 15 min, the fish were removed and placed in AHAB systems. Water temperature in all fish systems were set at 20 degrees Celsius. Sculpins were held in stand-alone AHAB recirculating systems, with 15 fish in each 9-L acrylic tank. Largemouth bass were held in recirculating AHAB systems with one fish in each 9 L tank. Aquaria and juvenile rearing systems at WSSNFH, White Sulphur Springs, West Virgina, were used to hold the fish as well as to collect juveniles.

*Culture of Juvenile Mussels*

Juvenile mussels excysted from host fish beginning after 2 wk and through 4 wk, post-infestation. Once juvenile mussels began excysting from the host fish, sieves from the self-siphoning AHAB aquaria were checked every 1-2 d to recover the juveniles. Excysted juvenile mussels were collected and held in 'Bucket of Muckets' (Barnhart Buckets) juvenile culture systems (Barnhart 2006) for 2 wk prior to initiating the experiment. This initial culture period allowed sub-viable juveniles to die and thus avoid initial mortality during the experiment. Each bucket culture system consisted of two nested buckets, a 18.9 L bucket serving as the bottom and a 11.4 L bucket nested inside of it (Figure 1). The inner bucket had six, 5.0 cm holes drilled into it to hold mesh containers for holding juvenile mussels. The mesh containers were made from nylon mesh glued onto a 4.4 cm section of 5.0 cm schedule 40 polyvinyl chloride (PVC) pipe. The pipe and mesh then were pressed into a 5.0 cm PVC coupling, forming a cup. The two cups were pressed together to create the containers for holding juvenile mussels. In this experiment, since juveniles were ~200 µm in length at the start of the experiment, the mesh size used on the juvenile cups was 200 µm. Six containers were fitted into the drilled holes in the inner bucket of the culture systems. A total of 18 L of water was recirculated between the two buckets by pumping water through the mesh containers using a small submersible pump (Marineland, Inc., Blacksburg, Virginia), creating a down-welling effect.

Juvenile mussels were fed one of three concentrations of algae; 30,000, 80,000, and 140,000 cells $mL^{-1}$, referred to as low, medium, and high, respectively. Algal density in the culture systems was recorded daily using a Beckman Coulter Counter, (Beckman

Coulter, Inc., Brea, California) and recorded as cells $mL^{-1}$ and $\mu m^3$ $mL^{-1}$ for the 60-d experiment. In addition to algal density counts, culture water from the buckets was filtered, dried, and ashed to calculate ash-free-dry weight and organic content within the culture water.

The experimental design consisted of three buckets per treatment, and three chambers containing juveniles for each species per bucket (Figure 2). Therefore, 6 chambers per bucket were occupied with juveniles, three with juveniles of *A. ligamentina* and three with juveniles of *E. t. rangiana*. In total, nine buckets and 54 containers of juveniles were used in the experiment (Figure 2). During experimental set-up, 200 juveniles of each species were placed in labeled juvenile containers, and 10 juveniles per chamber were measured using a microscope and ocular micrometer.

Mesh screens on the juvenile containers were sprayed off and cleaned twice daily with a household garden sprayer to avoid the clogging of juvenile holding chambers, especially given the juvenile densities per bucket system. Weekly, each bucket received a water exchange, replacing the culture water with clean, filtered (<30 µm) spring water. Juveniles were sampled every 15 d, with growth and survival recorded during each sample event. Sample events consisted of removing the containers from the bucket and inspecting and measuring the juveniles with the microscope. Growth (length in mm) and percent survival were evaluated for all containers and treatments. In addition, every 15 d the nylon mesh screens were changed in all of the juvenile chambers to prevent clogging. Juveniles were cultured throughout the experiment at 20-21° C, and temperature was maintained and recorded daily in each bucket by a heated water bath. A recirculating

trough system that included a heater and chiller was used as a temperature-controlled water bath for all buckets.

*Feeding Juvenile Mussels*

Juveniles were fed live algae cultured at WSSNFH containing the following mix of 4 species: *Bracteacoccus grandis, Neochloris oleoabundans, Phaeodactylum tricornutum,* and *Oosystis polymorpha.* Juveniles were fed continuously by an automated feeder system, controlled by setting timers and solenoids to release a pre-determined amount of algae into each bucket to maintain target concentration. All three concentrations were established by diluting known concentrations of feed mix to reach the treatment concentrations of the low, medium, and high treatments. After the concentrations were obtained, cell counts of each treatment were conducted every 5 min for 2 h. After 30 min, treatment concentrations dropped below 50%, so the timers were set to dispense algae at 30-min intervals. Since algae mix concentrations varied throughout the experiment, all feed was diluted to ~5,000,000 cells $mL^{-1}$ or 150,000,000 $\mu m^3$ $mL^{-1}$ to be fed to the mussels. The volume of feed delivered every 30 min to maintain the desired treatment density was approximately 3 mL for the low, 36 mL for the medium, and 90 mL for the high concentrations.

*Dry Weight and Ash-Free Dry Weight*

The feed mix was analyzed for dry and ash-free dry weight to evaluate organic content of the juvenile diet. In addition, a water sample from each juvenile bucket was analyzed for dry and ash-free dry weight. Samples were collected on GF/F 25 mm filters (Fisher Scientific, Inc., Pittsburgh, Pennsylvania) and first ashed in a muffle furnace at 232º C for 24 h and weighed. Coulter counts (Beckman Coulter) in cells $mL^{-1}$ and $\mu m^3$

mL$^{-1}$ for each sample filter also were recorded. Feed mix and juvenile buckets were filtered using a vacuum pump with previously ashed filters. The volume of sample water filtered was recorded in mL for each sample. Filters were then dried in a muffle furnace for 24 h at 15.6º C and then weighed to obtain dry weight (g). Filters then were ashed again at 232º C for another 24 h to get the ash-free dry weight for each sample. Each bucket and feed mix were evaluated twice (n=2) using this method during the experiment.

*Statistical Analysis*

All statistical analyses were completed in JMP, version 10.0 (SAS Institute 2010). I compared percent survival of juveniles among the three feed concentration treatments every 15 d during the 60-d experiment. Survival is reported as a percentage of survival during 15-d intervals for the 60-d experiment. Differences in mean shell length over 60 d also were compared among the three treatments. One-way analysis of variance (ANOVA) and student *t*-tests were used to analyze growth and survival of juveniles. All statistical tests conducted in this study were considered significant at an alpha level of 0.05 or less.

**Results**

*Juvenile Growth and Survival*

Mean percent survival of juveniles of *Epioblasma. t. rangiana* for the low medium, and high feed concentrations were 39.1 (95% CI: 30.7 to 47.4), 20.7 (95% CI: 12.8 to 28.6), and 12.7 (95% CI: 4.82 to 20.5), respectively. There were significant differences ($p<0.01$) observed between the low concentration and the medium and high concentrations (Figure 3). Student's *t*-test also showed a significant difference in mean percent survival between the low concentration and medium ($p<0.0001$) and high ($p<0.01$) concentrations; however, a significant difference was not observed between the

medium and high treatments ($p$=0.15). Mean shell lengths of juveniles of *E. t. rangiana* at 60 d for the low, medium, and high concentrations were 1.58 mm (95% CI: 1.49 to 1.66), 1.30 mm (95% CI: 1.19 to 1.40), and 1.01 mm (95% CI: 0.936 to 1.08), respectively, and all values were significantly different from each other ($p$<0.0001)(Figure 4). The low concentration exhibited the highest juvenile growth, whereas the high concentration had the lowest growth (Figure 4).

Mean percent survival of juveniles of *Actinonaias ligamentina* in the low, medium, and high concentrations were 46.8 (95% CI: 35.2 to 58.4), 24.6 (95% CI: 15.1 to 34.0), and 10.7 (95% CI: 5.45 to 15.9), respectively (Figure 5). Significant differences were observed between the low concentration and the medium and high feed concentrations ($p$<0.01). There was no significant difference between the medium and high feed concentrations ($p$>0.05). Mean lengths of juveniles for the low, medium, and high feed concentrations were 1.15 mm (95% CI: 1.08 to 1.22), 0.994 mm (95% CI: 0.930 to 1.06), and 0.83 mm (95% CI: 0.770 to 0.896), respectively, with significant differences ($p$<0.0001) among all three concentrations (Figure 6).

No significant differences were observed in survival between mussel species at the low ($p$=0.56), medium ($p$=0.80), or high feed concentrations ($p$=0.83) (Figure 7). However, significant differences were observed in shell lengths between species in the low ($p$<0.0001), medium ($p$<0.0001), and high ($p$<0.0001) concentrations (Figure 8).

*Cell Counts*

Mean cell counts $mL^{-1}$ for the low, medium, and high concentrations were 29,592.4 (95% CI: 18,910 to 40,275), 77,241.7 (95% CI: 66,604 to 87,879), and

139,908.3 (95% CI: 129,271 to 150,546), respectively (Figure 9), and all significantly different from each other ($p<0.0001$).

*Dry and Ash-free Dry Weights of Treatments*

Dry weights of algae in the treatment buckets were all significantly different ($p<0.0001$). The low feed concentration had a mean dry weight of 1.90 mg $L^{-1}$ (95% CI: 1.04 to 2.76), medium was 4.89 mg $L^{-1}$ (95% CI: 2.91 to 6.87), and high was 9.88 mg $L^{-1}$ (95% CI: 7.20 to 12.6) (Figure 10). Ash-free dry weights (AFDW) also were significantly different among treatments, with means for the low, medium, and high of 2.05 mg $L^{-1}$ (95% CI: 1.85 to 2.27), 5.55 mg $L^{-1}$ (95% CI: 5.34 to 5.75), and 9.67 mg $L^{-1}$ (95% CI: 9.49 to 9.85), respectively ($p<0.0001$) (Figure 11)

**Discussion**

Results of this study indicate that a continuous low density of algae (<30,000 cells $mL^{-1}$) provided to juvenile mussels in the Barnhart Bucket culture system yielded higher growth and survival than juveniles cultured at higher feed concentrations. Limited hatchery propagation work had been done with *A. ligamentina* because of its common abundance and status; however, the growth and survival reported in this study is comparable to those in other studies with common mussel species (Beck and Neves 2003; Liberty et al. 2007). Mean percent survival in this study for the low feed concentration was 39.1 % and 46.8% for *E. t. rangiana* and *A. ligamentina*, respectively. When this experiment was completed in 2007, no survival to 60 d had been reported for *E. t. rangiana*; however, survival for other *Epioblasma spp*. typically has ranged from 0.15-29.6% at 60 d (Zimmerman 2003; Jones et al. 2005; Barnhart 2006; Vincie 2008). Cell counts for these studies either were not made or ranged from 15,000-30,000 cells $mL^{-1}$.

Even though cell counts were close to the low concentration in my experiment, with the exception of Barnhart (2006), cell counts were not measured daily and mussels were not fed continuously in previous studies. Growth of *E. t. rangiana* at 60 d was higher in this study than in previously published studies on *Epioblasma spp*. Mean length in this study ranged from 1.01-1.58 mm, whereas previous studies recorded mean lengths ranging from 0.40-1.19 mm for *Epioblasma capsaeformis* at 60 to 70 d (Zimmerman 2003; Jones et al. 2005; Vincie 2008).

Higher growth and survival in this experiment, especially for the low algae concentration, could be explained by several factors. One such factor could be adequately monitoring cells in the culture system. Prior to 2004, food concentration in culture water was checked visually by assessing water color and on occasion could be counted manually using a hemocytometer. During my study, however, cell counts were performed daily to ensure that concentration treatments remained at target levels, and this practice maintained a relatively stable concentration of food in each culture system throughout the day. In earlier experiments, algae typically were added to the culture system once or twice daily (Gatenby 1996; Rodgers 1999; Jones et al. 2005). On a pre-trial experiment, I found that over half of the feed had either been consumed or had settled within 30 min. Therefore, it appears that by batch-feeding instead of continuous feeding, mussels were subjected to a high food concentration followed by hours of low food concentration until the next feeding.

Absorption efficiency and filtration rate of the juvenile mussels may be affected in high concentration treatments. One study showed that adult *Epioblasma spp*. fed a low algae concentration (20,000 cells $mL^{-1}$) had significantly higher absorption efficiency rate

than medium (40,000 cells $mL^{-1}$), high (80,000 cells $mL^{-1}$), and very high (120,000 cells $mL^{-1}$) feed rations (Bush 2008). With the freshwater fingernail clam, *Sphaerium striatinum*, Hornbach et al. (1984) found that as particle numbers increased, filtration rates decreased. Several marine studies also have investigated food concentration and filtration rate during various concentrations of algal feed. In these studies, filtration rate increases until a maximum amount of food was ingested (Winter 1978; Navarro 1982). Once the maximum ingestion rate was reached, filtration rate decreased continuously until the production of pseudofaeces began, and if concentration further increased, filtration and ingestion rate declined (Winter 1978; Navarro 1982).

It also has been shown that an increase in organic matter decreases clearance rate or filtration in marine bivalves (Bacon et al. 1998). In short, to control food intake, bivalves expend energy to produce pseudofaeces by decreasing absorption efficiency and reducing filtration rates; therefore, when bivalves are in contact with a surplus of food, the energy left over for growth and reproduction decreases (Navarro 1982).

In addition to the high feed concentration impacting growth by using energy for filtration/pseudofaeces production instead of growth, water quality parameters, in particular nitrogen in the form of ammonia, also could limit growth and survival in young juvenile mussels. Juveniles and adult freshwater mussels have been shown to be highly sensitive to ammonia (Augspurger et al. 2003; Wang et al. 2011). As the live concentrated algal paste begins to decompose, it can produce a high ammonia level with high feed concentrations. As well, the commercial diets that are used today to culture juvenile freshwater mussels have higher ammonia levels, another reason to feed mussels

the least amount as necessary, without limiting their growth. These factors could explain the low growth and survival in the higher feed concentrations in this study.

**Conclusions**

Results from my study showed that growth and survival of juveniles of *A. ligamentina* and *E. t. rangiana* was higher in the low (30,000 cells $mL^{-1}$), continuous, algae concentration of the WSSNFH mix in the bucket culture systems. Use of this density should improve growth and survival for these and likely other species. Overfeeding was shown to drastically decrease rearing success in this study; therefore the goal is to have the lowest possible food ration to maintain the highest growth and survival of juveniles. The need for facilities to closely monitor food levels in culture systems was demonstrated by this study. I recommend that facilities monitor culture concentrations at a minimum of weekly, preferably three times per week. I also suggest an automatic feeder to feed juveniles a target concentration of 30,000 cells $mL^{-1}$ throughout the day.

Additional research is needed to further improve propagation and culture technology, such as developing a suitable for different ages of juveniles, as food concentration requirements may change as juveniles grow. Also, comparing growth and survival of young juveniles (<2 mo) with lower algae concentrations, 5,000 to 30,000 cells $mL^{-1}$, should be tested. Lastly, a suitable food ration defined in mg of algae per g mussel tissue should be identified.

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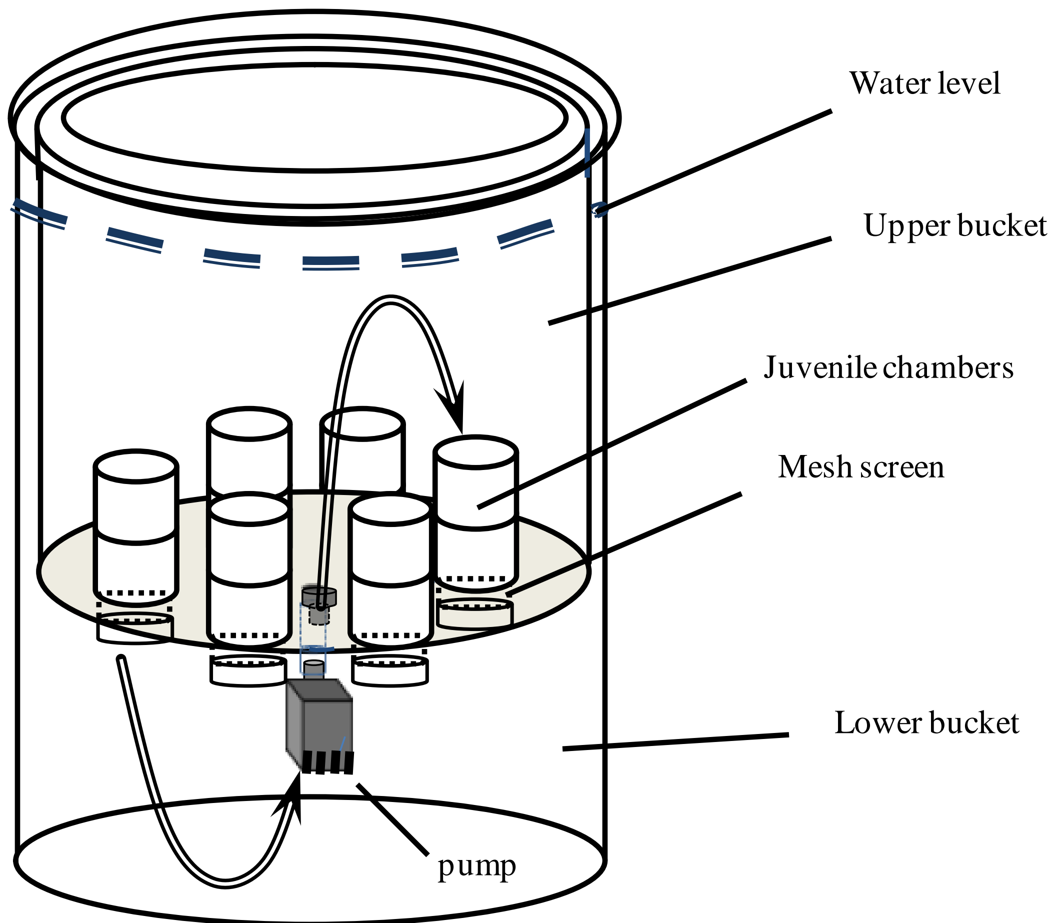



Figure 1. Diagram of bucket system. Lower bucket recirculates water by moving it into the upper bucket via pump. Water from the upper bucket gets pulled down through the juvenile chambers into the lower bucket (downweller).

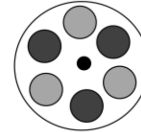
## Experimental Design

**Chambers**

= **200 *Epioblasma t. rangiana* juveniles**

= **200 *Actinonaias ligamentina* juveniles**

**Bucket System**



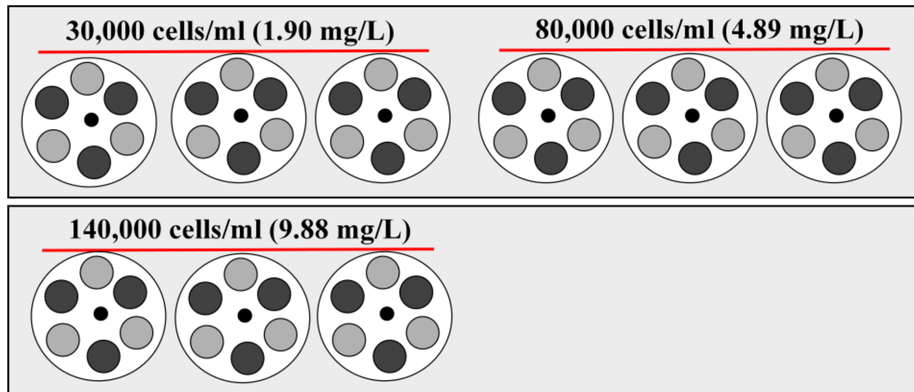



Figure 2. Experimental design to compare growth and survival of mussels among three feed concentrations, 30,000, 80,000, and 140,000 cells $mL^{-1}$. Two mussel species are represented by black or grey circles in each bucket system. In parentheses are the mean dry weights of the feed concentrations in mg $L^{-1}$.

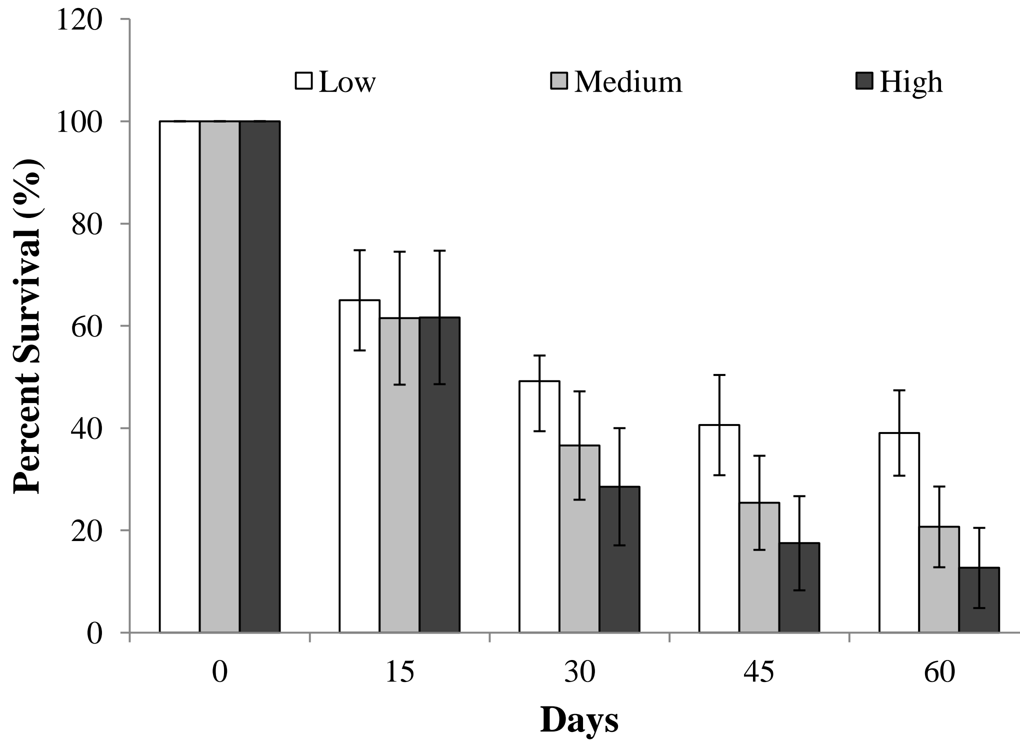



Figure 3. Percent survival (%) of juveniles of *Epioblasma t. rangiana* at 15-d intervals for 60-d. The low concentration had significantly higher survival than the medium and high concentration (Mean ± 95% CI; $p<0.01$).

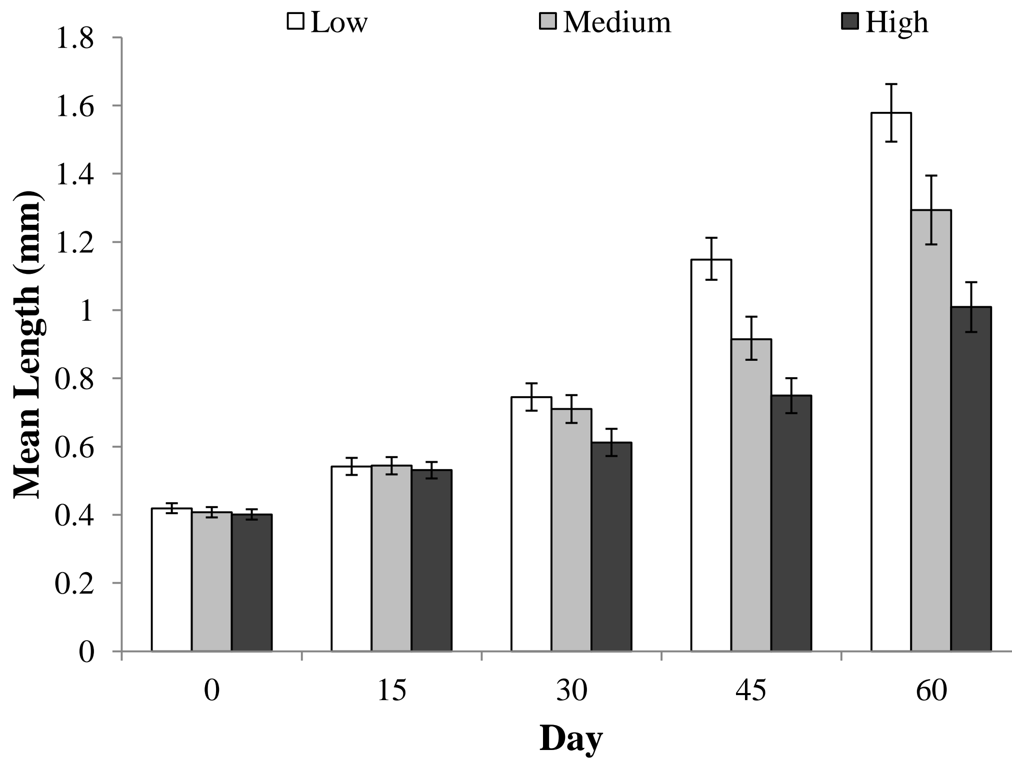



Figure 4. Shell length (mm) of juveniles of *Epioblasma t. rangiana* juveniles at 15-d intervals for the 60 d at three feed concentrations of low, medium, and high. All concentrations were significantly different from each other, and the low concentration exhibited the highest growth (Mean ± 95% CI; $p<0.0001$).

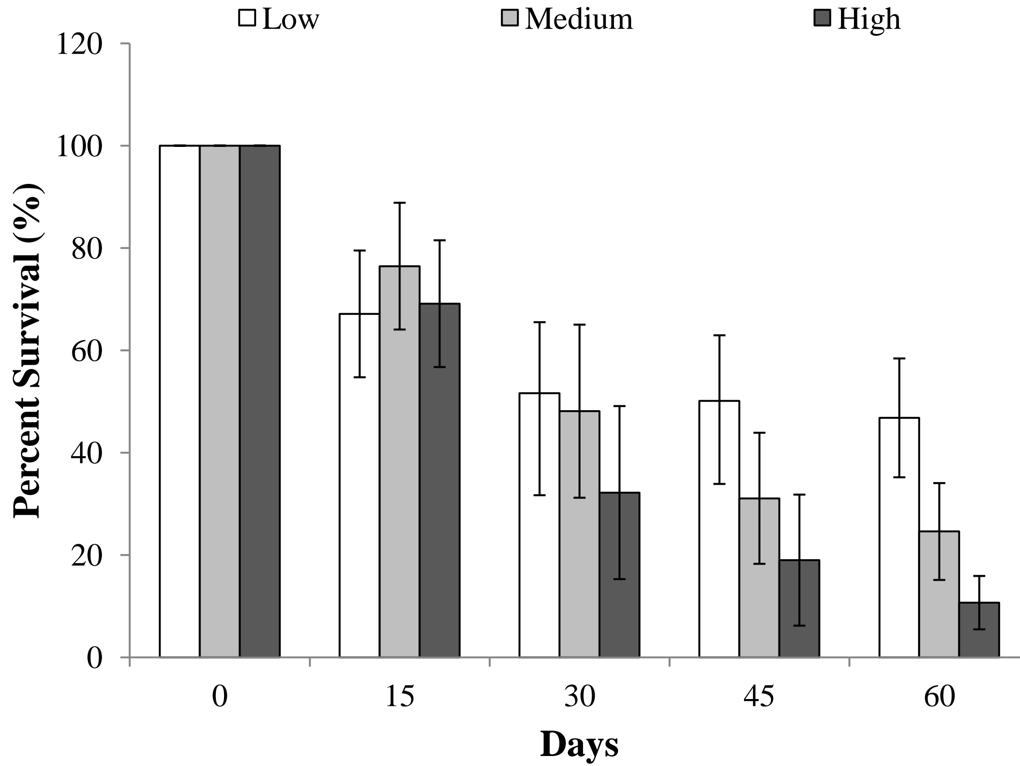



Figure 5. Percent survival of juveniles of *Actinonaias ligamentina* at 15-d intervals for 60 d at three feed concentrations of low, medium, and high. The low concentration was significantly higher than the medium and high concentrations (Mean ± 95% CI; $p<0.01$).

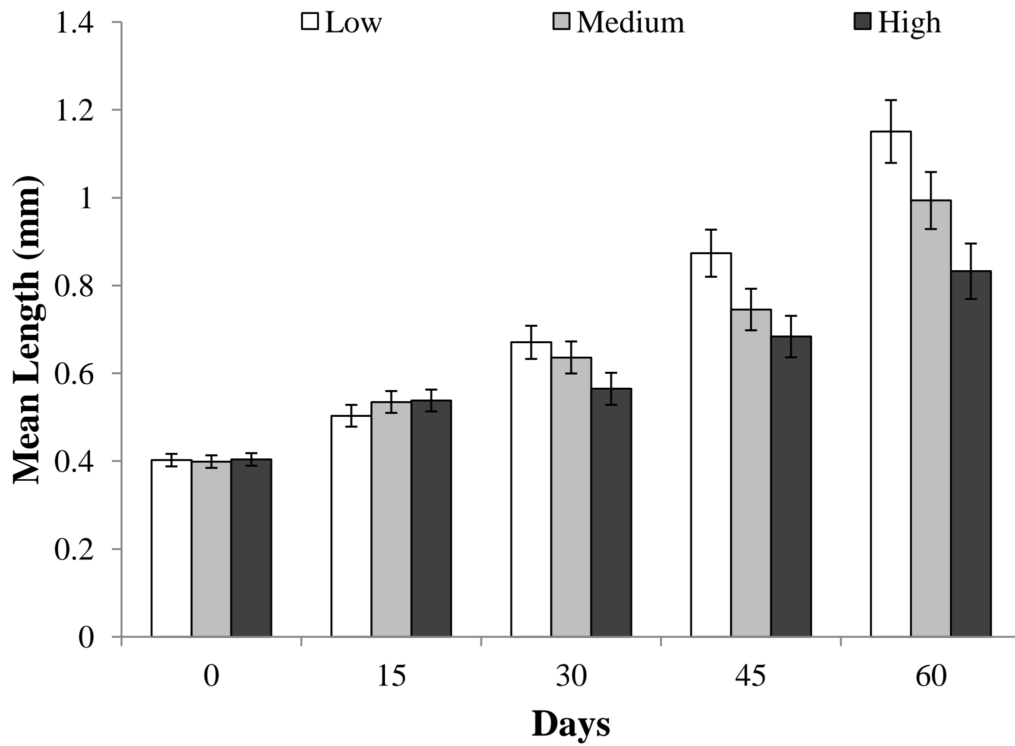



Figure 6. Shell length (mm) of juveniles of *Actinonaias ligamentina* at 15-d intervals for 60 d at three feed concentrations of low, medium, and high. All three concentrations were significantly different and the low concentration exhibited the greatest length (Mean ± 95% CI; $p<0.0001$).

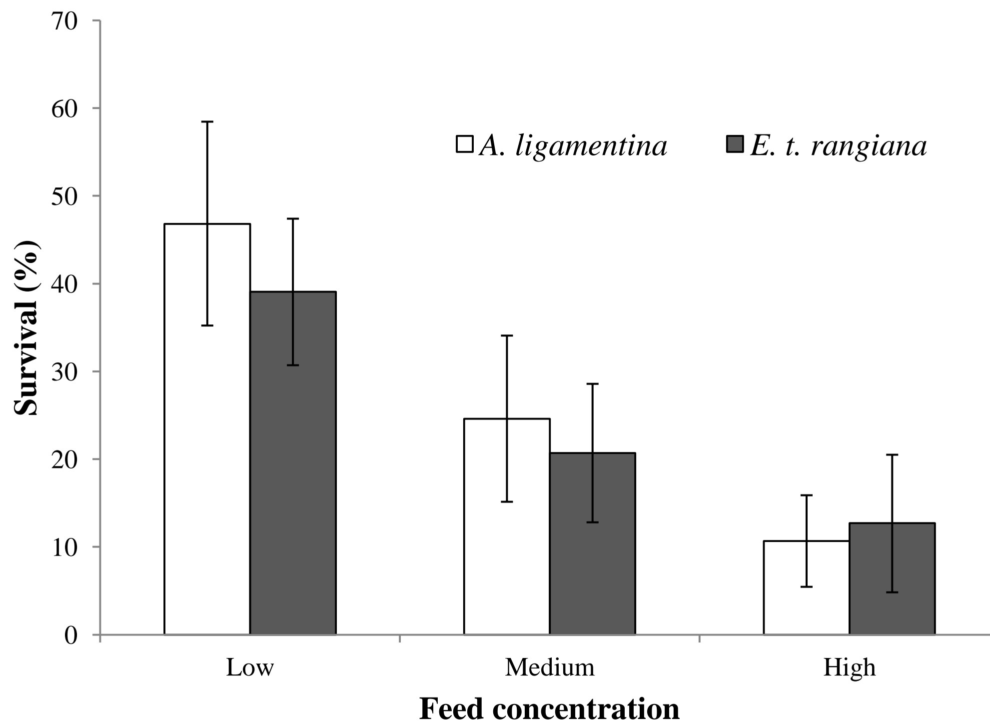



Figure 7. Survival (%) at 60 d of juveniles of *Actinonaias ligamentina* and *Epioblasma t. rangiana* for all three feed concentrations. There were no significant differences between the two mussel species (Mean ± 95% CI; $p$=0.84).

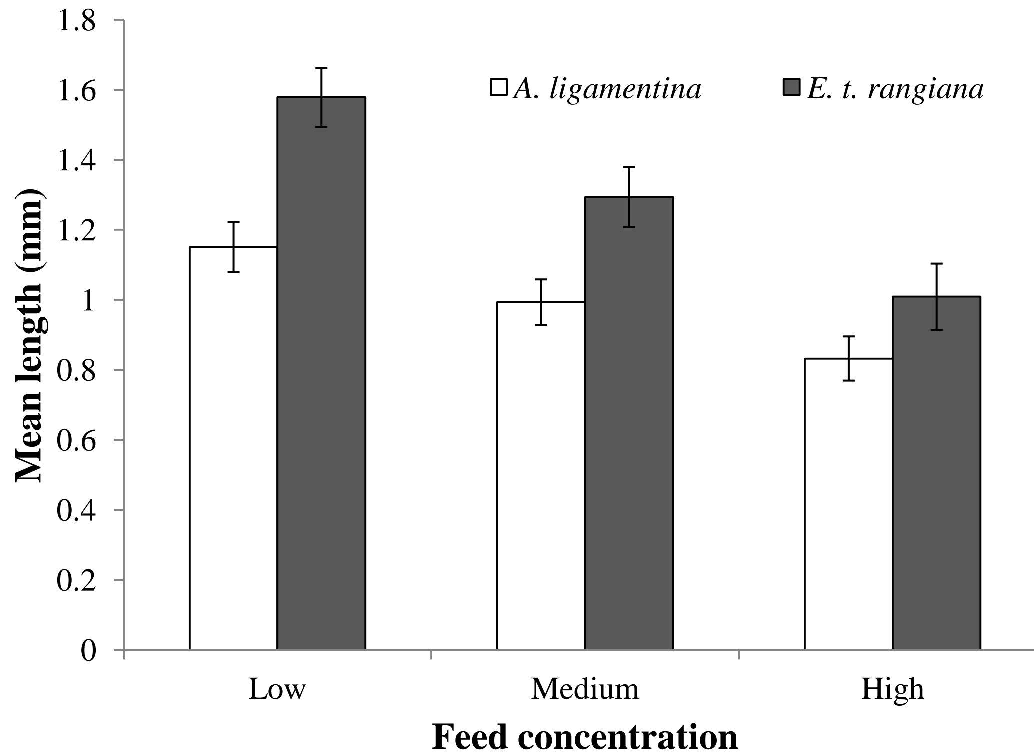



Figure 8. Shell length (mm) at 60 d for *Actinonaias ligamentina* and *Epioblasma t. rangiana* for all three feed concentrations. Juveniles of *E. t. rangiana* had significantly greater lengths than juveniles of *A. ligamentina* (Mean ± 95% CI; $p$<0.0001).

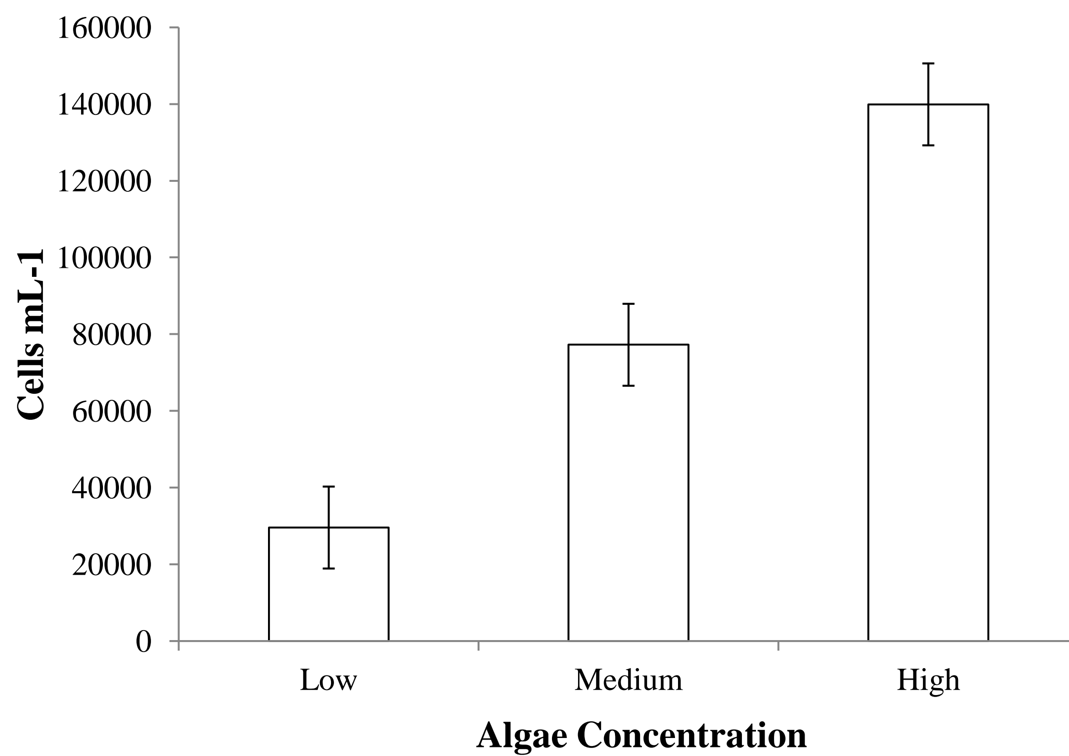



Figure 9. Cell counts (cells $mL^{-1}$) for the three feed concentrations during the 60-d experiment. Low, medium, and high concentrations were all significantly different (Mean ± 95% CI; $p$<0.0001). Cell counts for culture buckets were recorded daily.

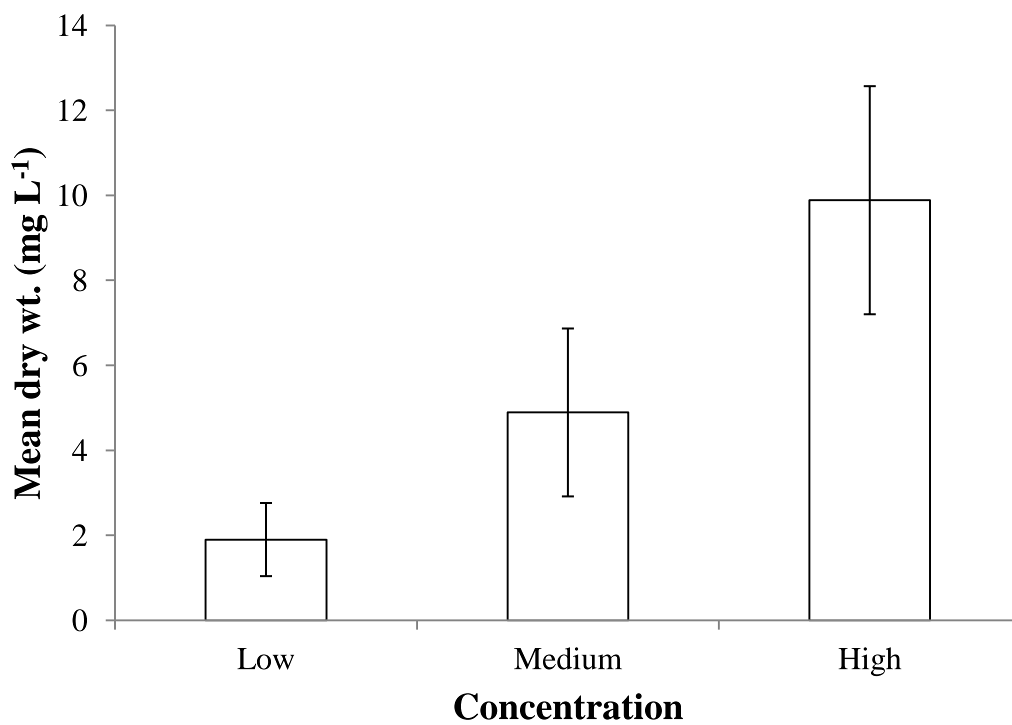



Figure 10. Dry weights (mg $L^{-1}$) for the three feed concentrations during the 60-d experiment. Low, medium, and high concentrations were all significantly different (Mean ± 95% CI; $p<0.0001$).

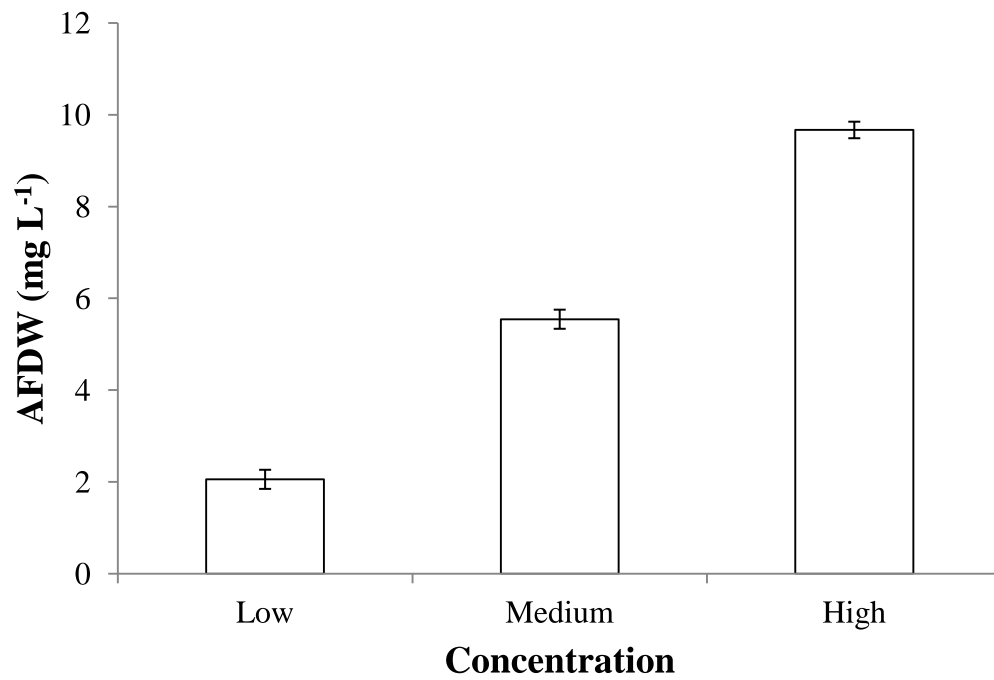



Figure 11. AFDW (mg $L^{-1}$) for the three feed concentrations during the 60-d experiment. All concentrations were significantly different (Mean ± 95% CI; $p$<0.0001).

## Chapter 4. A comparison of three recirculating aquaculture systems for rearing juvenile freshwater mussels at White Sulphur Springs National Fish Hatchery, WV.


ABSTRACT

To improve growth and survival of larger-sized juvenile mussels (>5 mm), three culture systems were tested; the Buckets, Pans, and Upwellers. After a 60-d experiment, mussel survival was higher for juveniles of northern riffleshell, *E. t. rangiana,* in the Upwellers than in the Bucket systems ($p$=0.02) and Pans ($p$=0.05). Percent survival for the Pans, Buckets, and Upwellers were 91.7 (95% CI: 87.4 to 96.0), 90.0 (95% CI: 80.6 to 99.4), and 100 (95% CI: 100 to 100), respectively. A significant difference was detected between the Upwellers and Pans ($p$=0.05). Mean total increment growth (length in mm) of juveniles of *E. t. rangiana* at 60 d in the Pans, Buckets, and Upwellers was 1.19 mm (95% CI: 0.746 to 1.62), 1.05 mm (95% CI: 0.608 to 1.49), and 2.07 mm (95% CI: 1.63 to 2.51), respectively. Mean total growth was significantly higher in the Upwellers compared to the Pans and Bucket systems ($p$=0.03). Growth rates calculated from mean total growth at 60 d for juveniles of *E. t. rangiana* in the Pans, Buckets, and Upwellers were 19.8 µm $d^{-1}$ (95% CI: 12.5 to 27.0), 17.5 µm $d^{-1}$ (95% CI: 10.1 to 24.8), and 34.5 µm $d^{-1}$ (95% CI: 27.1 to 41.7), respectively, and Upwellers exhibited significantly higher growth than in the Pans and Bucket systems ($p$=0.03).

Survival of juveniles of the mucket, *Actinonaias ligamentina,* at 60 days in Upwellers was significantly higher than in the Buckets ($p$<0.0001), but not when compared to the Pans ($p$=0.99). Mean percent survival of juveniles of *A. ligamentina* in the Pans, Buckets, and Upwellers was 100 (95% CI: 100 to 100), 86.7 7 (95% CI: 74.0 to 99.4), and 100 (95% CI: 100 to 100), respectively. Mean incremental growth of juveniles of *A. ligamentina* at 60 d reared in Pans, Buckets, and Upwellers was 1.96 mm (95% CI:

1.03 to 2.9), 0.88 mm (95% CI: 0.048 to 1.80), and 2.46 mm (95% CI: 1.537 to 3.38), respectively. No significant difference in total mean incremental growth was observed ($p$=0.06). Mean growth rates for juveniles of *A. ligamentina* at 60 d in the Pans, Buckets, and Upwellers were 32.7 µm $d^{-1}$ (95% CI: 17.29 to 47.94), 14.7 µm $d^{-1}$ (95% CI: 14.6 to 29.9), and 41.0 µm $d^{-1}$ (95% CI: 14.6 to 56.3), respectively, with no significant differences among systems ($p$=0.06). Results showed greater growth and survival of mussels reared in the Upweller culture systems.

## Introduction

To rear freshwater mussels, one must have knowledge of suitable host fish, appropriate diet for juveniles, location of adult broodstock, and a culture system for grow-out (Hanlon 2000; Henley et al. 2001; Beck and Neves 2003; Barnhart 2006). Initial efforts to culture juvenile mussels in the United States began in the early 1900s by scientists with the U. S. Bureau of Fisheries, the precursor agency to the United States Fish and Wildlife Service, who cultured mussels to mitigate anticipated damage from the commercial button industry (Coker et al. 1921). Results of these early experiments were somewhat successful; however, these mussel propagation and culture efforts were discontinued due to decreased demand for pearl buttons following the development and widespread use of plastic buttons. Early mussel culture experiments used floating crates, aquaria, tanks, troughs, cement and earth ponds, and pens to grow juveniles (Howard 1922), which were prototypes of methods developed since the late 1990s. For example, recent culture systems include floating cages in ponds, lakes, and rivers developed by biologists at the Genoa National Fish Hatchery, Wisconsin (Brady et al. 2011). Recently, a novel method developed by Barnhart (2006), Missouri State University simulates a

downweller system which was adapted from the marine bivalve culture industry. Even though the Barnhart bucket systems have increased survival of young juveniles, these systems are not ideal for rearing mussels to larger sizes (e.g., >10 mm). There are many advantages to the bucket systems, to include low cost, ease of sampling, small footprint, ability to culture several mussel species per bucket, and hence they are ideal for achieving high replication in culture experiments.

After using the bucket systems for several years at WSSNFH (see Chapters 1 and 2), I became familiar with their performance and observed that juvenile growth would drastically decrease when mussels reached approximately 4-5 mm in length. Thus, for the mussels to continue growing, an even larger culture system was needed for the next phase of grow-out.

Noticing the large production capability of marine bivalve facilities, I began researching the different systems used and visited several facilities. I visited a commercial clam hatchery on the eastern shore of Virginia, the Cherrystone Aqua-Farms, and the Virginia Institute of Marine Science. These site visits helped generate ideas for designing a new freshwater mussel culture system to improve grow-out success. After observing marine bivalve production, both facilities used upweller systems at that time to rear clam and oyster seed. The upweller appeared to work exceptionally well for marine bivalves which warranted testing this system for use in freshwater mussel culture.

The objective of this experiment was to compare growth and survival between three culture systems, including the new Upweller system, Pan system, and Bucket system. Ultimately, my goal was to identify a mussel culture system with the greatest potential to grow larger-sized juvenile mussels for federal and state recovery efforts.

**Methods**

To compare growth and survival of older juveniles (> 5 mm), juveniles of *Epioblasma t. rangiana* and *Actinonaias ligamentina* were reared in bucket culture systems for 10 mo prior to the experiment to achieve sizes above 5 mm in length. At the start of the experiment, juveniles of *A. ligamentina* ranged from 5-14 mm with a mean length of 8.04 (SE±0.20). Juveniles of *E. t. rangiana* ranged from 3.6 to 8.4 mm and had a mean length of 6.17 mm (SE±0.03).

Three culture systems were tested for differences in growth and survival among juveniles of *A. ligamentina* and *E. t. rangiana*: Pan system, Bucket system, and Upweller system. All juveniles were numbered with a permanent 'Fisherbrand' marker (Fisher Scientific, Inc., Pittsburgh, Pennsylvania) to identify and track growth of individuals (Figure 1; Figure 2).

The bucket system was discussed in detail in Chapters 1 and 2 (Figure 3A and 2B). Two bucket systems were used (n=2); each contained three culture chambers holding juveniles of each species, totaling six chambers per bucket. Each chamber held 10 individually numbered juveniles >5 mm in length. The mesh screens on the chambers were sprayed and cleaned daily with a household garden sprayer to avoid clogging. In addition, every 15 d, the nylon mesh screens were changed in the juvenile chambers to prevent clogging.

The Pan systems were developed by staff at the Aquatic Wildlife Conservation Center (AWCC), Virginia Game and Inland Fisheries (VDGIF) and were modified for use in this study. The Pan system consisted of six, 4.7 L plastic Duraflex pans (Miller Manufacturing, Inc., Glencoe, Minnesota), each with 1.3 cm bulkhead and 1.3 cm PVC

stand pipe that drains the water into a 150 µm mesh bag (Figure 4). The mesh bag drains into a reservoir sump located below the pan and catches any escaped juveniles (Figure 4B). A 0.25 hp submersible magnetic-drive pump (Danner, Inc., Islandia, New York) was used to circulate water from the sump into a 0.95 cm tygon tubing manifold and then to individual feed pans at a rate of 1-1.5 L $min^{-1}$. Flow in this system is circular, with a substrate of 5 to 7 mm of fine (<1 mm) sand. Ten juveniles of *E. t. rangiana* or 10 of *A. ligamentina* were placed in each pan, totaling 30 juveniles of each species per system. Sampling of the pans consisted of swirling the pans in a circular motion to lift the lightest materials and juveniles to the top of the substrate surface and then pour the contents through a 1 mm sieve. Water was added to the pans, and the swirling method was continued until all 10 juveniles were recovered from each pan.

Two Upwellers also were constructed and tested in this experiment. Each Upweller had an operating volume of 674 L (Figure 5). The polyethylene rectangular tank and oval-shaped sump had an operating volume of 344 L and 330 L, respectfully, and dimensions were 213 x 51 x 56 cm and 122 x 86 x 56 cm. Each rectangular tank had six juvenile chambers or sieves made from 28 cm length of 15 cm schedule 40 PVC pipe. A 5 cm hole cut was cut into it 5 cm from the top of the pipe (Figure 5). One mm nylon mesh was glued to the bottom of the PVC, making one juvenile chamber. Each rectangular tank had six chambers, with three for each species. Each chamber had a hole drilled in the upper portion of the plastic bucket to accommodate a 3.8 cm PVC bulkhead which connects to another bulkhead installed in the side of the rectangular tank. From there, a 90º PVC elbow drained the tank water to a 7.6 cm PVC pipe sending the water to the sump. A 240 L $min^{-1}$ pump (Reeflo Pumps, Inc., Colorado Springs, Colorado)

recirculated the water from the sump to the tank. The sump also had a 1.9 cm PVC air manifold to keep oxygen level at saturation and the algae in suspension. A 1 kW submersible heater (Innovative Heat Concepts, Inc., Homestead, Florida) and a 0.5 horsepower titanium coil chiller (Aqua Logic, Inc., San Diego, California) were kept in the sump to maintain water temperature at 20-21° C (Figure 5). As with the other two treatments, juveniles were individually numbered to track growth. Ten juveniles of either species were placed in each pan, totaling 60 juveniles per system.

Juveniles were fed the WSSNFH mix at the concentration of 30,000 cells $mL^{-1}$, obtained from results in Chapters 1 and 2. Two system replicates were used, and each system had three chambers of each mussel species (Figure 3). Only two systems of each treatment were constructed and used during the experiment. Sample events occurred every 15 d for 60 d. During experimental set-up, juveniles were counted and placed in juvenile chambers. All 10 juveniles per chamber were measured using a microscope and ocular micrometer; thereafter, a dial metric caliper was used to measure lengths. Twice weekly, the Pan system and Upweller system received a 30% water exchange, replacing the culture water with clean, filtered (<30 µm) spring water. The bucket systems received a 100% water change weekly. The amount of water changed varied between systems for several reasons. First, I knew that the Bucket systems were successful with a 100% water change weekly, and I wanted to test the system along with its known husbandry methods. Second, the Buckets are only 18.9 L compared to the larger volume systems of the Pans and Upwellers. I did not believe that 30% water change on the Bucket systems would be enough, which could cause possible water quality issues.

Sample events consisted of removing juveniles to record survival and growth every 15 d. Incremental growth (measured as the change in length over time in mm) and percent survival were recorded for mussels within all chambers and treatments. Length was measured as the longest distance from anterior to the posterior of the shell. Juveniles were cultured at 20-21° C. Water temperatures were maintained in each Bucket system by using a temperature-controlled water bath, and for the other systems, a heater and chiller were installed. Temperature and algal cell counts were recorded daily in all systems. Algae concentration in the culture systems was counted daily using a Beckman Coulter Counter, (Beckman Coulter, Inc., Brea, California) and recorded as cells $mL^{-1}$ and $\mu m^3$ $mL^{-1}$ for the 60-d experiment.

*Juvenile Mussel Diets*

Juveniles were fed live algae cultured at WSSNFH containing the following mix of four species: *Bracteacoccus grandis, Neochloris oleoabundans, Phaeodactylum tricornutum,* and *Oosystis polymorpha.* Algae were grown in photo-reactors known as Biofences (http://www.variconaqua.com/, Varicon Aqua Solutions, Ltd., Malvern, United Kingdom) and then centrifuged into a thick paste, vacuum-sealed and refrigerated. Algae paste was refrigerated for a maximum of 4 wk for use in these experiments (see Chapters 1 and 2 for details).

*Statistical Analysis*

All statistical analyses were conducted in JMP, version 10.0 (SAS Institute 2010). I compared survival of juveniles among the three systems (n=2) using a Generalized Linear Model (GLM) on the data collected at the end of the 60-d experiment. Additionally, because each individual mussel was numbered, I recorded total length for

each mussel and over time and tracked incremental growth (change in length over time, in mm) for each individual. Mean incremental growth (mm) was compared among the three systems. One-way analysis of variance (ANOVA), Repeated measures ANOVA, and student *t*-tests were used to analyze both length and survival values. I assessed growth of mussels that were 280 d old at the start of the experiment; thus to compare juvenile growth to other published studies of younger mussels (e.g. 0-140 days old), mean growth rate in $\mu m\ d^{-1}$ was calculated by dividing length increase by time. All statistical tests conducted in this study were considered significant at an alpha level of 0.05 or less.

## **Results**

### *Juvenile Growth and Survival*

Survival of *E. t. rangiana* at 60 d in the Upwellers was significantly higher than in the Buckets ($p$=0.018). Percent survival for the Pans, Buckets, and Upwellers were 91.7 (95% CI: 87.4 to 96.0), 90.0 (95% CI: 80.6 to 99.4), and 100 (95% CI: 100 to 100), respectively. Survival of juveniles in the Pan system and Upwellers was at the significance threshold ($p$=0.05). Mean growth of *E. t. rangiana* juveniles at 60 d in the Pans, Buckets, and Upwellers was 1.19 mm (95% CI: 0.746 to 1.62), 1.05 mm (95% CI: 0.608 to 1.49), and 2.07 mm (95% CI: 1.63 to 2.51), respectively, and was significantly greater in the Upwellers than in the other two systems ($p$=0.03) (Figure 6). Growth of juveniles in the Bucket and Pan systems was not significantly different from each other ($p$=0.54). Growth rates, calculated from mean total growth at 60 d for *E. t. rangiana* in the Pans, Buckets, and Upwellers were 19.8 $\mu m\ d^{-1}$ (95% CI:12.5 to 27.0), 17.5 $\mu m\ d^{-1}$ (95% CI: 10.1 to 24.8), and 34.5 $\mu m\ d^{-1}$ (95% CI: 27.1 to 41.7), respectively, and the

Upwellers had significantly greater growth rates than the Pans and Bucket systems (Figure 8).

Survival of juveniles of *Actinonaias ligamentina* at 60 d in Upwellers and Pans was significantly higher than in the Buckets ($p<0.0001$). Mean percent survival of juveniles of *A. ligamentina* in the Pans, Buckets, and Upwellers was 100 (95% CI: 100 to 100), 86.7 (95% CI: 74.0 to 99.4), and 100 (95% CI: 100 to 100), respectively. Mean growth of juveniles of *A. ligamentina* juveniles at 60 d in the Culture pans, Buckets, and Upwellers was 1.96 mm (95% CI: 1.03 to 2.9), 0.88 mm (95% CI: 0.048 to 1.80), and 2.46 mm (95% CI: 1.537 to 3.38) respectively, with no significant differences in juvenile growth among systems ($p=0.07$) (Figure 7). Mean growth rates for *A. ligamentina* at 60 d in the Pans, Buckets, and Upwellers were 32.7 µm $d^{-1}$ (95% CI: 17.29 to 47.94), 14.7 µm $d^{-1}$ (95% CI: 14.6 to 29.9), and 41.0 µm $d^{-1}$ (95% CI: 14.6 to 56.3), respectively, and there were no significant differences among systems (Figure 9).

*Cell Counts*

Mean cell counts $mL^{-1}$ for the Pans, Buckets, and Upwellers were $2.58 \times 10^4$ (SE$\pm 2.18 \times 10^3$), $2.08 \times 10^4$ (SE$\pm 2.18 \times 10^3$), and $2.13 \times 10^4$ (SE$\pm 2.18 \times 10^3$), respectively (Figure 10), and were not significantly different from each other ($p=0.6143$). Mean cell volumes in the Pans, Buckets, and Upwellers were $2.07 \times 10^6$ (SE$\pm 2.05 \times 10^5$), $1.71 \times 10^6$ (SE$\pm 2.05 \times 10^5$), and $1.76 \times 10^6$ (SE$\pm 2.05 \times 10^5$) and were not significantly different ($p=0.4175$) (Figure 11).

## **Discussion**

Few laboratory experiments have evaluated the growth in length and survival for long-term grow-out of young freshwater mussels. Most published studies have focused

on the first 0 to 140 d (Gatenby et al. 1996; Jones et al. 2005; Barnhart 2006; Liberty et al. 2007; Kovitvadhi et al. 2008), and mean growth rates of mussels in these studies ranged from 3.9 to 240 µm $d^{-1}$. Gatenby (1996) reported a mean growth rate for juveniles of *Villosa iris* that ranged from 10.9 to 25.0 µm $day^{-1}$, using fine silt as a substrate and possible food source, and feeding cultured algae at concentrations ranging from 10,000 to 500,000 cells $ml^{-1}$. Although this and several other studies discussed the importance of fine sediment in the culture of mussels, growth rates in her study were comparable to growth rates observed using the Bucket systems which had no substrate or sediment (O'Beirn et al. 1998; Rodgers 1999; Mummert 2001; Zimmerman 2003). Liberty et al. (2007) also used similar substrates of fine sediment, sand, or course sediment and reported higher survival in the fine and course sand substrates than in fine sediment. Growth rates in his study of *Villosa iris* ranged from 7.9 to 12.7 µm $d^{-1}$. Barnhart (2006) measured growth of six mussel species up to 84 d old in the bucket systems, and growth rates ranged from 4.7 to 12.2 µm $d^{-1}$. Algal cell concentrations in his study were maintained at 10,000 to 15,000 cells $mL^{-1}$, and water temperature was 22-23 ºC. Growth rates of juvenile mussels in the bucket system in my study were comparable to those of Barnhart (2006), and ranged from 14.7 to 28 µm $d^{-1}$, whereas Upweller and Pan system growth rates were higher (19.8 to 67.3 µm $d^{-1}$) at a temperature of 20-21 ºC.

Another study compared the performance of growth and survival of the freshwater pearl mussel, *Hyriopsis myersiana* in two recirculating culture systems (Kovitvadhi et al. 2008). Growth rates in the two culture systems ranged from 0.1 to 40 µm $d^{-1}$ in one culture system, and 20 to 240 µm $day^{-1}$ in the other system. Juveniles in this study were fed two species of cultured algae which was discovered and isolated from

the mussels digestive tract. The system which yielded significantly higher growth incorporated a biological filter using bioballs and macrophytes. Additionally, sand was used as a substrate for the juvenile mussels. Kovitvadhi et al. (2008) also had a culture temperature at 27 to 28 ºC, which likely contributed to higher growth rates when compared to previous studies. Several studies have used cages deployed in natural river or lake water to achieve excellent growth rates for *Lampsilis higginsi* in Lake Pepin, Upper Mississippi River, Wisconsin, with maximum growth rate of 242 µm $d^{-1}$ (USFWS 2002; Brady et al. 2011). Besides temperature, substrate, and food concentration, another consideration for evaluating a suitable culture system is water flow. Many studies have not taken into consideration water flow, or the systems were non-recirculating culture tanks. Flow in a Bucket system is difficult to measure because the mussels are in chambers between nylon mesh. However, an estimated water flow for the Bucket system was 0.9 L $min^{-1}$, 1 to1.5 L $min^{-1}$ for the Pan system, and 18.5 to 19.7 L $min^{-1}$ for the Upwellers. Kovitvadhi et al. (2008) culture systems had a juvenile container flow rate of 0.02 L $min^{-1}$.

Survival rate can assess the suitability of a culture system; however, survival was >90 % in my study, likely because of the older age of the juveniles. Mussels exceeding 5 mm in length are fairly hardy and so growth rate was a better way to assess the performance of the three culture systems in the experiment.

Maintenance and sampling ease differed for each type of culture system. The Bucket systems generally required more maintenance than the other two systems; screens were sprayed daily and the mesh changed every several weeks. Additionally, the buckets were cleaned, and a 100% water exchange was conducted weekly to maintain water

quality. There are however, several positive attributes of this system. The systems are isolated from each other which can prevent any disease or water quality issue from affecting all juvenile mussels. Species also can be separated easily and reared together in the same system but in separate chambers. Lastly, this system allows easy replication for culture experiments and has a small footprint.

Pan systems have initially more maintenance to sieve and clean the sand; however, they are relatively simple to maintain and only require a quick cleaning every month. If you have juveniles that exceed 5 mm, sampling is also relatively quick and trouble-free. The Upwellers are the easiest to set up and sample or to move juveniles. The sides of the rectangular tank need to be cleaned weekly or as needed to prevent the mesh on the bottom of the chambers from becoming clogged. After using the Upwellers throughout and after my experiment, I learned that if you grade juveniles by length and keep similar-sized mussels in each chamber, their growth rate will increase.

## Conclusions

Results from this study confirmed that the Upweller had the highest growth and survival of the three systems tested for both mussel species. Results also demonstrated that *E. t. rangiana* did best in the Upweller system instead of the Pan system; however *A. ligamentina* performed equally well in both systems. This suggests that different mussel species may prefer different culture systems. My study also showed a downward trend in growth rate (µm $d^{-1}$) over time in all systems, which could indicate that these mussels require more food or higher flow, substrate, or perhaps a new method that includes outdoor rearing.

Future research should include evaluation of system-level species preferences. Additionally, more research is needed to identify suitable water flow, optimal culture temperature, and substrate preferences for various sizes and ages of juvenile mussels.

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Figure 1. Individually numbered juveniles of *A. ligamentina* from the Pan system.

Figure 2. Individually numbered juveniles of *E. t. rangiana* from the Upweller system before (left) and after (right) the 60-d experiment.

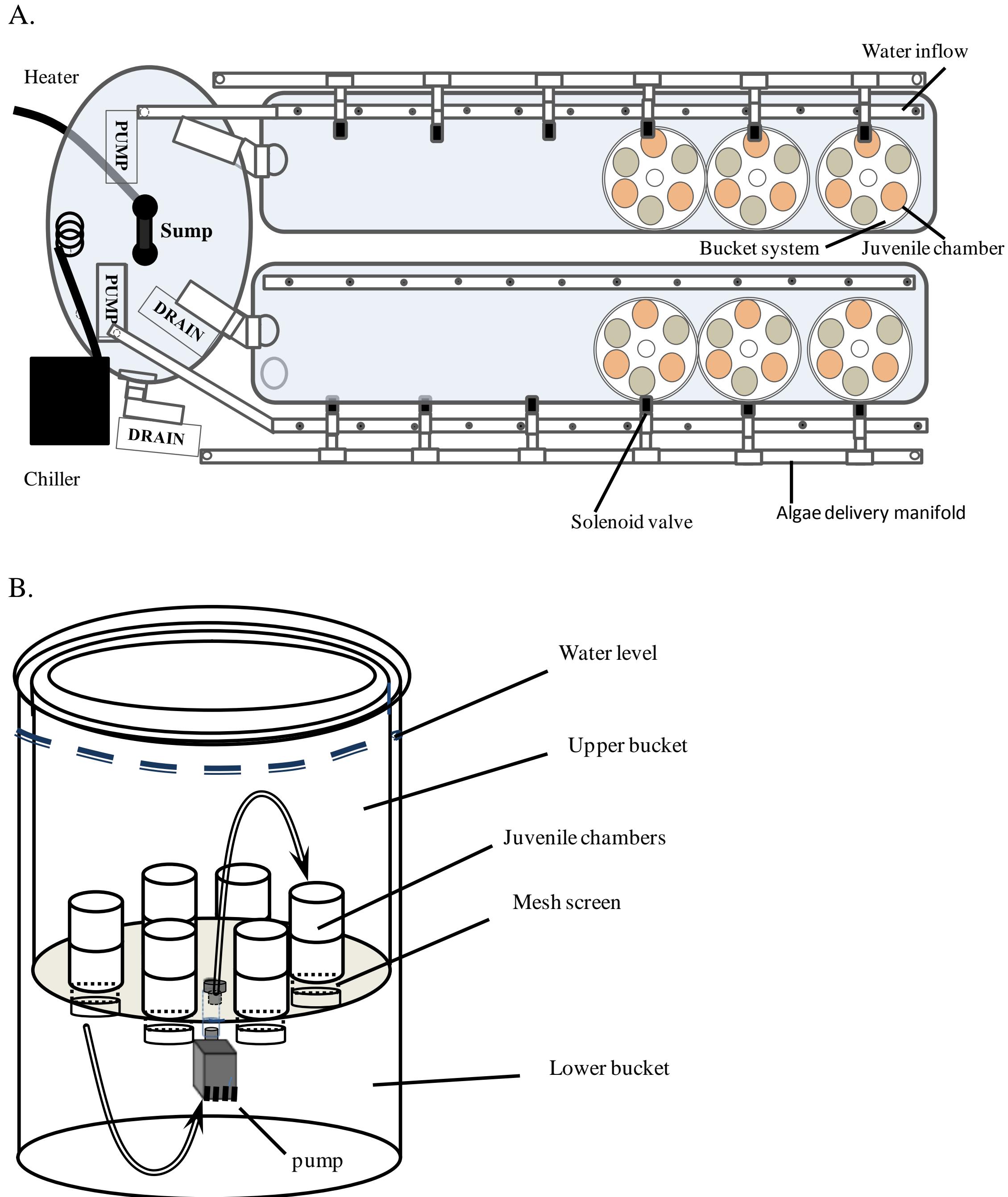
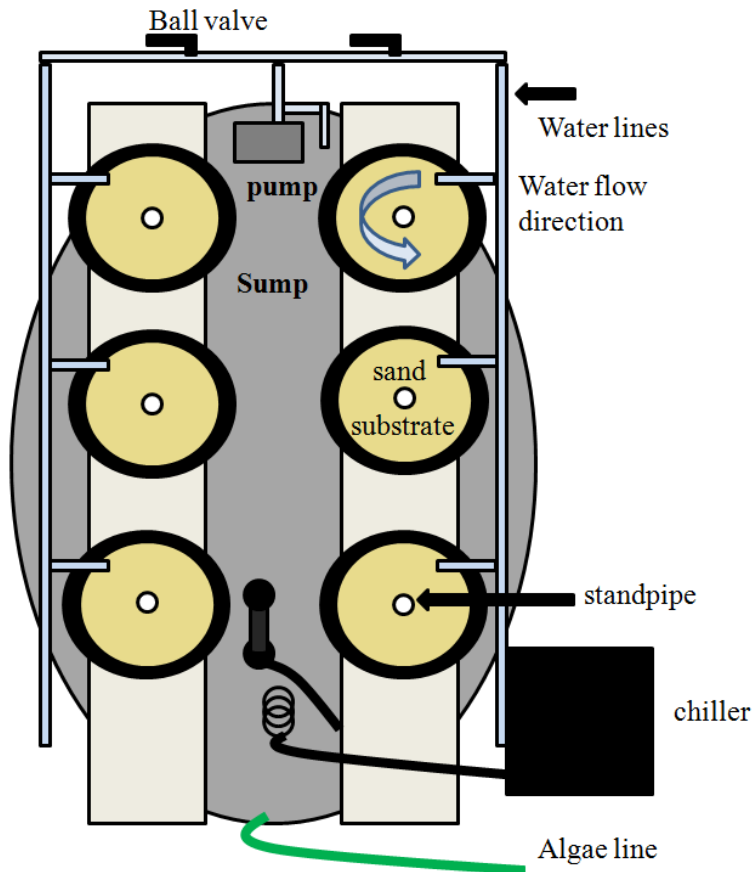



Figure 3. A. Top view of Bucket system with temperature-controlled water bath trough and algae delivery system. Water was recirculated from the sump into the trough and then returned to the sump. The algae manifold circulated algae from a main tank (not shown) to the bucket system by an open solenoid valve. B. Side view diagram of bucket system, the top bucket recirculates water with the lower bucket by submersible pump. The water flows through the nylon screens in the juvenile chambers in the top bucket into the bottom bucket is pumped back to the top bucket.

A

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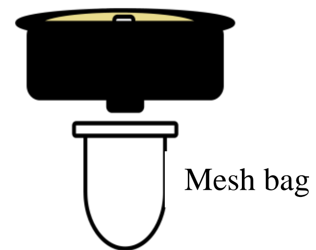


Figure 4A. Top view of Pan system. Water is pumped from the sump into the water line and is distributed to each pan which flows through a nylon mesh bag and drains to the sump. Temperature is controlled with a chiller and heater. B. Side view of pan and mesh bag.

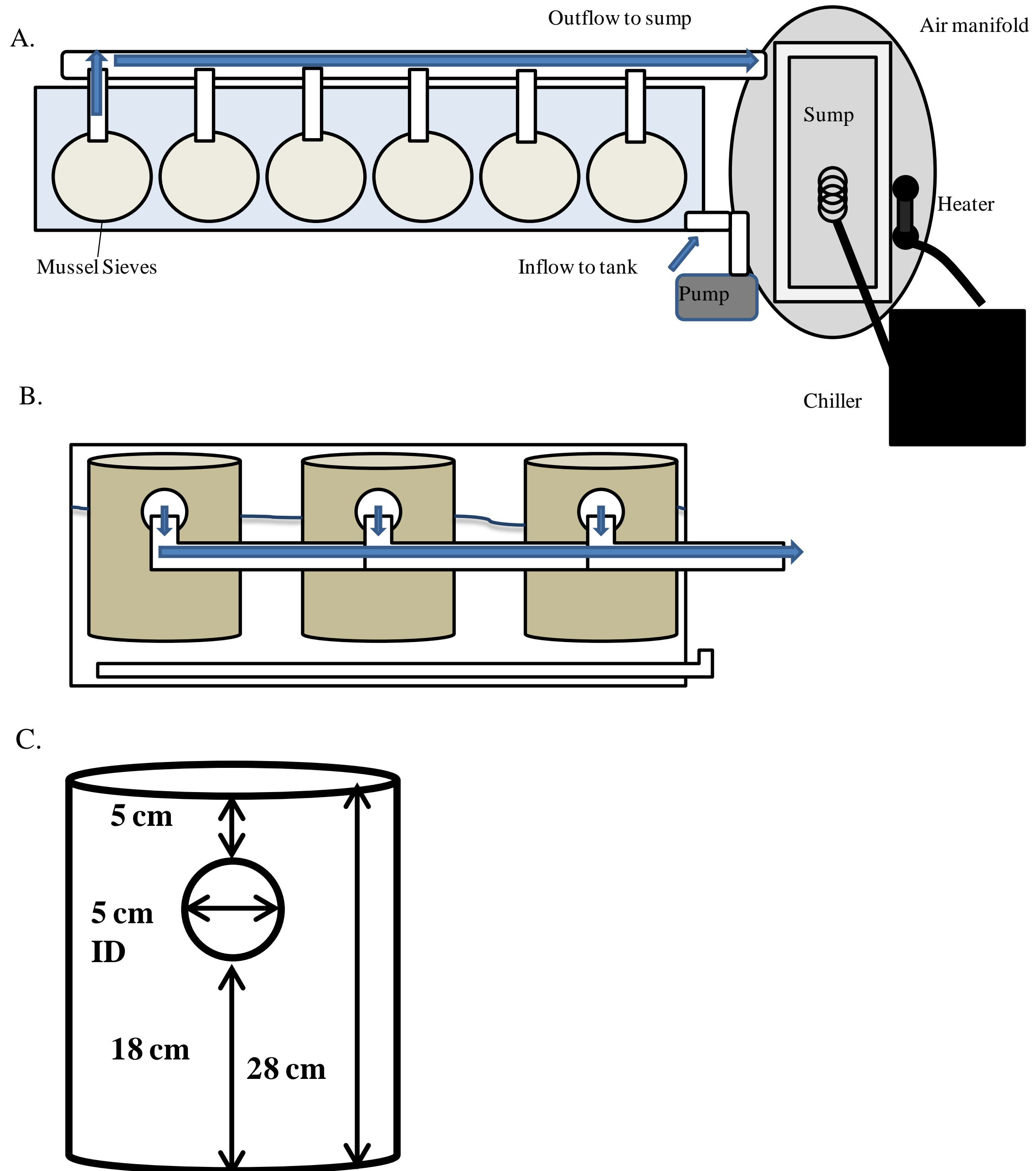


Figure 5. Diagram of Upweller system. A. Top view of the Upweller system. Temperature-controlled water is recirculated from the sump into the rectangular tank. Each mussel sieve chamber drains back into the sump. An air manifold keeps the algae in suspension. B. Side view of the Upweller system. Water is pushed up into the mussel chambers and then out the side of the chamber where is drains to the sump. C. Mussel chamber with approximate dimensions.

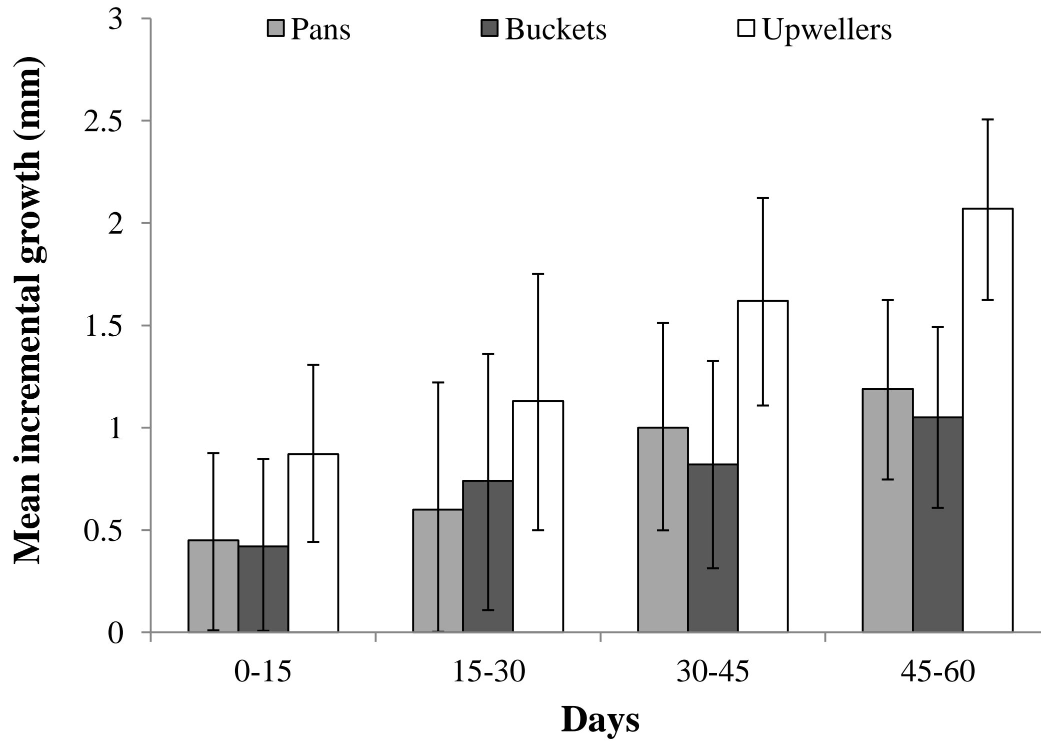


Figure 6. Incremental growth (length in mm) for juveniles of *Epioblasma t. rangiana* at 15-d intervals during 60-d experiment for the three culture systems of Pans, Buckets, and Upwellers. Upwellers growth was significantly higher than in the Bucket and Pan systems (Mean ± 95% CI; $p$=0.03). Culture systems were sampled and mean lengths assessed and recorded every 15 d.

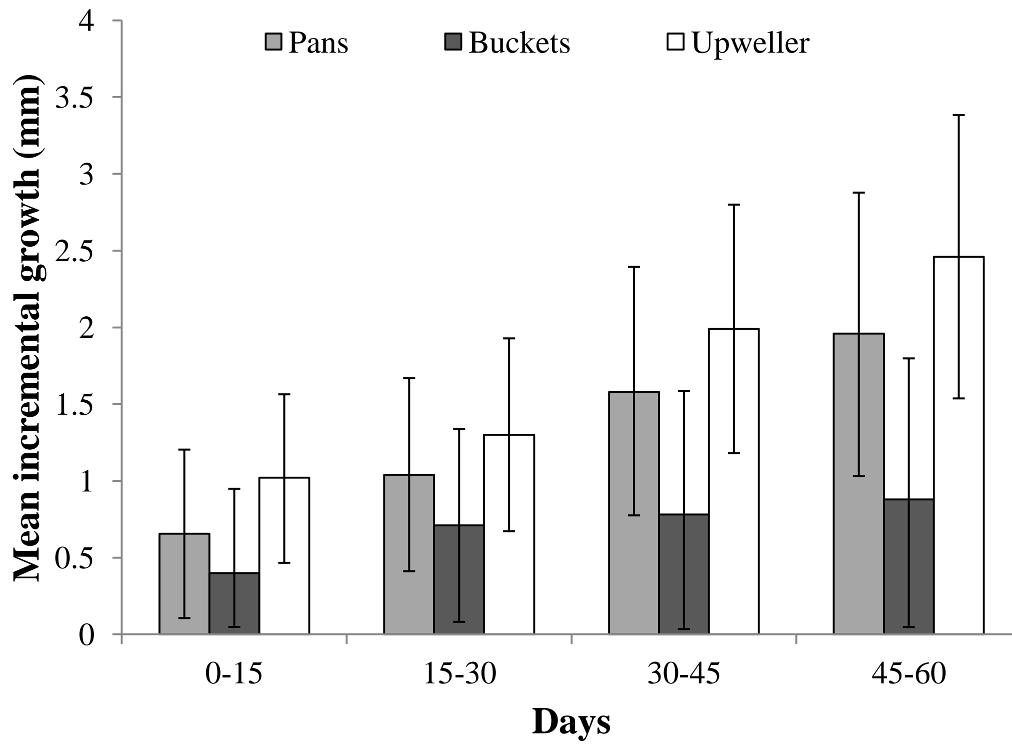


Figure 7. Incremental growth (length in mm) for *A. ligamentina* juveniles at 15-d intervals for 60 d experiment in the three culture system treatments of Pans, Buckets, and Upwellers. No significant difference was observed among systems (Mean ± 95% CI; $p$=0.06). Culture systems were sampled and mean lengths assessed and recorded every 15 d.

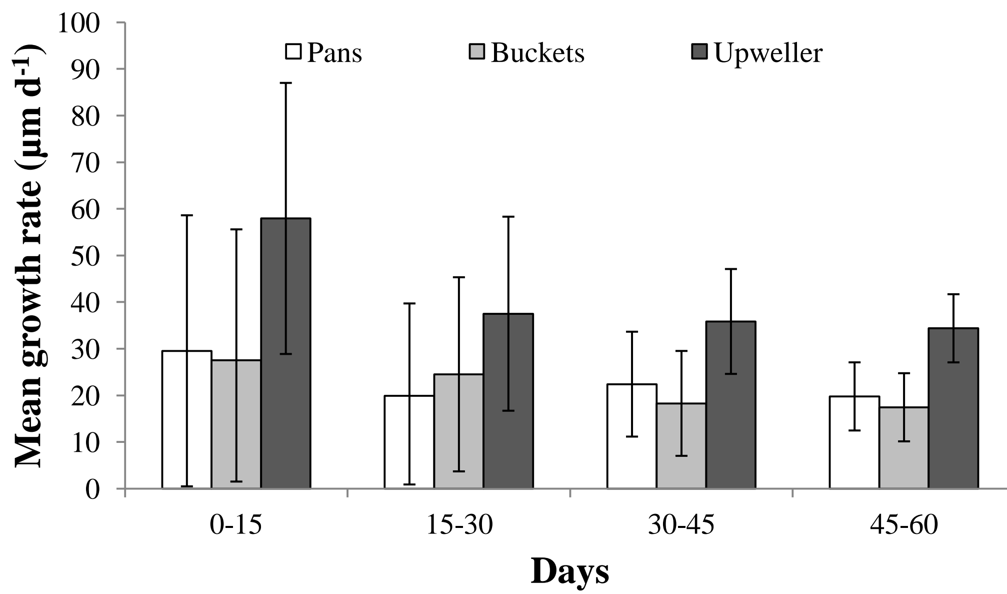


Figure 8. Growth rate (μm $d^{-1}$) for juveniles of *Epioblasma t. rangiana* at 15-d intervals for 60 d in the three culture systems of Pans, Buckets, and Upwellers. Upwellers had a significantly higher growth rate than that in the Pans and Buckets (Mean ± 95% CI; $p$=0.03).

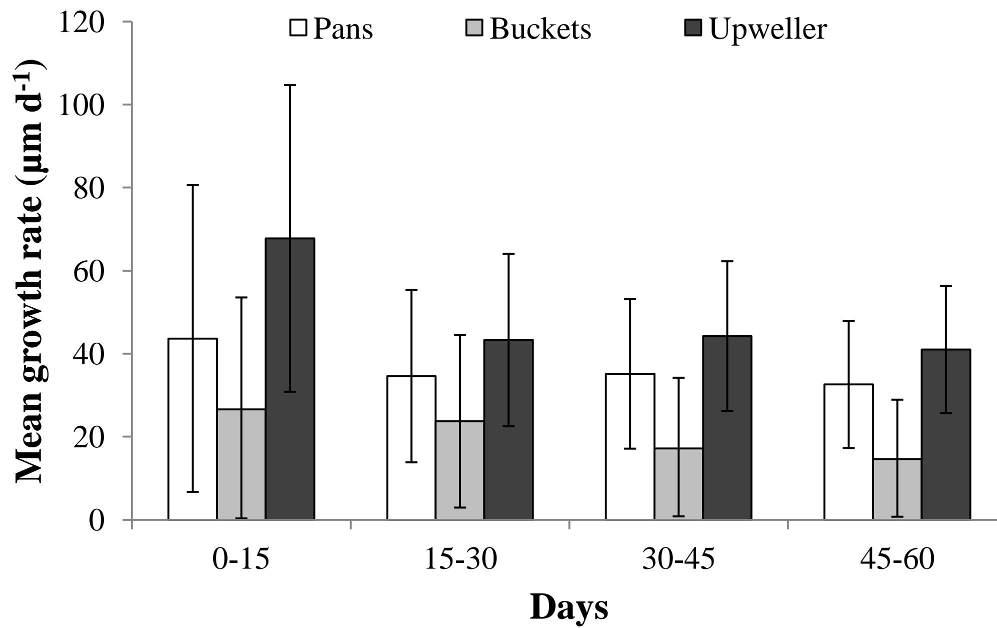


Figure 9. Growth rate (μm $d^{-1}$) for juveniles of *Actinonaias ligamentina* at 15-d intervals for 60 d in the three culture systems of Pans, Buckets, and Upwellers. No significant differences in growth rate were observed among the systems (Mean ± 95% CI; $p$=0.06).

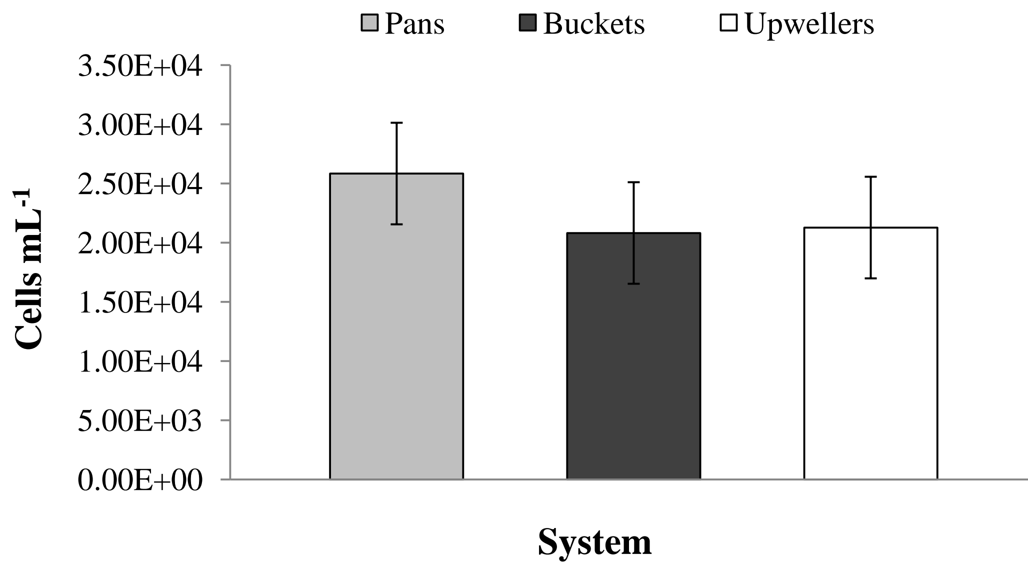


Figure 10. Cell counts (cells $mL^{-1}$) for the three culture systems during the 60-d experiment. Cell counts were not significantly different among systems (Mean ± 95% CI; $p$=0.20). Cell counts for all culture systems were recorded daily.

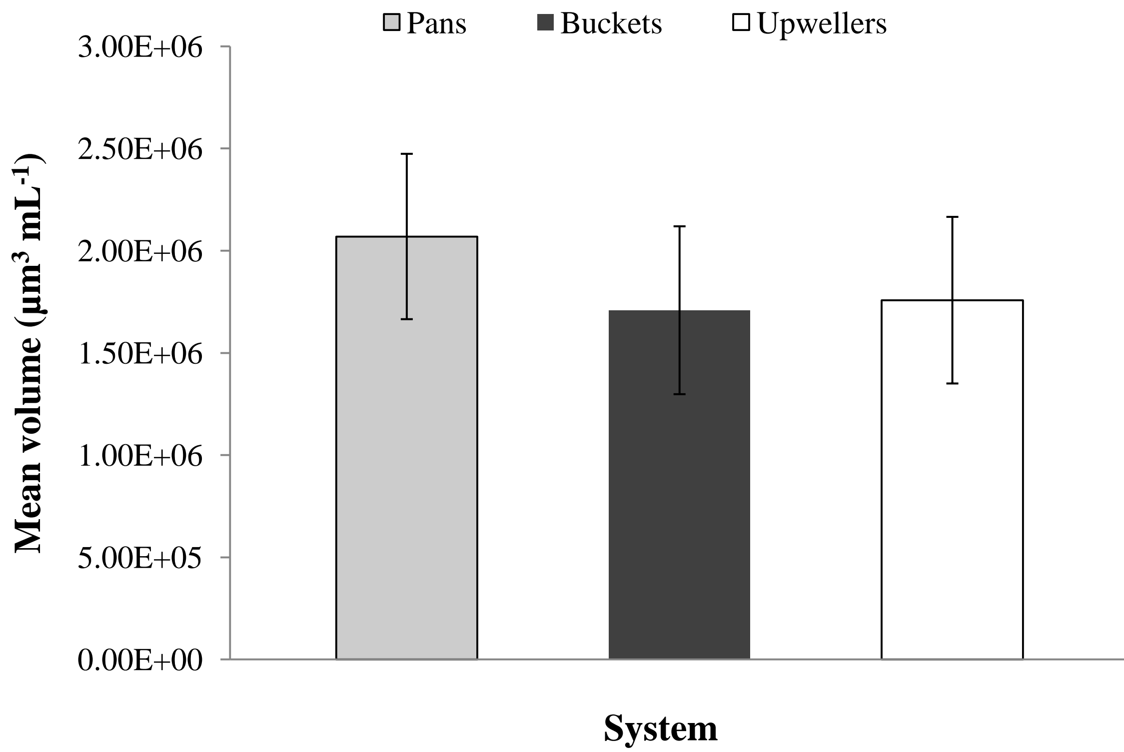


Figure 11. Cell count volume (cells $\mu m^3$ $mL^{-1}$) for the three culture systems during the 60-d experiment. No significant differences were observed among systems (Mean ± 95% CI; $p$=0.41). Cell counts for all culture systems were recorded daily.

## Chapter 5. General Conclusions

Freshwater mussel conservation includes propagation as a method to offset continued declines and to increase mussel abundances by stocking of juveniles. At the time of my experiments in 2007 and 2008, achieving high growth and survival in young (<2 mo) juveniles was a difficult task. My goal was to improve the success and consistency in rearing young juveniles and identifying a suitable culture system for longer-term grow-out.

My research centered on improving growth and survival among juvenile mussels using three experiments to compare diet, algae concentration, and culture system. Based on the diet experiment results in Chapter 2, a diet of live algae can significantly increase growth and survival of juvenile mussels. Growing multiple species of live algae, capable of supplying food to a large mussel facility, is expensive and time limiting. However, my research shows that the additional expense to culture multiple species of algae for juvenile culture is justified.

Since many mussel propagation facilities do not have the space, staff, expertise, or infrastructure to grow large quantities of algae, Shellfish Diet is a suitable alternative for feeding juvenile mussels. I would recommend that a supplement of live algae or a natural water source such as pond or lake water be included in the diet. The WSSNFH mix was comprised of four species of live algae high in proteins, carbohydrates, and lipids, and provided juveniles with adequate nutrition for growth and survival. Lipids, in particular highly unsaturated fatty acids (HUFA), are known to be absolutely essential for all stages of aquatic organisms and are directly linked to successful bivalve culture (Gallager et al. 1986; Chu et al. 1987; Napolitano et al. 1990; Gatenby 1994; Helm and

Bourne 2004). HUFA's were slightly lower in the Shellfish Diet; therefore, I suggest that live *Neochloris oleoabundans* or another algal species high in polyunsaturated fatty acids, be added to the Shellfish Diet, if feeding a completely live diet is not an option.

Additional research on dietary requirements is necessary to build upon the improvements in growth and survival made in this study. Studies that evaluate the biochemical requirements of all life stages of mussels are needed. Once we know the biochemical requirements, new diets can be constructed that are based on appropriate levels of proteins, carbohydrates, and lipids. More research is also needed to evaluate potential supplements that could be added to commercial diets. This could improve the growth and survival of juveniles reared in commercial diets and could reduce the operational costs of growing algae. More research is also needed to evaluate diets for appropriate shelf life, and storage methods of live and commercial diets by analyzing the degradation of biochemical composition over time.

The literature on feed concentration to rear mussels is highly varied and ranges from 10,000 to 500,000 cells $mL^{-1}$(Gatenby 1994, Gatenby et al. 1997; Rodgers 1999; Henley et al. 2001; Barnhart 2006). Overfeeding increases system maintenance time, staff time to culture algae, and operating costs. Therefore, the lowest possible feed ration is desired while maintaining adequate growth and survival. To narrow the large concentration range in the published literature, I examined growth and survival of juveniles among three algal concentrations.

The results of this study show that juvenile mussels cultured at high feed concentrations exhibit decreased growth and survival compared to juveniles cultured with a lower feed concentration. The lowest feed ration in this study showed the highest

growth and survival, making 30,000 cells $mL^{-1}$ a suitable concentration for feeding juvenile mussels. In addition, feeding a low feed ration will conserve algae resources and system maintenance time. Therefore, I suggest that facilities monitor their food ration and algal concentrations several times per week and install an automatic feeder to feed the low density of algae throughout the day. Freshwater studies have validated bivalve mariculture experiments that used low feed rations (10,000 to 15,000 cells $mL^{-1}$) to feed clam and oyster larvae and juveniles (Taylor and Brand 1975; MacKay and Shumway 1980; Heasman 2001; Helm and Bourne 2004).

Also, algae concentrates and alternative diets were shown to be unsuitable as complete replacements to live algae for marine bivalves, and juvenile growth and survival were reduced when compared to fresh algae. (Heasman 2001; Ponis et al 2003).

Additional research to increase juvenile rearing success includes comparing growth and survival of juveniles fed different algae concentrations for multiple juvenile sizes and ages. For example, a 15 mm juvenile may require more than 30,000 cells $mL^{-1}$. Conversely, a one-day old juvenile might require a concentration less than 30,000 cells $mL^{-1}$.

My last experiment used larger juveniles (> 5 mm) of two mussel species to evaluate three culture systems. This study showed that Upweller systems are better for freshwater mussel rearing because they had the highest growth and survival of the three systems tested for both mussel species. This study also showed a decrease in growth rate (µm $d^{-1}$) over time in all systems, which suggests several hypotheses. A decrease in growth rate over time could indicate that larger mussels require more food or higher flow, substrate, or perhaps a new method that includes outdoor rearing. In mariculture, after

the clams in Upwellers reach 10 mm, they are transferred into trays with sand and moved into estuaries for continued grow-out. Future research should include species preferences for culture system and studies that evaluate suitable water flow, rearing temperature, and substrate preferences for various species and ages of mussels.

This study and future propagation research may increase mussel recovery success by stocking more and larger juveniles, enlarging populations because of higher survival of stocked juvenile mussels. Release of larger mussels is advantageous for several reasons. First, a shellfish tag or pit tag could be glued on larger mussels and used to monitor survival in the future. Additionally, small juveniles are easily consumed by fish and other aquatic organisms; by stocking larger juveniles, predation will be reduced. Lastly, monitoring and tracking of individuals in rivers will give aquatic biologists detailed information about growth rates, survival, and reproductive activity of stocked mussels. Improving propagation and culture technology will likely increase the success of mussel recovery efforts by releasing more, larger-sized mussels back into restoration areas. This research has improved juvenile mussel production at WSSNFH through the successful use of Upweller systems for long term grow-out of over 15 species of freshwater mussels by feeding juveniles the WSSNFH mix at a maintained concentration of 30,000 cells $mL^{-1}$. Thus the application of methods developed in this study will improve growth and survival of juvenile mussels when cultured in recirculating systems at any propagation facility.

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