DEVELOPMENT OF A SUITABLE DIET FOR ENDANGERED JUVENILE OYSTER MUSSELS, *EPIOBLASMA CAPSAEFORMIS* (BIVALVIA: UNIONIDAE), REARED IN A CAPTIVE ENVIRONMENT

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Development of a Suitable Diet for Endangered Juvenile Oyster Mussels, *Epioblasma capsaeformis* (Bivalvia: Unionidae), Reared in a Captive Environment

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(ABSTRACT)

Epioblasma capsaeformis, commonly named the oyster mussel, once occupied thousands of miles of stream reaches, but has now been reduced in range to small, isolated populations in a few river reaches. Due to this significant decline in population numbers, a study was conducted to develop a diet for propagating this endangered species under captive conditions, in hope of eventually restoring extirpated populations. Oyster mussel juveniles were collected from several sites on the Clinch River and sacrificed for gut content and biochemical composition analyses in summer. Feces and pseudofeces from live river-collected juveniles were examined seasonally for algae, detritus, and bacteria to qualitatively determine diet of specimens. Two feeding trials also were conducted in this study to evaluate effect of diet (commercial and non-commercial diets), on growth and survival of oyster mussel (*E. capsaeformis*) juveniles.

From examination of gut contents, fecal and pseudofecal samples, it was apparent that algae and a large amount of detritus (~90%) composed wild juvenile diets. *E. capsaeformis* juveniles (1-3 y of age) could have fed on particles up to 20 µm in size, indicating that they were capable of ingesting particles within the 1.5-12 µm size range. Protein content of sacrificed juveniles ranged from 313 to 884 mg/g and was highly variable. Glycogen content ranged from 49-171 mg/g. Caloric content of four juveniles ranged from 2,935.10 to 4,287.94 cal/g, providing a preliminary baseline range for future energetic studies on freshwater mussels.

Growth was significantly higher in those juveniles fed the triple concentration algae-mix (62,076 cells/ml) than all other diets tested in trial 1. Results of both feeding trials indicated that survival of juvenile oyster mussels was enhanced when fed an algal diet supplemented by bioflocs.

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Introduction

Declines in Freshwater Mussel Populations

Freshwater mussels are found worldwide and are most diverse in North America (Williams et al. 1993). The world's greatest diversity of pearly mussels, which include nearly 300 species, resides in the continental United States (Turgeon et al. 1988). Presently, approximately 70% of these species are classified as threatened, endangered or extinct (Neves 1999). More specifically, 35 are presumed extinct (Turgeon et al. 1998), while 70 species are listed as federally endangered or threatened in the United States under the Endangered Species Act. During this century alone, more than one in ten mussels may have gone extinct (Williams et al. 1993; Master et al. 1998). When compared to other organisms, The Nature Conservancy recognizes 55% of North America's mussels as extinct or imperiled versus only 7% of the continent's avian and mammalian species (Master 1990).

Within the past fifty years, mussel populations have greatly declined due to natural and anthropogenic causes altering freshwater habitats, such as water pollution, impoundments, sedimentation, channelization and dredging of streams and rivers, and more recently the non-native zebra mussel (*Dreissena polymorpha*) (Neves 1997). In addition, muskrat predation, though not a cause of mussel decline, could inhibit recovery of endangered mussel species (Neves and Odom, 1989).

Importance of Freshwater Mussels

Freshwater mussels are ecologically important as a food source for aquatic and terrestrial animals. As suspension feeders, they play a critical role in maintenance of water quality and ecosystem stability. Indeed the diversity and abundance of other invertebrates and fish are higher around a bed of mussels (Vaughan et al. 2008). Because of their sensitivity to toxins, they can serve as an early indicator of water quality problems (National Native Mussel Conservation Committee 1998).

Classification

All freshwater mussels are classified in the order Unionoida (Unionacea) and two superfamilies, Unionoidea and Etherioidea, which are distinguished by larval forms. Those mussels of Unionoidea are all freshwater species having glochidia larvae and include Hyriidae, Margaritiferidae and Unionidae. Mussels of Etherioidea have lasidia larvae and include Etheriidae and Iridinidae.

Study Species, *Epioblasma capsaeformis*

Current Status

Epioblasma capsaeformis, commonly named the oyster mussel, was historically one of the most widely distributed Cumberlandian species.It was described by I. Lea (1834) from specimens collected in the Cumberland River near Nashville, TN (Jones et al. 2006).This riffleshell species was once found throughout the Cumberland and TN River systems, ranging in location from VA, Kentucky, TN, North Carolina, Alabama and Georgia. Once occupying thousands of miles of stream reaches, populations have been decimated and now exist only as small, isolated populations in a few stream and river reaches in two states; the Duck River, TN, Clinch River, TN and VA, and the Nolichucky River, TN (Parmalee and Bogan 1998). This species and its habitat have been impacted by excessive sediment loads, changes in turbidity, increased suspended solids, from non-point source loading, poor land-use practices, lack of best management practices (BMPs), and water pollution from pesticides. Of the 25 described species of *Epioblasma*, 24 are recognized as extinct, endangered or threatened. *Epioblasma capsaeformis* was first listed as endangered throughout its entire range on January 10, 1997 (USFWS 2004). A draft recovery plan for the species was published in June 1998 by USFWS, in response to the relict populations. It was determined that the best way to prevent extinction of critically endangered mussels involves establishing captive populations to augment extant populations and restore extirpated populations (USFWS 2004). It will only be possible to restore federally endangered species if juveniles can be mass-produced for reintroduction and augmentation. This species was selected for study in this research project, to determine a suitable diet for cultured juvenile oyster mussels in the laboratory, for release into rivers to restore populations.

Reproductive Information

Oyster mussels vary morphologically (i.e., shell and mantle appearance) based on the river system inhabited. In the Clinch River, female oyster mussels display a mantle that is a bluish/white. Duck River specimens display a grayish/blackish; and a brownish-colored mantle occurs in the Big South Fork Cumberland River (Ortmann 1924). At the posterior connection of the mantle pads, there are two protrusions (micro-lures) used to attract host fish, and these are morphologically different among rivers. Based on molecular, morphological, and life history data, there is evidence that the *E. capsaeformis* populations in these rivers are actually distinct species (Jones et al. 2006).

Preferred Habitat

Oyster mussels usually inhabit gravel and sand substrates in medium-sized streams to larger rivers (Parmalee and Bogan 1998). They tend to aggregate in shallow riffles and shoals and can be associated with water willow (*Justicia americana*) beds, found primarily along the shoreline or islands in rivers (Ortmann 1924, Parmalee and Bogan 1998). According to Neves (1991), oyster mussels also inhabit coarse sand to boulder substrate and can be found in pockets of gravel between bedrock ledges in areas experiencing swift current.

Reproductive and Early Life History

In freshwater mussels, bivalve reproduction typically occurs by release of glochidia as a parasite on a host fish. Early observations of freshwater mussel glochidia described them as bivalve parasites (Rathke 1797). Oyster mussels must rely on attracting a host fish to complete the life cycle. Reproduction begins when a male mussel releases sperm into the water column. A female will siphon in the sperm and her eggs will become fertilized. Glochidia develops from fertilized eggs and are located in water tubes referred to as marsupia within a female's gills. When the marsupia are filled, they are visibly swollen and the female is gravid. The female oyster mussel must then attract a host in order for the reproductive cycle to be completed. In some species, glochidia are attached to the female in a mucous strand (conglutinate), which mimics the size and shape of a small fish to attract a host fish (Haag et al. 1995). In other species, the mantle display or conglutinates may resemble macroinvertebrates or crayfish. Once encysted on a fish, glochidia will develop into juveniles over a certain period of time (typically several weeks) and eventually drop off to suitable habitat in streams.

For many North American species of freshwater mussels, little is known of their hosts, and some species have no host information available (Williams et al. 1993). Glochidia of *E. capsaeformis* have been found on the following native host fish: wounded darter (*Etheostoma vulneratum*), redline darter (*E. rufilineatum*), bluebreast darter (*E. camurum*), dusky darter (*Percina sciera*), banded sculpin (*Cottus carolinae*), black sculpin (*C. baileyi*), and mottled sculpin (*C. bairdi*) (Yeager and Saylor, 1995). Of these fish, the banded scuplin has been used as a suitable host fish for laboratory infestations (Neves, 1991; Yeager, 1987).

Glochidia can be held for a few weeks or up to several months by females, depending on the species (Heard 1998). They can be triangular, spherical or hatch-shaped (Lefevre and Curtis 1910), and the number of glochidia released from a single female depends on the size of

the female and size of glochidia produced. They consist of two valves having low calcium content (Kondo et al. 1997), and they often have a hook allowing them to attach to the host fish gills or body. Glochidia without hooks usually attach to the gills. The adductor muscle controls closure of the valves and can make rapid contractions and relaxations. There are sensory hairs that help in the quick contraction of the adductor muscle upon tactile stimulation. Once larvae attach to a host, the adductor muscle remains contracted, holding the larva to the fish for an extended amount of time. This muscle will be replaced by a new pair of adductor muscles during metamorphosis. After glochidia are released, their survival can be anywhere from 2-14 days (Zimmerman and Neves 2001). The rate of successful host infestation is crucial to a parasite, therefore; glochidia must come in contact with a host fish shortly after release from the marsupia. Glochidia attach to the host fish either by surface contact or by water current transport through the gills. Once attached to the fish, host fish tissue forms a cyst over the larva, and this cyst provides protection and nutrients during the metamorphosis into a juvenile. Feeding during the parasitic stage occurs by microvilli of the larva's mantle cells. An increase in carbon content occurs while the larva is within the cyst. The amount of time spent in this parasitic stage in unionids varies from 3 d to 10 mo, depending on species and environmental conditions.

While encapsulated in the cyst, larvae experience many changes during metamorphosis from the parasitic larval stage to the free-living juvenile stage. These changes include replacement of a single adductor muscle with a pair of muscles, formation of mantle tissue involved in food uptake and shell secretion, formation of the digestive system and nervous system, and finally the appearance of a foot and paired gill apparatus. The foot is involved initially in the uptake of food by way of pedal-sweep and pedal-locomotory feeding behaviors, but is also used for burrowing into the substrate. The valves of glochidia remain for an extended amount of time even as the juvenile shell grows. Juveniles have a pair of adductor muscles (anterior and posterior) for closing the two valves, and a layer of epithelium grows over the larval mantle on the interior of the shells. The juvenile intestinal system consists of a ciliated mouth opening, large digestive gland, stomach, and an uncoiled intestine. This entire system has developed by the end of the mussel's parasitic stage.

It is thought that the combination of thinning of the capsule wall and movement of the juvenile mussel aids in escapement of the mussel from the cyst on the host fish (Waller and Mitchell 1989). After release, it is crucial that juveniles find suitable habitat. Mussels may be

able to time their release from the cyst with the current temperature, increasing their chances of finding suitable habitat (Hruška 1992). Juveniles sink to the bottom and move using their foot, making them much more mobile than adults. After finding suitable habitat, they secrete a byssal thread to attach to the substrate (Wächtler et al. 2001), and they remain there for further development.

Significance of Research

In response to the declines in freshwater mussels, a National Strategy for the Conservation of Native Freshwater Mussels was developed by the National Native Mussel Conservation Committee in 1998. One of the many goals of this strategy was to develop diets for artificially propagating juvenile mussels. Efforts to culture mussels in captivity have had some successes, but there are still limitations in growth and survival, and these most likely include inadequate diet (Barnhart 2006). Feeding and rearing juveniles to a size suitable for release is crucial to their survival in the wild. Technology for feeding juvenile mussels requires further development, and much of the technology has only been tested on a few species. Diets must be adapted to large-scale operations in order for propagation to become a significantly beneficial management tool in conservation aquaculture.

For the most part, knowledge of bivalve feeding physiology and their nutritional requirements comes from the marine literature for commercial shellfish. Because mussel species most likely vary in feed requirements and environmental conditions, knowledge of diet requirements by species and age is crucial to adequately care for and rear them in captivity (Gatenby et al. 1996, Gatenby 2003).

Objectives

The purpose of this project was focused on developing a diet and culture protocol for rearing juveniles of *Epioblasma capsaeformis* in a laboratory setting that would support higher growth and survival than previously attained. Results from this study should be applicable for propagation of not only other *Epioblasma spp*, but perhaps other endangered mussel species and could also provide further refinements to nutritional studies. The specific objectives of this project were to determine: 1) gut content and body composition of wild juveniles, 2) whether the addition of bacteria would enhance the micronutrient content of the diet necessary for growth and survival of juveniles, and 3) whether the addition of macronutrient supplements to the diet would enhance growth and survival in juveniles. Because culturing of algae requires

considerable labor and expense, and is economically infeasible for many facilities (Starkey et al. 1998), this project aimed at developing diets for juvenile mussels that do not require live, cultured algae.

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Chapter 1: An evaluation of the biochemical composition and gut content of wild juvenile oyster mussels, *Epioblasma capsaeformis* (I. Lea, 1834).

Introduction

It is generally known that bivalves feed on suspended particles such as bacteria, phytoplankton, microscopic zooplankton, detritus and even dissolved organic material (DOM). Freshwater mussels are omnivores, feeding across trophic levels from the water column and sediment with ciliated gills (Vaughn et al. 2008). Various studies have determined their feeding habits to include diet composition, particle size, and selection of particles by mussels. Yeager et. al (1994) found that gut contents of juvenile *Villosa iris* consisted of mostly particles in the 2-5 µm range. Results of their feeding chamber study showed that juveniles burrow quickly into the sediment and feed interstitially rather than from the water column. During early development of juveniles, particle size may influence selectivity, where larger particles are rejected and smaller particles are accepted. Smaller particles were found to be most common in the gut contents of adult rainbow mussels (*Villosa iris*), while in the gut content of juveniles, only small particles were present (Beck and Neves 2003). Patterson et al. (1998) found that the guts of *Amblema p. plicata* and *Quadrula p. pustulosa* collected from the Ohio River contained significant amounts of detritus and algal cells, including diatoms and green algae ranging in cell size from 4 to 80 µm in length. Planktonic algae such as Chlorophyta (green algae) and Bacillariophyceae (diatoms) have been confirmed as food for freshwater mussels in previous studies (Gatenby et al. 1996, Parker et al. 1998, Nichols and Garling 2000). However, very few works have examined the diet of juvenile mussels, particularly those of endangered species.

Data obtained at White Sulphur Springs National Fish Hatchery, West VA (Patterson, pers. comm) suggest that survival alone is not a reliable indicator of mussel condition for those held in captivity. A more accurate method of measuring condition is obtained by conducting various analyses on a sacrificed specimen, using protein, lipid, carbohydrate, glycogen and calorie content methods. Although there is not a standard method for measuring the condition of juvenile freshwater mussels at this time, glycogen is the primary storage form of carbohydrates in bivalves (Naimo et al. 1998) and is useful in determining physiological condition of unionids (Patterson et al. 1999).

Domestic selection can occur when cultured mussels become accustomed to laboratory conditions and may not be capable of surviving well in the wild. If diets are developed to more

accurately include what mussels consume in nature (specific to individual species), then laboratory-cultured mussels would likely be better adapted to reintrouction. Greater understanding of natural diets would allow for the development of cultured diets that better meet juvenile mussel nutritional demands.

Clinch River, Tennessee

Originating in Tazewell County, southwestern VA, the Clinch River flows for 483 km through the Great Appalachian Valley. It pairs with one of its important tributaries, the Powell River, eventually joining the Tennessee River in eastern TN. The Clinch River has been characterized by its unique mussel biodiversity, which is unlike any other mussel assemblage in the world. The Clinch River in southwestern VA and eastern TN contains roughly 45 species of mussels. At one time, mussels inhabiting the Clinch River provided an important food source for Native Americans and contributed to the success of the freshwater pearl industry in the late 19th and early 20th centuries (Parmalee and Bogan 1998). Unfortunately, the natural habitat of the Clinch was dramatically changed after construction of Norris and Melton Hill dams by the TN Valley Authority (TVA). Due to damming, coal mining, and unsustainable agricultural practices along the river in the last century, water quality and substratum have been negatively impacted, causing a decline in native mussel populations. In spite of their past history, the Clinch and Powell river watersheds have been classified by The Nature Conservancy (1990) as one of the "last great places." Today, the Clinch Valley and its rivers are home to 30 federally listed threatened or endangered species. Conservationists and biologists are currently making efforts to sustain and augment native mussel populations in hopes of recovering the Clinch River's biodiversity.

Study Objectives

The objectives of this research were to determine the gut contents of river-collected juveniles, chemical composition of the body, and finally algal densities in the Clinch River (wild juvenile habitat).

Materials and Methods

Sample Collections

Because this research involved an endangered species, the number of juveniles that could be collected was limited by federal permit. To assess gut content and body composition, 15 river-collected juvenile mussels, ages 1-3 years, were collected by snorkeling from several sites (Frost

Ford, Kyle's Ford, and Honey Hole) on the Clinch River, TN in late summer and fall 2007. Site location, river conditions, and other observations were recorded at collection times.

In mid-August 2007, 8 of the 15 juveniles were collected from the Frost Ford site on the Clinch River, TN and immediately sacrificed and held in ice water for 24 h. Wet weight, length, age and sex of juveniles were determined and recorded. Each juvenile was then pried open, and the visceral mass was removed, placed in 15 ml plastic tubes, capped and stored at -20 ° C until body composition analyses. Prior to body composition analyses, mussel tissue was freeze-dried (Labconco freeze drier) for 36 h to ensure complete drying of the tissue.

In mid-October 2007, 7 of the15 juveniles were collected from the Frost Ford site on the Clinch River, TN and immediately placed in ice water to prevent further digestion. After 1 h, the juveniles were carefully pegged open and placed in 10% neutral buffered formalin to fix the juvenile body for later gut content analysis (Patterson et al. 1998).

In November 2007, seven juveniles were collected from the Kyle's Ford and Honey Hole sites on the Clinch River, TN and held in a container of sterile water. Eleven juveniles were later collected from the Frost Ford site on the Clinch River in May 2008 to make comparisons between winter and spring food habits. Juveniles were allowed to purge their guts for 24 h in the water, after which feces and pseudofeces were collected from the bottom of the container. Feces and pseudofeces were combined. Feces/pseudofeces were stored in a glass vial with the addition of a small amount of 10% neutral buffered formalin to preserve contents for later examination. Juveniles were returned to the wild once feces/pseudofeces had been collected. This method proved to be a successful, non-lethal way of obtaining gut content information in the laboratory.

In late January 2008 and May 2008, 2 L water samples were obtained from the Kyle's Ford and Frost Ford sites on the Clinch River, TN in proximity to juvenile sampling sites. The sample water was stored in a Nalgene bottle and fixed within 24 h of collection with Lugol's solution for later analysis.

Gut Contents

Because gut content and biochemical composition analyses had never been conducted on juveniles of this species; juveniles of *Villosa iris* cultured in the pond at Virginia Tech's Freshwater Mollusk Conservation Center (FMCC) were sacrificed to develop methods (dissection and gut content analysis) prior to this assessment.

To obtain gut contents from the juveniles, each sacrificed individual was opened to remove the visceral mass. The mussel tissue was then cut at the digestive gland and opening of the stomach, and this area was then rinsed with distilled water into a vial. Several drops of 10% neutral buffered formalin were added to each vial to preserve the gut contents for later analysis (Patterson et al. 1998). The gut content analysis also included examination of feces and pseudofeces of several river-collected juveniles. A semi-qualitative gut content analysis was conducted on seven sacrificed juveniles and feces/pseudofeces of seven live juveniles using a compound microscope (Olympus BH-2 Phase Microscope). Dr. Bruce Parker, retired phycologist from Virginia Tech, identified the different genera of algae and other gut inclusions.

Determination of Presence of Bacteria in Gut

Two sacrificed individuals were dissected, and their gut contents were rinsed into vials using distilled water and fixed with several drops of 10% neutral buffered formalin. A 250 µl sample of the fixed gut contents solution was placed into a 2 ml microfuge tube. One microliter of DAPI (4'-6-diamidino-2-phenylindole) stain was added to the gut contents and then allowed to sit in the dark for approximately 30 min to allow the stain to penetrate and attach to any DNA present in the gut contents. Small, visible pieces of gut content were then removed from the solution with forceps and rinsed with the formalin water. A droplet of this solution was placed on a slide, and a cover slip was mounted and sealed onto the slide using wax. The slide was then examined using an epifluorescence microscope (Nikon Microphot-SA) at 100X magnification in oil immersion to increase magnification. Each of the individuals was examined on separate slides. The presence of bacteria in the gut contents, which fluoresced white against the blue background, was determined.

Clinch River Water Content

To determine density and relative abundance of algal genera present in the water column, the water sample was fixed in Lugol's solution and then poured into a 100 ml settling chamber for 48 h to allow particulate matter to settle to the bottom of the chamber onto a cover slip-thin glass surface. At the end of 48 h, the settling chamber column was carefully removed and the settled material was examined for algal composition and density using an inverted microscope (Wild M40-58583 Inverted Microscope). Various genera of algae, including notation of non-algae matter in the sample, were identified by Dr. Bruce Parker. Algal genera were identified using the dichotomous key of Prescott (1978). At each field of view, all algal cells within the

ocular grid were counted. A total of 51 grids for winter water and 137 for spring water (98.17 is the mean of all grids) were examined randomly, and the algal density of the sample was determined using the equation from Vollenweider (1969):

cells ml $^{-1}$ = (((# of cells in a grid X 130) # of grids counted $^{-1}$) X 98.174) 100^{-1}

Biochemical Composition

Body composition analysis of river-collected juveniles included determination of protein, glycogen and calorie content of homogenized mussel tissue.

Protein content

Total protein content from eight individuals was determined spectrophotometrically using a Pierce test kit (BCA 23225), based on the procedure of Smith et al. (1985) and standardized with bovine serum albumin (BSA). In sample preparation, 5 to 6.5 mg (0.0050 to 0.0065 g) of freeze-dried, pulverized mussel tissue was weighed and placed into a reinforced glass homogenizer tube. Freeze-dried mussel tissue was assumed to be homogenized before weighing each sample. Two ml of 0.1 N NaOH was added to the tube and then homogenized with an electric drill and teflon drill bit for 1 min to suspend and digest all tissue. Contents were then transferred to a 15 ml disposable tube. The homogenizer tube was rinsed with 6 ml of 0.1 N NaOH and then transferred into the 15 ml disposable tube, bringing the total volume to 8 ml.

Working reagents and BSA standards were prepared according to test kit protocol. For the test tube procedure, 0.05 ml (50 µl) of each standard and unknown sample replicate was pipetted into a labeled test tube, using a new pipet tip for each sample. One ml of the working reagent was added to each tube and vortexed with a Vortex-Genie. Test tubes were capped and allowed to sit for 2 h at room temperature. After zeroing the spectrophotometer (Genesys 8™ Thermo Spectronic) to the blank made with the standards, the absorbance of all samples was measured within 10 min at 562 nm. A standard curve was created, and the protein of the sample (in µg/ml) was determined using the equation generated from fitting a line to the standards. The protein in mg/g was found using the equation below:

mg/g protein = ((µg/ml/1000) X 8)/tissue weight

Glycogen content

Total proximate glycogen content of mussel tissue from eight individuals was determined based on a modified procedure of the Upper Midwest Environmental Services Center, La Crosse, Wisconsin, with modifications by Ostby (2007). In sample preparation, approximately 3.4-4.9

mg of freeze-dried, pulverized tissue samples were weighed and recorded, and then the tissue was added to 2 ml cryovials. Freeze-dried tissue was assumed to be homogenized before weighing each sample. Standard solutions were prepared based on standard protocol using a glycogen standard powder (*Mytilus edulis* Sigma Type VII). In standard solution preparation, 250 µl of each standard was added to 2 ml cryovials. There were two replicates for each individual tissue sample and standard solution. Each sample and standard cryovial received 500 µl of 30% KOH (potassium hydroxide) and was capped tightly. All cryovials were placed in a 100° C water bath (Precision) for 20 min. Cryovials were then removed and shaken for 30 sec, after which they were placed on ice for 5 min. Then, 750 µl of 95% EtOH (ethyl alcohol) was added to each cryovial, and vials were capped tightly again and shaken. All cryovials were placed in the same water bath for 15 min. After this step, cryovials could be frozen to await subsequent analytical steps at a later time.

In the remaining analyses, cryovials were vortexed with a Vortex-Genie, and the liquid was transferred to 15 ml disposable tubes. Cryovials were rinsed 2-4 times with a total of 5 ml distilled water. An additional 350 µl of distilled water was added to sample tubes, and 100 µl of distilled water added to standard tubes to bring the total volume to 6.6 ml. Tubes were then shaken, and 2 ml aliquots of the solution was added to glass tubes in replicate. Under a fume hood, 100 µl of 80% phenol was added to each glass tube. Five ml of concentrated H_2SO_4 (sulfuric acid) was slowly added to each glass tube to initiate the acid-base reaction. Glass tubes were capped and carefully inverted twice. After these reagents were added, the tubes were allowed to sit under the fume hood for 30 min at room temperature. Two ml from the glass tubes were then pipetted into 2 ml cuvettes. After zeroing the spectrophotometer (Genesys 8™ Thermo Spectronic) to the blank made with the standards, the absorbance of all samples was measured within 10 min at 490 nm. A standard curve was created, and the total glycogen content of the samples (in mg/g) was determined using the equation generated from fitting a line to the standards.

Calorie content

Total calorie content of mussel tissue from four specimens was determined using standard bomb calorimeter methods. Before mussel tissue samples were measured for calorie content, benzoic acid pellets were placed in the bomb calorimeter (Parr 1271) to calibrate the machine (benzoic acid reading was close to 6,318 cal/g). The bomb calorimeter was then

prepared for sample runs. A metal capsule was placed on the calorimeter's scale and pre-weighed. Approximately 0.2-0.3 g of freeze-dried, pulverized mussel tissue was placed in the capsule and post-weighed. The scale was then tared again, once the weight information was sent to the bomb, and approximately 0.36-0.4 g of mineral oil was slowly added to the sample. A 10 cm piece of string was attached to the wire on the bomb head, and the capsule containing the sample was placed under the wick, allowing it to touch the mineral oil. The bomb head was secured on the machine and the lid was closed. Each sample took 8 min to process, after which calorie data in cal/g were derived.

Results

Gut Content

A complete list of algal genera determined from all samples taken (water, gut, feces/pseudofeces) for this objective is presented in Table 1. Shell lengths of sacrificed juveniles for gut contents can be found in Table 5 and for biochemical analyses in Table 6. Shell lengths ranged from 21.21-30.59 mm.

From examination of both the feces and pseudofeces samples, and the gut content samples, a total of 10 genera of algae was identified, with overlap in composition among the samples as expected. Diatoms (Bacillariophyta), green algae (Chlorophyta), and blue-green algae (Cyanoprokaryota) were found in the samples. In the fall feces and pseudofeces samples, the most abundant genus was *Navicula*, while *Diatoma* was most abundant in the spring samples (Table 2, Figure 2). A total of four genera was found in fall samples, and seven genera in spring samples. The spring feces and pseudofeces samples contained significantly more diversity, and the overall numbers of algal cells were higher than in fall samples, attributed to greater algal production in the spring of the year and less so in the fall. Several genera were different between fall and spring samples. The diatoms *Cocconeis*, *Cyclotella*, *Cymbella*, *Diatoma* and *Gomphonema* were present in the spring samples, but not in the fall. The blue-green algae *Lyngba* and *Oscillatoria*, though scarce, were only found in the fall samples. The most abundant algae in the gut content samples were *Chlorella* and *Oscillatoria* (Table 3, Figure 1). Algae found in the gut content samples that were present but uncommon included *Chlamydomonas*, *Coscinodiscus*, *Cyclotella*, *Fragilaria* and *Trachelomonas*. In both gut content and feces and pseudofeces samples, a significant amount of detritus was observed (~90%).

By examining the gut contents of two individuals using DAPI stain and epifluorescence microscopy, an abundance of bacteria was confirmed. Rod-shaped bacteria were seen in the individual guts, and were approximately 1-2 µm in length and 0.5 µm in width. Bacteria were more abundant in one specimen than the other.

Clinch River Water

A total of 13 genera of algae was identified in the winter water sample, and 11 in the spring sample (Table 4, Figure 3). Several algae occurred in the gut contents and feces and pseudofeces samples that were not in either of the Clinch River water samples, including *Chlamydomonas*, *Coscinodiscus*, and *Lyngbya*. All other genera identified also occurred in the water samples. *Chlorella*, the smallest of the algae, was the most abundant genus in the winter sample (76% of total cells), with *Navicula* as second most abundant. *Fragilaria* was the most abundant genus in the spring sample (63% of total cells), with *Navicula* as second most abundant.

Biochemical Composition

Protein content for the eight river-collected juveniles collected in August 2007 ranged from 313-884 mg/g, (Table 6, Figure 4). Although these juveniles were collected from the same site on the Clinch River, the protein content among individuals was highly variable. Glycogen values of four individuals ranged from 49-171 mg/g (Table 6, Figure 5). Caloric levels of three juveniles ranged from 2,935.10-4,287.94 cal/g (Table 6).

Table 1. Classification of algal genera determined from all samples including gut contents, fecal/pseudofecal, and Clinch River water samples.

Phylum Cyanoprokaryota (blue-green algae)
- Class Cyanophyceae
- Order Chroococcales
- Family Chroococcaceae
  - *Chroococcus*
- Order Oscillatoriales
- Family Oscillatoriaceae
  - *Oscillatoria*
  - *Lyngbya*

Phylum Chlorophyta (green algae)
- Class Chlorophyceae
- Order Tetrasporales
- Family Oocystaceae
  - *Chlorella*
- Order Chaetophorales
- Family Chaetophoraceae
  - *Desmococcus*

Phylum Heterokontophyta
- Class Bacillariophyceae (diatoms)
- Order Achnanthales
- Family Cocconeidaceae
  - *Cocconeis*
- Order Centrales (Biddulphiales, centric diatoms)
- Family Coscinodiscaceae
  - *Coscinodiscus*
  - *Cyclotella*
- Order Pennales (Bacillariales, pennate diatoms)
- Family Fragilariaceae
  - *Diatoma*
  - *Fragilaria*
  - *Synedra*
  - *Tabellaria*
- Family Naviculaceae
  - *Navicula*
  - *Pinnularia*
- Family Gomphonemaceae
  - *Gomphonema*
- Family Cymbellaceae
  - *Cymbella*

Phylum Cryptophyta (cryptophytes)
Class Cryptophyceae
Order Chroomonadales
Family Chroomonadaceae
Chroomonas

Phylum Euglenophyta (euglenoids)
Class Euglenophyceae
Order Euglenales
Family Euglenaceae
Trachelomonas

Table 2. Algal genera counts in fecal/pseudofecal samples from live juveniles of *Epioblasma capsaeformis* collected in October 2007 and May 2008 on the Clinch River, TN.

Genus	**Number of cells present**	**Composition (%)**	**Number of cells present**	**Composition (%)**
	Fall	Fall	Spring	Spring
Cocconeis	0	0	14	9.72
Cyclotella	0	0	1	0.69
Cymbella	0	0	2	1.38
Diatoma	0	0	92	63.88
Gomphonema	0	0	16	11.11
Lyngbya	1	14.29	0	0
Navicula	3	42.86	18	12.50
Oscillatoria	2	28.57	0	0
Pinnularia	1	14.29	1	0.69
Total # of genera	**4**		**7**	

Table 3. Algal genera counts in gut contents from eight sacrificed juveniles of *Epioblasma capsaeformis* collected in October 2007 from Frost Ford, Clinch River, TN.

Genus	**Number of algal cells present per individual**	**Composition (%)**
Chlamydomonas	1	4.76
Chlorella	6	28.57
Coscinodiscus	1	4.76
Cyclotella	1	4.76
Fragilaria	1	4.76
Navicula	4	19.05
Oscillatoria	6	28.57
Trachelomonas	1	4.76
Total # of genera	**8**	

Table 4. Density of algal genera in Clinch River samples collected in January 2008 and May 2008.

Genus	**Number of cells present**	
	January 2008	May 2008
Chlorella	169	0
Chroomonas	4	0
Chroococcus	2	0
Cocconeis	0	1
Cyclotella	1	0
Cymbella	2	1
Desmococcus	2	0
Diatoma	9	11
Fragilaria	0	170
Gomphonema	2	3
Navicula	19	65
Oocystis	0	9
Opephora	0	2
Oscillatoria	1	0
Pinnularia	4	2
Synedra	2	5
Tabellaria	6	0
Trachelomonas	0	1
Total # of cells	223	270
Total # of genera	13	11
Total # of grids counted	51	137

Table 5. Shell lengths of juveniles of *Epioblasma capsaeformis* collected for gut content at Frost Ford on the Clinch River, TN in October 2007 used for gut content work.

Individual	Length (mm)
1	28.49
2	30.59
3	30.55
4	30.45
5	29.75
6	28.14
7	29.80

Figure 1. Pie chart of algal genera found in the gut contents of juveniles of *Epioblasma capsaeformis* collected in October 2007 at Frost Ford on the Clinch River, TN.

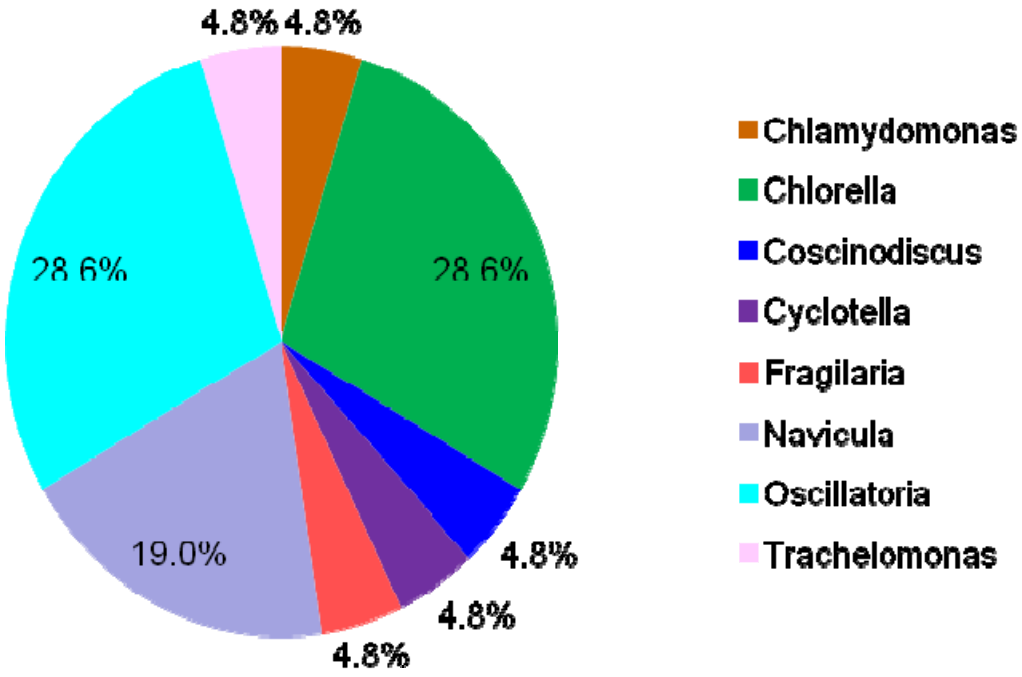
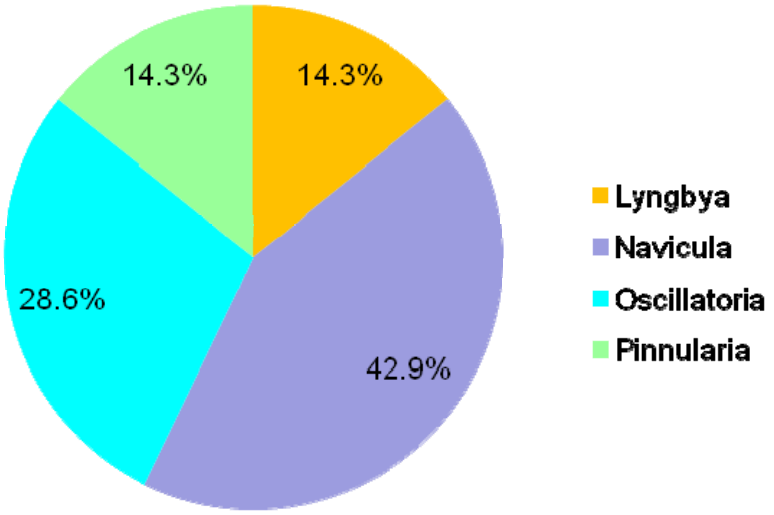


Figure 2. Pie chart of algal genera found in the feces/pseudofeces of juveniles of *Epioblasma capsaeformis* collected in October 2007 and May 2008, respectively, at Frost Ford on the Clinch River, TN.



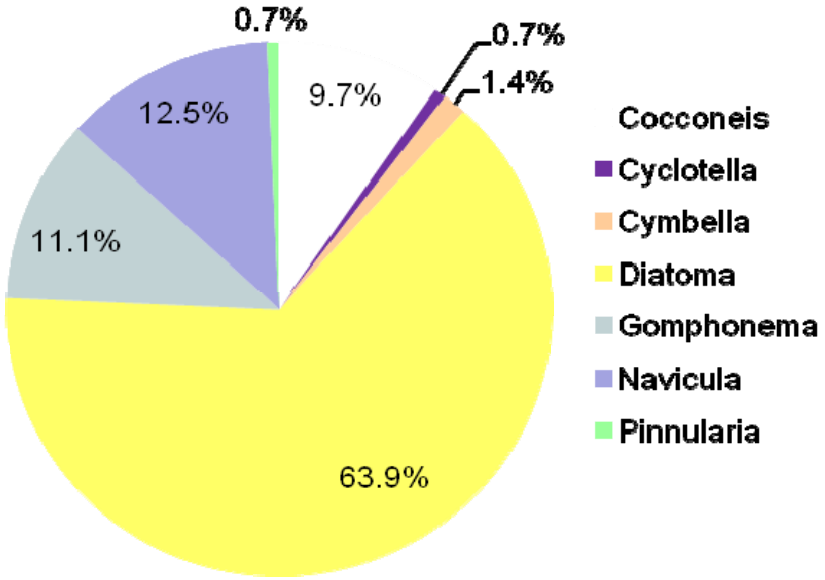
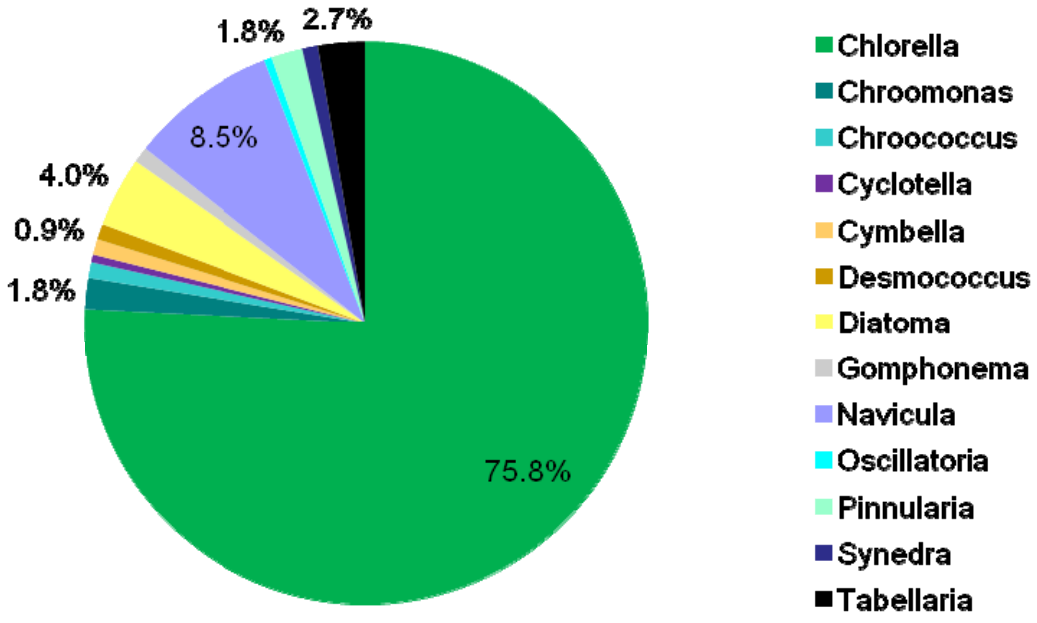


Figure 3. Pie charts of algal genera found in Clinch River water samples collected in January 2008 and May 2008, respectively.



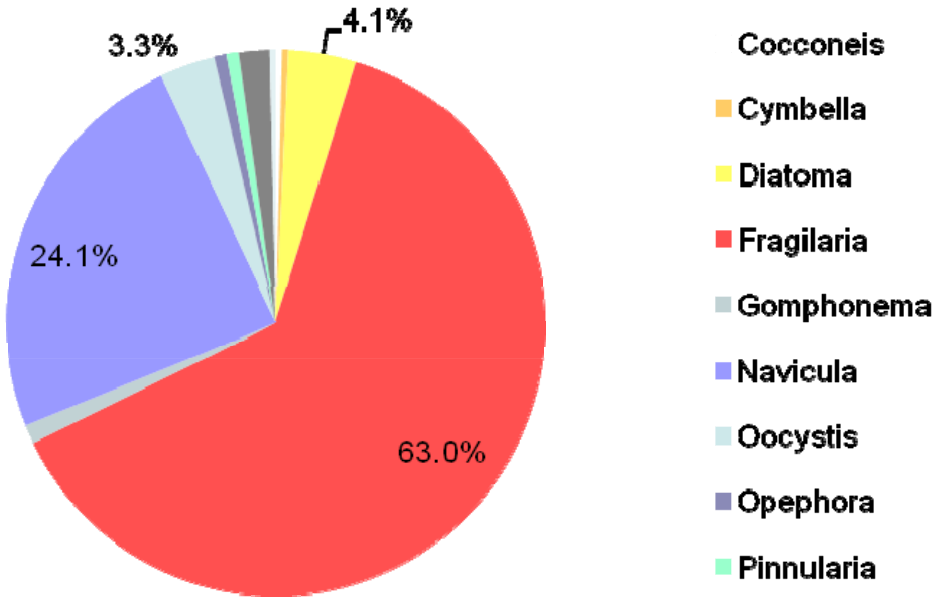


Table 6. Total length (mm), and protein (mg/g), glycogen (mg/g) and calorie content (cal/g) of juveniles of *Epioblasma capsaeformis* (visceral mass) collected at the Frost Ford on the Clinch River, TN in August 2007.

Individual	Length (mm)	Protein (mg/g)	Glycogen (mg/g)	Calorie (cal/g)
1	28.85	545.191	171.911	4,287.94
		555.446	152.823	
2	28.19	425.386	143.111	
		428.103	142.537	
3	na	576.012	55.205	3,236.18
		568.283	49.060	
4	27.98	313.146	61.011	
		314.555	90.302	
5	23.64	572.595		2,935.10
		600.314		
6	21.21	639.280		
		608.242		
7	na	840.073		
		884.351		
8	21.62	590.894		
		575.716		
Mean		**564.85**	**108.245**	**3,486.407**
Standard Deviation		**±153.22**	**±49.715**	**±710.285**

Figure 4. Protein content (mg/g dry tissue) of wild juveniles of *Epioblasma capsaeformis* (visceral mass) collected at the Frost Ford site on the Clinch River, TN in August 2007.

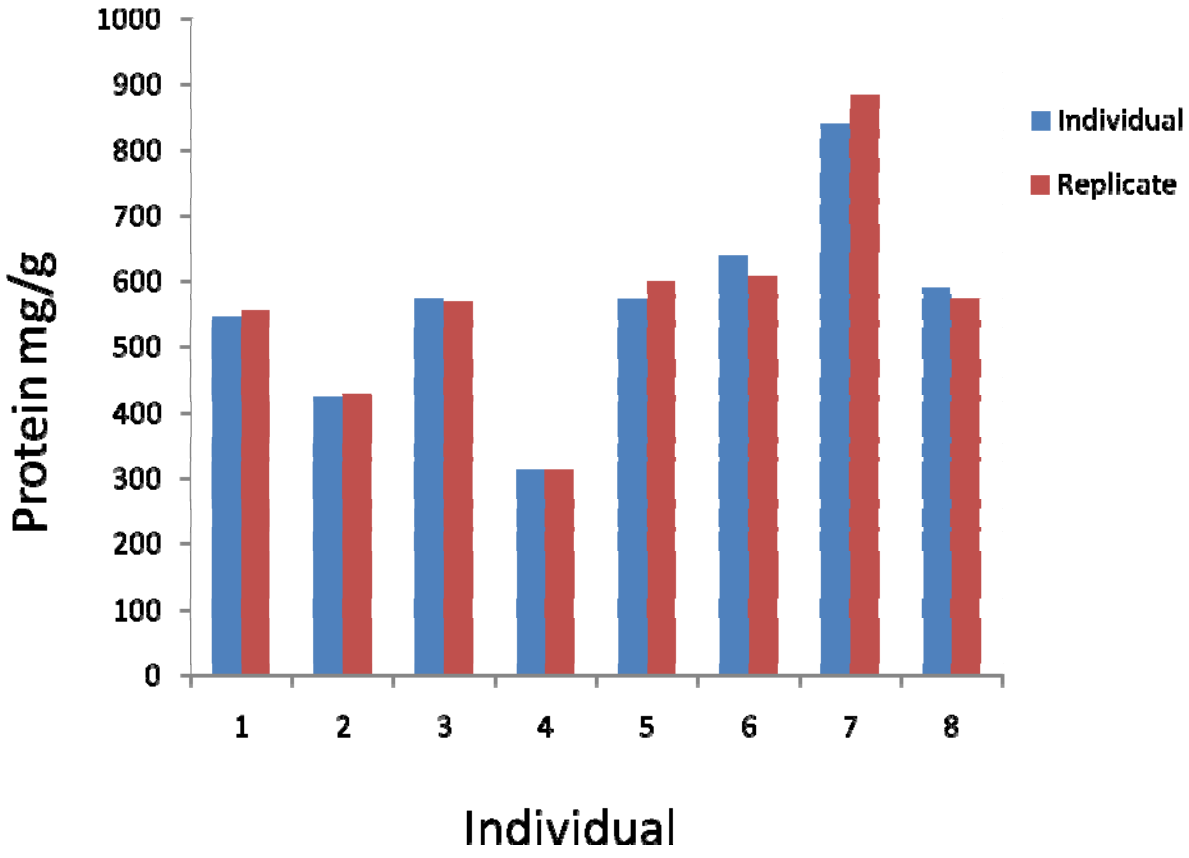
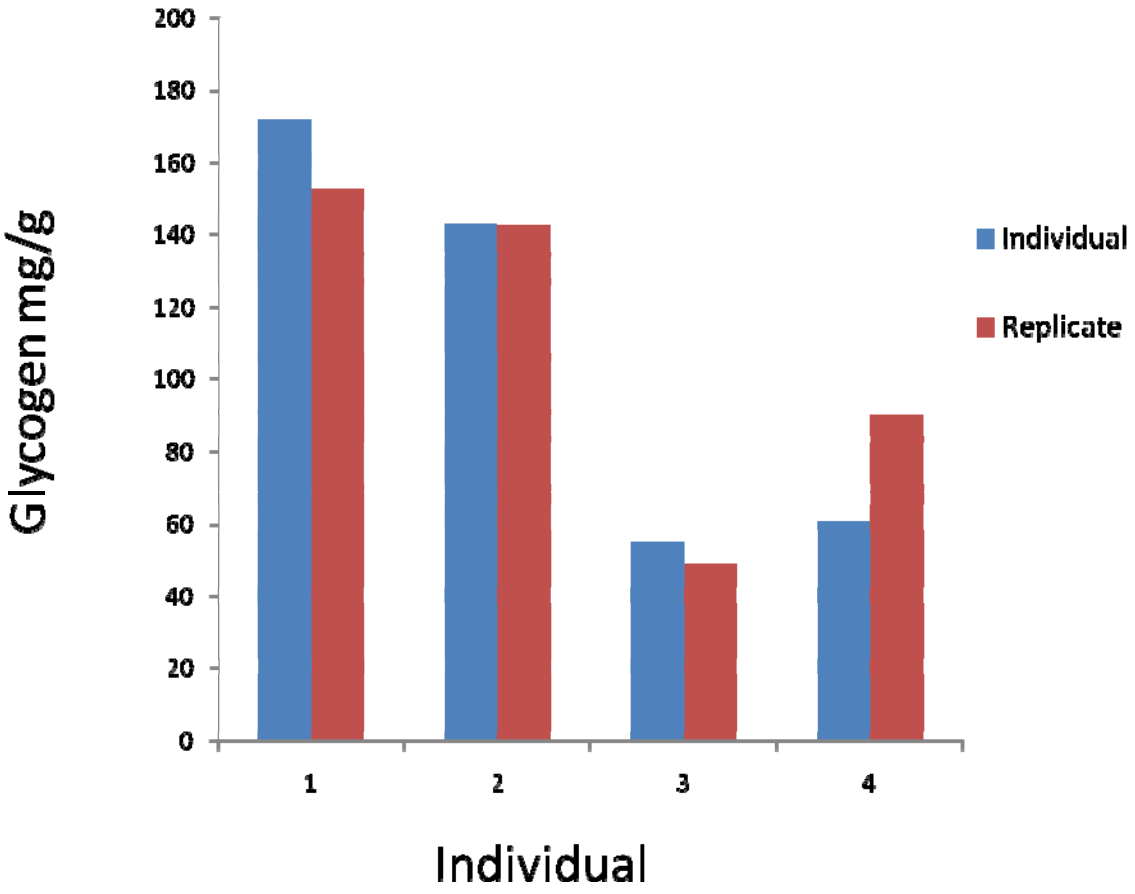


Figure 5. Glycogen content (mg/g dry tissue) of wild juveniles of *Epioblasma capsaeformis* (visceral mass) collected at the Frost Ford site on the Clinch River, TN in August 2007.



Discussion

Gut Content

Algae data for the Clinch River, TN were not found in the published literature; however, algae information was compiled from the National Water Quality Assessment Program (NAWQA) provided by the USGS on the Clinch River above Tazewell, TN. These data, collected in the summers of 1996 and 1998, confirmed algae identified from my samples (Table 7), particularly algae found in the spring samples of juvenile fecal/pseudofecal and Clinch River water samples. These genera included *Cocconeis*, *Cyclotella*, *Cymbella*, *Diatoma*, *Fragilaria*, *Gomphonema*, *Lyngbya*, *Navicula*, *Oscillatoria* and *Synedra*.

Some assessment can be made from examining the Clinch River samples for winter and spring. The most abundant genus in winter was the green alga *Chlorella*, whereas the most abundant alga in spring was the centric diatom *Fragilaria*. *Navicula* was the second most abundant alga in both winter and spring water samples and was more abundant in spring than winter. Both *Chlorella* and *Fragilaria* are typically found suspended in the water column; therefore, juvenile mussels may have obtained at least some of these algae through suspension or possibly interstitial feeding. Results from Yeager et al. (1994) showed that juveniles burrow quickly into the sediment and feed interstitially rather than from the water column. *Navicula* commonly occurs on the substratum or sometimes in suspension, and juveniles could employ pedal-feeding or suspension-feeding behavior to obtain this taxon. As seen from the gut content and feces/pseudofeces samples, juveniles were ingesting two of the most abundant algae in the river, *Chlorella* and *Navicula*. Additionally, juveniles were feeding heavily on *Oscillatoria* and *Diatoma*, both of which were not abundant in the water samples. Perhaps juveniles collected in this study are specifically selecting for these algae, while they are also feeding on algae that are abundant and readily available in the water column. Some general knowledge of the algal genera found in samples including algal type, cell size, and habitat are summarized in Table 8.

In comparing the algal genera found in gut contents and fecal/pseudofecal samples, only *Cyclotella*, *Navicula* and *Oscillatoria* occurred in both samples and in Clinch River water samples. If algae was identified in the fecal/pseudofecal samples, it can be assumed that these algae were not digested otherwise one would no longer be able to identify them. All other algal genera differed between feces/pseudofeces samples and gut content samples. Although *Cyclotella* was found in the gut, spring feces/pseudofeces and water samples, this genus was

scarce (only one cell per sample) in comparison to other algae identified in the samples. I conclude from this information that *Cyclotella* is not an important genus for nutrition of these juveniles, nor was it abundant in the Clinch River when samples were collected. Because *Navicula* and *Oscillatoria* were much more abundant in all samples and found in both feces/pseudofeces and gut contents, it seems that *E. capsaeformis* juveniles may be selecting for and ingesting these algae in the river.

Navicula was most abundant in the fall feces/pseudofeces samples, *Diatoma* in the spring samples, and *Chlorella* and *Oscillatoria* were most abundant in the gut content samples. *Navicula* and *Diatoma* are diatoms, *Chlorella* is a green alga, and *Oscillatoria* is a blue-green alga. *Navicula* is an epiphyte typically found living on leaves, blades and stems of submerged macrophtyes in the benthos (Pentecost, 1984). *Chlorella*, *Cyclotella* and *Oscillatoria* often occur suspended in the water column (Pentecost, 1984), and if juveniles were ingesting these three algae, then they were most likely able to suspension-feed. Since juveniles collected for this objective were between one and three years of age, they were most likely capable of suspension-feeding behavior and were relying less on pedal-feeding as a means of obtaining food.

Previous observations have found that juveniles of *E. capsaeformis* probably ingest particles in the size range of 2-8 µm (Jess Jones, pers. comm.). Cell width sizes of *Navicula*, *Diatoma*, *Chlorella* and *Oscillatoria* range from 1.5-12 µm, though *Oscillatoria* can be as large as 20 µm. In examining all algae in either the gut or feces/pseudofeces samples, particle width size-range was 1-20 µm, with the exception of *Coscinodiscus* (up to 70 µm in diameter). Most likely then, *E. capsaeformis* juveniles (1-3 y of age) could have fed on particles up to 20 µm in size and probably fed on particles within the 1.5-12 µm size range.

Many of the diatoms in gut content and feces/pseudofeces samples did not have protoplasm, suggesting that they were digested. Diatoms are more informative than other algae in determining what juveniles digested because their exoskeleton is left behind after digestion occurs. In comparison to other algal types, diatoms were more frequent and in higher amounts in the juvenile guts and feces/pseudofeces. This may be due to a juvenile dietary preference or because the Clinch River contains an abundance of diatoms, readily available to juveniles. In either case, diatoms retain essential nutrients in the food chain that would otherwise be lost. One diatom cell is usually filled with as much as 11% by volume of oil droplets, and these cells are basically a capsule of stored food, making them an excellent source of nutrients for growing

juveniles (Prescott, 1984). A study of the biochemical composition of two green algae species and one diatom species revealed that protein was the most abundant component, followed by carbohydrate and then lipid (Gatenby et al. 2003). Polyunsaturated fatty acids (PUFA) and sterols are present in green algae and diatoms (Gatenby et al. 2003), and PUFAs are presumed an important part of freshwater mussel diet as they are important to all organisms. Perhaps *E. capsaeformis* juveniles select for diatoms because of their richer nutrient content.

In addition to algae, grains of silt and detritus (~90%) were found in the gut and feces/pseudofeces samples. Detritus, consisting of plant material, is most likely one of the main organic materials consumed as a food source. Bacteria also were found in juvenile samples by both epifluorescent and compound microscopy; hence, bacteria may provide nutrients to these juveniles. Results from this study show similar results to those of Yeager et al. (1994); gut contents of 3-5 d old *Villosa iris* juveniles contained mostly flagellated bacteria (2-5μm) and detritus in the same size range.

Other gut content studies completed on freshwater mussels have reported similar results. Yeager et al. (1994) found diatoms (*Fragilaria* sp.) and *Chlorella* in the gut contents of 3-5 d old *V. iris* juveniles. Gut content analysis of these *V. iris* juveniles showed that they were ingesting bacteria, detritus, algae, and colloidal particles from the interstitial water. Patterson et al. (1998) found that the guts of adult *Amblema p. plicata* and *Quadrula p. pustulosa* collected from the Ohio River contained significant amounts of detritus and algal cells, including diatoms and green algae ranging in cell size from 4 to 80 μm in length. Some of the dominant algal genera in these adult mussels, *Chlorella*, *Cyclotella* and *Navicula,* were found in the juveniles of this study.

By collecting gut content and feces/pseudofeces samples, I was attempting to obtain as much data as possible on the types of algae consumed. By using both sets of data, a more accurate assessment can be made on algae consumption in the river. Because feces and pseudofeces within a sample were not separated, I can only draw basic inferences as to what algal genera were ingested; however, this method is a non-lethal way of obtaining feeding information on river-collected juveniles. The gut content samples likely provided more accurate data because these samples were obtained from dissected juveniles by rinsing their guts. By collecting river water samples, more information was obtained and provided insight into what types of algae and other organic matter are available to juveniles.

Biochemical Composition

Protein composition of juveniles (ranging from 30% to almost 90% protein content of dry tissue) was most likely variable among individuals perhaps due to differences in their sex. In a study completed by Marin et al. (2003), they found that reproductive cycle and physiological features influence biochemical composition in clams (*Tapes philippinarum*). Season can also affect biochemical composition in mussels. A study examining seasonal differences in composition and physiology in the freshwater mussels *Amblema plicata* and *Actinonaias ligamentina* found that both species had low protein and high carbohydrate content in early summer; however, larval brooding did not affect biochemical composition of these mussels. Although my juveniles were relatively the same size, age may have also been a factor in the variation of protein content among individuals.

By conducting bomb calorimetry on the visceral mass of several individuals, a range of 2,935.10 to 4,287.94 cal/g was determined. The calorific values among individuals were likely variable because they were all separate individuals with different amounts of visceral mass as I was attempting to use as much remaining tissue per individual as possible to conduct this analysis. This range is a preliminary assessment that can serve as a foundation for future calorimetric work conducted on juvenile mussels. As a comparison, mussels of the genus *Perna perna* cultivated for human consumption were analyzed for proximate composition and caloric value each month for a year, and the study determined mussels had a caloric value of 118 kcal 100 g^{-1} (Tavares et al. 1998). It would be beneficial to repeat these analyses with a larger number of individual mussels (this may require the use of a non-endangered species), to determine a much more accurate baseline calorific range for freshwater mussels.

Table 7. Algae data from the National Water Quality Assessment Program (NAWQA), provided by the USGS of the Clinch River above Tazewell, TN in June 1996 and July 1998. Those algal genera with an asterisk (*) are the same as those of this study.

Genus
Achnanthidium
Amphora
Caloneis
Cladophora
Cocconeis *
Cyclostephanos
Cyclotella *
Cymbella *
Diatoma *
Encyonema
Encyonopsis
Fragilaria *
Gomphonema *
Karayevia
Leibleinia
Lyngbya *
Mayamaea
Melosira
Navicula *
Nitzschia
Oedogonium
Oscillatoria *
Phormidium
Planktolyngbya
Planothidium
Reimeria
Rhoicosphenia
Sphaerocystis
Staurosira
Staurosirella
Surirella
Synedra *
Thalassiosira

Table 8. The algal genera found in wild juveniles of *Epioblasma capsaeformis* gut content and feces/pseudofeces samples, including general information about each genus. Information about these algal genera was found in Pentecost (1984) and Prescott (1964).

Genus	Algal type	Algal cell width (µm)	Typical habitat
Cocconeis	Diatom	1.2-2.2	Benthic, epiphytic
Coscinodiscus	Diatom	20-70	Planktonic
Cyclotella	Diatom	6-13	Benthic, planktonic
Cymbella	Diatom	5-8	Benthic
Diatoma	Diatom	3-10	Benthic
Fragilaria	Diatom	5-20	Benthic, planktonic
Gomphonema	Diatom	5-8	Benthic, epiphytic
Navicula	Diatom	4-8	Benthic
Pinnularia	Diatom	5-8	Benthic
Chlamydomonas	Green alga	12	Pools in stream
Chlorella	Green alga	2.5-12	Planktonic
Oscillatoria	Blue-green alga	1.5-20	Benthic, planktonic
Lyngbya	Blue-green alga	12	Planktonic
Trachelomonas	Euglenoid	1-2.5	Benthic, epiphytic

Conclusions and Recommendations

Juveniles of *E. capsaeformis* from the Clinch River fed on particles mostly 1.5-12 μm in size. Juveniles consumed a large number of diatoms in their diet. In addition to diatoms, *Chlorella* and *Oscillatoria* were abundant algal genera in juvenile gut content samples and seem to be important components of their natural diet. Detritus and bacteria are also major components of the juvenile diet. Examination of the juvenile diet using both gut content and feces/pseudofeces samples were helpful methods in assessing ingestion of food, and these same methods are applicable to other species of freshwater mussels. Biochemical composition analyses showed variable results of which few definite conclusions can be drawn at this time; however, these results can serve as a baseline for other studies. Below are several management recommendations that have been determined from the research completed.

1) Juveniles of *E. capsaeformis* juveniles fed on particles mostly 1.5-12 μm in size. In formulating a diet for cultured juveniles, this size range should be applied.
2) Juveniles in the river consumed a large number of diatoms in their diet. According to Gatenby, diatoms should be a major component of the diet fed to cultured juveniles, as they are rich in unsaturated fatty acids which are needed for growth and production of hormones in juvenile mussels.
3) In addition to diatoms, *Chlorella* and *Oscillatoria* were abundant algal genera in juvenile gut samples, and these could be beneficial components in a diet fed to cultured juveniles because they are high in protein and abundant in the wild.
4) Based on the types of particles found in the guts and feces/pseudofeces samples, it seems likely that the juveniles (ages 1-3 y) were capable of both suspension and pedal-feeding.
5) Collection and examination of gut content and feces/pseudofeces samples can provide useful data for determining the diet of river-collected juveniles. Additional studies to analyze gut content samples from juveniles of various species would better identify their diet in natal rivers.
6) The condition of juveniles cultured at facilities should be assessed by conducting protein, lipid, carbohydrate or glycogen assays to determine whether diets are suitable.
7) The protein range for juvenile mussel dry tissue was 313-884 mg/g. This range can serve as a foundation for future protein work conducted on this and other freshwater mussel species.

8) The glycogen range for juvenile mussel dry tissue was 49-171 mg/g . This range can serve as a foundation for future glycogen work conducted on this and other freshwater mussel species.
9) The calorific (energy) range for juvenile mussel dry tissue was 2,935.10 to 4,287.94 cal/g. This range can serve as a foundation for future calorimetric work conducted on this and other freshwater mussel species.

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Chapter 2: An evaluation of various diet treatments to enhance growth and survival of juvenile oyster mussels, *Epioblasma capsaeformis* (I. Lea, 1834), reared in a captive environment.

Introduction

Nutritional Requirements

The specific food habits of *Epioblasma capsaeformis*, as with most freshwater mussel species, are unknown. In general, bivalves feed on suspended particles such as bacteria, phytoplankton, microscopic zooplankton, detritus and dissolved organic material (DOM). Information on bivalve diets has been gained from studies of gut contents (Patterson 1998, Beck and Neves 2003), measurement of isotopic enrichment or depletion in tissues (Bayne and Hawkins 1992), or by analysis of digestive and absorptive efficiencies. Isotope data completed for *Velesunio ambiguus* suggest that most carbon found in their diets is derived from algae rather than benthic detritus (Bunn and Boon 1993, Nichols and Garling 2000).

Results from many studies indicate that algae are a suitable food source for rearing early juvenile mussels. Previous studies have indicated that mussels may be successfully reared and held in captivity on algal-based diets (Hudson and Isom 1984, Gatenby et al. 1996, Gatenby et al. 1997). The algal *Neochloris oleabundans*, having a diameter of 2-20 μm, has been shown to be a suitable diet for both adult and juvenile mussels, particularly for *Villosa iris* (Gatenby et al. 1997, Gatenby et al. 2003) and for *Epioblasma capsaeformis* (Bush 2008). Previous diet studies utilizing this algal species have shown adequate growth and survival rates for juvenile mussels (O'Beirn et al. 1998, Gatenby et al. 2003). Gatenby et al. (1997) also found that an algal diet high in oils is best for culturing recently metamorphosed juveniles. When developing a suitable algal diet for mussels, the nutritional value and the concentration of algae must be considered.

Juvenile Feeding

Recently metamorphosed juveniles begin benthic life with three pairs of poorly developed, posteriorly positioned gill filaments (Lasee 1991). Though there is development of an exhalant aperture in one to two-week old juvenile unionids (Wright 1995), the development of functional inhalant and exhalant apertures awaits further growth and differentiation (Dimock 2000).

Observations have determined that juveniles exhibit pedal-feeding behavior, the collection of food via sweeping motions of the foot (Reid et al. 1992), while adults suspension-feed by remaining sedentary and drawing water and particles through an incurrent aperture. From Gatenby's (1994) observations, it appeared that juveniles pedal-feed for approximately 120 days, depending on the species.

Importance of Substrate

Historically, juvenile mussels propagated in an aquaculture environment did not survive for longer than a few weeks (Lefevre and Curtis 1912, Howard 1917). Low survival in juveniles after excystment from the fish host is likely due to missing characteristics of their habitat and ecology (Isley 1911). Substrate is a critical part of the habitat, serving as protection and as a means for employing pedal-feeding behavior. Many studies have examined the effects of substrate on the diet and habitat for freshwater mussels reared in captive environments. In one study of the presence of sand within the cage system, *Margaritifera margaritifera* (eastern pearlshell) juveniles were cultured in an increased surface area for colonization by micro-organisms and by filtering out particulate organic matter, both of food value (Buddensiek 1995). *Mytilus edulis*, the marine blue mussel, fed with unicellular algal cells within the range of optimal food concentration, was highly stimulated by low quantities of suspended silt; though the mussel was filter-feeding versus pedal-feeding, the study supports the use of substrate particles in a diet (Winter 1978). Previous studies on *M. margaritifera* suggest that 95% of juveniles leaving the host fish die before they are able to establish in the substrate, but once established, mortality is low (Young and Williams 1984).

In examining studies completed on freshwater mussels, juveniles of *Lampsilis fasciola* and *Villosa iris* cultured in silt grew faster and produced a byssal thread for attachment to the substrate. Those mussels in containers without the presence of silt did not grow as fast (O'Beirn et al. 1998). Gatenby (1994) found that 45-day-old juveniles of *V. iris* and *Pyganodon grandis*, fed algal diets in addition to silt, showed an increase in shell length and better survival, while juveniles fed algal diets without silt showed no increase in shell length, with survival varying among species. The silt most likely served as a physical substratum for juvenile pedal-feeding and likely provided some nutritional value. Barnhart (2006) concurred that suspended silt could be a source of nutrition. Finally, survival and growth of both the rainbow mussel (*V. iris*) and the oyster mussel (*Epioblasma capsaeformis*) were significantly greater when cultured in a substrate

than in no substrate (Jones et al. 2005). Because substrate was beneficial to *E. capsaeformis* juveniles, it was crucial to include a sediment substrate in culture conditions during feeding trials for this study.

Other Factors in Developing Freshwater Mussel Diets

For successful rearing of mussels in aquaculture, it is important to recognize optimal conditions for growth, including quality and quantity of the food (Winter 1978). In examining ingestion in bivalves, the amount of food ingested (per unit of time) is based on body size, temperature, and food concentration. Relative amounts of food ingested by smaller bivalves are significantly higher than that in larger individuals. The amount of food ingested increases in smaller bivalves due to their high metabolic rate; therefore, juvenile mussels will ingest and require greater amounts of food particles (Winter 1978). The assimilation efficiency should also be considered when developing a diet for freshwater mussels. Assimilation efficiency, defined as the amount of nutrients absorbed by the body after digestion and expressed as a percentage of the amount of food ingested, depends on quality and quantity of the food ingested (Winter 1978). At high cell densities, the assimilation efficiency is low and vice versa (Widdows and Bayne 1971).

Knowledge of maintenance ration, as a function of body size and temperature, is very important to aquaculture efforts. As long as the amount of food in the diet is above the level of maintenance ration, growth will occur. Some production of pseudofaeces is normal, but excessive amounts would signify that juveniles are receiving a higher food quantity than what is necessary for adequate nutrition.

Winter and Langton (1976) found that growth is not only influenced by the total amount of food presented, but also how often food is provided to bivalves. In feeding experiments where constant food concentration is maintained and cell concentration remains high enough, there are no periods of rest in feeding, no formation of pseudofeces, and there is steady food intake and food digestion. These characteristics provide the most suitable dietary situation for mussels reared in aquaculture systems (Winter 1978).

Diets for *Epioblasma* Species

There are only two studies that have been conducted on determining a suitable diet and feed environment for *Epioblasma* species, and these include studies by Jones et al. 2005 and Bush 2008. Bush examined potential seasonal differences in diets of *Epioblasma* species reared in captivity and found that adult *E. capsaeformis* mussels should be fed a diet high in energy

(carbohydrates and lipids) in the spring and summer, when mussels were limited in energy. Mussels should be fed a diet high in protein during the winter, as mussels seemed to be deficient in this macro-nutrient during that time of year. Bush's results supported Gatenby et al. (2003), in that the alga *Neochloris oleoabundans* provides sufficient dietary protein for *E. capsaeformis*. Bush also concluded that all adult *Epioblasma* species and each sex could be fed equal rations. Currently, the most updated knowledge for the feed requirements of *E. capsaeformis*, as well as other *Epioblasma* species, is provided by Bush (2008).

Study Objectives

Holding and propagation of adult and juvenile mussels occurs at several federal fish hatcheries, including Genoa National Fish Hatchery, Genoa, WI; Neosho National Fish Hatchery, Neosho, MO; Warm Springs National Fish Hatchery, Warm Springs, GA; and White Sulphur Springs National Fish Hatchery, White Sulphur Springs, WV. Although several of these hatcheries have attempted to propagate juveniles of the federally endangered *Epioblasma capsaeformis* in a captive environment, this species continues to be one of the most challenging to rear because of high mortality in the first 2 mo of culturing. I selected this species to provide recommendations on diet and nutrition for these juveniles to hatcheries in raising this and perhaps other juveniles of endangered mussel species in captivity. My efforts were focused on developing a diet for juveniles of *E. capsaeformis* that will support growth and survival in the laboratory setting and be applicable for propagation of other *Epioblasma spp.*). The specific objectives of this study were: 1) whether the addition of bacteria will enhance the micronutrient content of the diet necessary for growth and survival of juveniles, and 2) whether the addition of macronutrient supplements will enhance the diet necessary for growth and survival in juveniles. By evaluating test diets over time in feeding experiments, a more suitable diet for this species can be formulated.

Materials and Methods

Before conducting the feeding trials for this project, necessary field and laboratory work was completed and is described in the following paragraphs.

Sample Collection

There were two basic tasks of the field work conducted before the feeding trials: 1) obtain several gravid females of *E. capsaeformis* for the fish infestation and production of juveniles, and 2) obtain *Cottus carolinae* (banded sculpin) as host fish for the infestation.

Females of *E. capsaeformis* typically become gravid in early spring; therefore, they were collected from Frost Ford on the Clinch River, TN by snorkeling in May 2007. Displaying females were carefully examined for abundance and maturity of their glochidia. The number of mussels collected, locality data, and relevant notes were recorded. Females were transported to the laboratory in a cooler containing aerated river water and then held at the Freshwater Mollusk Conservation Center (FMCC) in a Living Stream containing substrate and water (50% conditioned municipal and 50% conditioned well water). They were fed once daily with a laboratory-protocol algae diet consisting of Nanno 3600 and Shellfish Diet 1800 [Reed Mariculture Inc]. Water temperature was maintained at 15° C before infestations to prevent premature glochidial expulsion. Infestations provided juveniles for a pilot study and for feeding trial 1.

Banded sculpins (*Cottus carolinae*) were selected as the host fish for *E. capsaeformis* infestations based on previous studies (Yeager and Saylor 1995). Host fish were obtained by electrofishing using a Smith-Root, INC LR24 Electrofisher in riffle habitats of the Middle Fork and South Fork Holston rivers (May and July 2007) in Smyth and Washington counties and Sinking Creek (November 2007) in Giles County, VA, transported to the laboratory in coolers containing aerated river water, and then held in quarantine tanks to acclimate at least 1 wk prior to infestation. The number of fish collected, site location, locality data, relevant notes and how long fish were held in captivity were recorded.

Fish Infestation

Because survival and growth of *E. capsaeformis* are significantly greater when propagated in the spring when glochidia were mature (Jones et al. 2005), most of the infestations for this project were completed in spring to ensure healthy juveniles. The female mussel was carefully pried open, and glochidia were removed from marsupial gills by injection of water into the gill with a syringe and hypodermic needle. All glochidia were then rinsed into Petri dishes. To determine whether the glochidia were viable, samples were tested with salt (NaCl) and examined microscopically. If the valves of the glochidia rapidly closed upon exposure to NaCl, the glochidia were assumed to be mature and used for infestation (Zale and Neves, 1982).

Approximately 50 banded sculpins were placed in 1-2 L containers of aerated water. Three to four Petri dishes with glochidia were poured into the containers, and fish were left for 1 hr undisturbed for the infestation. Based on previous laboratory infestations, 1 hr is sufficient

time for glochidial attachment to the fish. Host fish were then transferred and held in a recirculating tank system maintained at 19-20° C and fed a diet of bloodworms once daily. Fish mortality was checked daily.

Juvenile Culture

Recently metamorphosed juveniles were siphoned from the bottom of the fish tanks using 150 and 300 micron sieves, counted using a microscope, and then transported to a recirculating juvenile culture system containing 2 mm of sieved sediment and filled with sieved (100 μ) pond water, prior to the feeding trials. The pond water was obtained from the outdoor pond at the FMCC and was changed daily to maintain good water quality. Fine sediment was collected from McCoy on the New River, Montgomery County, VA, sieved to 200 μm, autoclaved, and then aerated in conditioned water for 1 wk before use. Sediment was added to the bottom of the juvenile culture tanks and allowed to settle for several days before the addition of juveniles. This sediment was selected as substrate in order to provide a habitat for juveniles and aid in pedal-feeding; it was added to each culture container of every treatment. Organic content of the sediment used in the feeding experiments was analyzed (Appendix J).

Juveniles were cultured from the time of drop-off from the host fish to 2 wk after drop-off, before the feeding trial began. The decision to use pond water and sieved, autoclaved sediment, as well as the culture period, was based on previous laboratory protocol for the culture of this species that has resulted in the healthiest juveniles. Before feeding trials commenced, juveniles were fed a mixed commercial algae [Reed Mariculture Inc.] daily of Shellfish Diet 1800 and Nanno 3600. This diet is described in more detail in Appendix A and B.

After the 2-wk culture period, juveniles were acclimated for 1 wk prior to the feeding trial, to transition from pond water to conditioned water used in the experiment. Some juveniles continued to be held in pond water; these would be sampled for use in the pond water treatment. Juveniles were held in culture containers with 3,000 ml of pond-water with flow and sieved, autoclaved sediment. With the exception of the pond water batch of juveniles, 25% of the water in sump tanks was changed from pond water to conditioned water each day. All juveniles were fed three times daily on the algae mix diet utilized previously during the culture period. Average age of juveniles was 21 d old at the time of initiating feeding trials.

General Feeding Trial Methods

Feeding Trial System

A recirculating aquaculture system (RAS) at the FMCC was selected for the feeding experiments. This system contains six plastic sump tanks (replicates), and each sump tank has its own water source, providing flow for potentially four 500-ml plastic containers for juveniles. Aeration tubes of polyvinyl chloride (PVC) entered at the top of each culture container to provide recirculation and adjustable flow within the container. Bag sieves located underneath each dish prevented escapement of juveniles from culture containers into the sump tank below, and these sieves were checked during feeding trials to return juveniles to their proper culture container. The RAS and its components were either replaced or acid-washed between feeding trials to provide a clean environment for juveniles.

Water Quality

Conditioned water in sump tanks was changed every 2 to 3 d, while pond water in sump tanks was changed daily. Sump-tank water in the commercial algae mix and probiotic diet was changed weekly so that nitrifying bacteria in the probiotic product could adequately establish a healthy aquatic environment. The water quality parameters of temperature, pH, dissolved oxygen (DO), nitrate (NO_3), nitrite (NO_2) and total ammonia nitrogen (TAN) were monitored in every sump tank weekly for the first feeding trial, to determine a reusable water quality protocol. These parameters were measured using Hach test kits. Based on a study completed by Augspurger et al. (2003), freshwater mussels can only withstand between 0.3 and 1.0 mg/L of total ammonia as N; therefore, this parameter in particular needed to be monitored.

Water quality was monitored less frequently in subsequent feeding trials based on results from trial 1. Water temperature was recorded daily to ensure a temperature range of 18-22° C. Mean temperature was calculated at the completion of each feeding trial, and was 21º C for both trial 1 and 2. Water quality of conditioned water and pond water remained within acceptable values for mussels throughout the duration of both feeding trials based on Hach test results (see Appendix H for water quality test results).

Preparation of Culture Container

Approximately 2-3 mm of sediment was added to each culture container prior to the addition of juveniles. Before initiating flow in the containers, the sediment was allowed to settle in the dishes under a drip flow for approximately 30 min. This water was then siphoned off, and

the container was refilled with water on a drip flow before juveniles were added. Juveniles were carefully rinsed by spray bottle from a Petri dish into the culture container. To prevent disturbance of juveniles, flow within the dish was not initiated until juveniles had settled to the bottom for approximately 30 min. These methods were based on previous FMCC protocols for preparing culture containers for juveniles. Figure 1 is a schematic of the culture container components.

Feeding Methods

Feeding treatments were initiated on the first full day after setup of the experiment. Food was added directly to the culture containers holding juveniles, and flow was temporarily removed to allow diets to settle to the substrate for several minutes before resuming water flow. All liquid feeds, including algae mix, bacteria floc, Culture Selco Plus, and Protein Selco Plus, were added to the containers in a circular motion using a Gilson pipetman. These feeds were refrigerated when not in use. Calculations used to determine the amount of feed for each treatment are shown in Appendix H. More specific details about commercial feeds are presented in Appendix A-G. A complete listing of all treatments used in both feeding trials is summarized in Table 3.

Feeding Trial 1 Treatments

Four different diet treatments were evaluated in feeding trial 1, with particular interest in the effects of bacteria-supplemented diets on juveniles. Each treatment was assigned to three systems (N=3). The diets were added directly to the water column in the culture containers with juveniles. The treatments for the feeding trial included:

1) commercial algae-mix and bacteria flocs
2) commercial algae-mix and probiotic
3) commercial algae-mix at triple concentration (3X the algae concentration currently fed to juveniles at FMCC)
4) pond water only

Algae mix

Current FMCC protocol for feeding juveniles at the laboratory is a 550 ml liquid diet of 10 ml of Shellfish Diet 1800, 10 ml of Nanno 3600 and 530 ml of conditioned water. Shellfish Diet 1800 and Nanno 3600 are commercial algae produced by Reed Mariculture [www.reed-mariculture.com]. A standard 30 squirts from this bottle (approximately 2.7 ml) is added to

juvenile culture tanks once daily, and flow was turned off in the tanks during feeding to allow settlement of algae. This diet was selected as a standard for comparison among treatments in feeding trials, and treatments were designed to supplement this current diet. By conducting feeding trials with this laboratory diet, it could be determined whether juveniles of *E. capsaeformis* were receiving adequate nutrition or perhaps would benefit from other nutritional components.

The algae-mix used in the above treatments was the same as the diet used in juvenile culture. The algae-mix used to feed juveniles at FMCC consists of one quart of Shellfish Diet 1800, a mixed diet of *Isochrysis*, *Pavlova*, *Thalassiosira weissflogii* and *Tetraselmis* algae species (size range of 5-20 µm with a cell count of two billion per ml), and one quart Nanno 3600, composed of the single *Nannochloropsis* species (size range from 1-2 µm with a cell count of 68 billion per ml). The amount of algae-mix fed to juveniles was determined from calculations of the volume of the system and on current protocol at FMCC (Appendix H). The algae-mix was added to each culture container of the treatment three times daily at 830 µl (2.5 ml per day). The diet was added directly to the tanks with juveniles, and flow was temporarily stopped to allow algae to settle to the substrate.

Both Nanno 3600 and Shellfish Diet 1800 were analyzed for protein content during the course of 40 d (average feeding trial duration), with samples taken at day 0 (when bottle was first opened), day 20 and day 40. These samples were taken to determine whether the protein level in algae used in feeding juveniles changed over time. Results of proximate protein analysis can be found in Appendix A.

Bioflocs

Bioflocs are a conglomeration of organisms including several bacteria species, fungi, filamentous organisms, algae, protozoans, metazoans and nematodes. The flocs can range in size from 50 to 500 µm, although they are typically 75-200 µm in size.

To create bioflocs, a 250 g bioreactor was filled with tilapia wastewater from tanks at the VA Tech Aquaculture Center in Blacksburg, VA. It is important to use freshwater fish wastewater because presence of salt will interfere with floc development. A PVC circular aeration unit was placed in the bottom of the tank to provide adequate oxygen and agitation of water. A recipe including Nupro fish feed and refined sugar was determined for feeding the flocs (David Kuhn, pers. comm.). Fish feed was checked for mold, to prevent introduction into the

bioreactor. The feed was added directly to the water column each day to provide a diet for the growing flocs. After approximately 1 wk, a healthy heterotrophic bacteria floc population was established and ready to be harvested. Once harvested, the bioflocs were frozen at -20° C to prolong the usability of this dietary treatment. Several floc samples were saved for determining total suspended solids (TSS), using the following equation (TSS results are summarized in Appendix C):

$$TSS = \frac{\text{final weight – initial weight (g)}}{\text{volume of water (ml)}} * \frac{1{,}000\ \text{mg}}{1\ \text{g}} * \frac{1{,}000\ \text{ml}}{1\ \text{L}}$$

The flocs were blended for varying times to determine floc breakdown into the smallest-size particles ingestible for juvenile mussels. It was determined that a blending time of 7 min would allow for the smallest size floc particles (mean size = 16.46 μm).

To prepare the bacteria floc treatment for feeding trials, the frozen bacteria flocs were allowed to thaw at room temperature for several hours. Once thawed, the flocs were then placed in a blender for 7 min to reduce particle size range. A fresh bottle of flocs was obtained from the freezer and blended every few days to ensure adequate nutrients within the treatment. The bacteria flocs were added to each culture container of the treatment three times daily at 830 μl (2.5 ml per day), the same volume as the algae treatment. A & L Eastern Laboratories (Richmond, VA) determined the composition of the bioflocs, and results of these analyses can be found in Appendix D.

Probiotic

Clear Flo 1002 is a commercial probiotic powder [Alken-Murray Corporation] and was recommended for this project. Clear Flo 1002 is specifically designed for aquatic organisms, and is a soluble, highly concentrated, dry blend of eleven natural spore-forming, gram-positive *Bacillus* species. The bacterial count is 20 billion CFU/g, and the bacteria are 1 μm in size. The bacteria in this product benefit cultured organisms in tanks by promoting better digestion and stronger immune systems in fish and crustaceans, reducing or eliminating turbidity in the water and sludge buildup, and promoting a healthy environment by reducing nutrient loads and producing many enzymes. Aerobic and facultative anaerobic strains of bacteria in this product oxidize deadly hydrogen sulfide and reduce buildup of nitrates. The probiotic is safe and requires only small doses added directly to the water column to become effective (Product Information

Sheet). Though this had not been specifically designed for or tested on freshwater mussels, it could provide substantial benefits to mussel digestive tracts, feeding ability, and improve overall habitat in the laboratory setting. This product had several system requirements including a dissolved oxygen level above 2 mg/L, temperature range of 5° to 35° C, and pH range of 4.5 to 8.5. All of these system requirements were met by the system used in the feeding trial.

The dose levels for this treatment were based on product instructions, with the exception that the concentration of the probiotic treatment for this feeding trial was twice that recommended in the instructions. The company recommended that for each 100 gallons (378 L), the initial dose be 0.5 g, followed by 0.2 g weekly for 3 wk and then 0.3 g thereafter, twice monthly. These instructions are more applicable for long-term use versus a short-term experiment; however, the instructions were followed as accurately as possible. Information regarding this probiotic product can be found in Appendix E.

To prepare the probiotic supplement (commercial algae-mix and probiotic) for this feeding trial, a small amount of powder was weighed and added directly to the water column in each of the three sumps. For the first week of the feeding trial, an initial dose of 107 mg of the probiotic powder was added once directly to the sump tanks of this treatment. In subsequent weeks of the feeding trial, a 22.2 mg dose was added weekly to the sump tanks.

Pond water

The pond water was obtained from the 0.25 acre man-made pond located on the FMCC property. Sump tanks for the pond water treatment were filled daily from the pond and sieved to 64 μm to limit aquatic organisms entering the system. Typically, pond water was changed during the mid-day or afternoon hours so that water temperature of sump tanks did not fluctuate greatly. During water changes, the average fluctuation in temperature was between 1 and 3° C. Pond water samples were collected at the start, middle, and completion of the feeding trial to test various water quality parameters, and whether or not they changed over the course of the feeding trial. The tests included determination of ammonia, chloride, nitrate, nitrite, orthophosphate, pH, total water hardness, and total organic carbon concentrations (Appendix I).

Feeding Trial 2 Treatments

Four different diet treatments were evaluated in feeding trial 2, with particular interest in the effects of rotifer-supplemented diets on juveniles. Each treatment was assigned to three

systems (N=3). The diets were added directly to the water column in the culture containers holding juveniles. The treatments for the feeding trial included:

5) commercial algae-mix and bacteria flocs (CA + BF)
6) commercial algae-mix and Protein Selco Plus rotifer supplement (CA + PSP)
7) commercial algae-mix and Culture Selco Plus rotifer supplement (CA + CSP)
8) commercial algae-mix only (CA)

Protein Selco Plus

This rotifer supplement is fortified with vitamin C and immunostimulants to increase stress resistance and ensure larval health and enriched with marine algae and vitamin B12 (Product Information Sheet). This supplement was selected as a treatment because of its easy application in a culture tank as well as for its various components beneficial to an aquatic organism in early life stages.

Culture Selco Plus

This rotifer supplement is a substitute for live micro-algae that also guarantees incorporation of essential nutrients. This supplement was selected based on its easy maintenance and its high protein content. Both Protein Selco Plus and Culture Selco Plus are rotifer-production aquaculture additives produced by INVE Aquaculture [www.inve.com]. They were refrigerated when not in use and were made fresh approximately every other day, based on the recommendations of INVE. See Appendix F and G for information on Protein Selco Plus and Culture Selco Plus.

Experimental Design

A sample size of juveniles (For trial 1, N=42; for trial 2, N=50) of comparable age (21-d old) was randomly assigned to each culture container. The sample size numbers used in the feeding trials were determined from the number of juveniles available for use (those alive after the acclimation period). There were two culture containers for each sump tank. Three separate sump tanks were assigned for one treatment to avoid pseudo-replication, with a total of six culture containers per treatment (252 juveniles per treatment for trial 1 and 300 juveniles per treatment for trial 2). See Figure 2 for general experimental setup and Table 1 for the experimental setup.

A preliminary feeding trial was initiated in late May 2007, but due to high juvenile mortality, the trial was terminated. However, this pilot study aided in determining preparation and feeding methods for future feeding trials.

Feeding Trial 1

The feeding trial for this objective was conducted from late August to early October 2007, and ran for 51 d. A sample size of 42 juveniles per culture container in triplicate per treatment (n=252) was selected based on the number of juveniles available from the cultured batch.

Feeding Trial 2

The feeding trial for this objective was conducted from late December 2007 to early February 2008, and ran for 40 d. A sample size of 50 juveniles per culture container in triplicate per treatment (n=300) was selected based on the number of juveniles obtained from the cultured batch.

Table 2 summarizes the experimental design and other information related to each feeding trial.

Evaluation of Diet Treatments

Initial valve measurements of 20 juveniles were taken just before placement in culture containers for the feeding trial. Juveniles were sampled approximately every 10 d, depending on feeding trial duration, to measure length and width of valves, with only length data analyzed statistically (Figure 3). Based on the work of Liberty et al. (2007), only 50% of each culture container was sampled, to prevent stress to juveniles and potential effects on feeding trial results. The survival rate of juveniles was determined at the completion of the feeding trial. Observations were recorded on overall juvenile health, based on level of activity and appearance.

Statistical Analysis of Feeding Trials

A priori hypothesis:

H_0= There is no difference among feed treatments.

H_A= There is difference among feed treatments.

All statistical analyses were conducted in SAS (SAS Institute, 2005). Juvenile lengths were compared between treatments, time, and the interaction of treatment and time using a repeated measures analysis of variance (ANOVA), assuming compound symmetry (PROC MIX; type=cs). Because high mortality occurred in one of the treatment replicates, missing values were

present in the data set. A repeated measures ANOVA test was therefore selected in testing these data because of the test's ability to properly analyze missing data in SAS. Least-squares means tests were used in making pairwise comparisons among treatments, time, and the interaction of treatment and time. In running this post-hoc test, a more specific null hypothesis could be tested; namely, to determine whether or not the addition of bacteria to juvenile diet contributed significantly to growth and survival.

Survival is reported as the percent of the initial number of juveniles placed in each treatment, recovered at completion of the feeding trial. For statistical analysis, survival was arcsine-transformed to ensure homogeneity of variance and a one-way ANOVA (PROC MIX) was run, followed by least-squares means tests to make pairwise comparisons.

Results

Statistical analysis of valve lengths included an ANOVA and a repeated measures test, along with several multiple comparison tests, to compare shell lengths and survival rate among treatments. The most suitable diet treatment is defined as the diet providing greatest growth and highest survival rate. Once a treatment (or treatments with similar results) was found to be most suitable, this diet was used in feeding trials for the following objective.

During both feeding trials, I observed that those juveniles fed the algal diet supplemented with bacteria flocs had guts that were dark in color (most likely filled with algae). Juveniles were so mobile that at times, it was difficult to measure them before they had already pulled themselves away with their foot.

Feeding Trial 1

Growth

In examining trial 1 data, it was noted that the sub-sampling was adequate in this feeding trial because the coefficient of variation was below 20%. Sampling effort had little effect on variability in juvenile length or survival.

There were significant differences among treatments (P=0.0657), a significant difference in time (P=0.0029), and no significant difference among treatment and time interactions (P=0.1726). In looking at the post-hoc test results, the triple concentration algae-mix was significantly better than all other diets tested in this feeding trial, with juveniles having a mean average length of 813 μm (Table 4). There was no significant difference between the other treatments tested (algae-mix supplemented with bacteria flocs, algae-mix supplemented with

probiotic, pond water), with lengths of 748 µm, 720 µm, and 790 µm, respectively (Figure 4). These results in mean lengths were higher than for cultured *E. capsaeformis* juveniles in previously published studies. In regards to time, there was a significant difference between sampling times with the exception of between sampling time 2 and 3.

Survival

Survival was significantly higher in those juveniles fed the algae-mix supplemented by bacteria flocs treatment, with a survival rate of 9.92% (Table 4). There was not a significant difference in survival among the other treatments for this trial (Figure 5). Survival rates for the triple concentration, pond water, and algae-mix supplemented with probiotic treatments were 4.37%, 5.16%, and 3.17%, respectively.

Feeding Trial 2

Growth

There was a significant difference among treatments (P=0.0096) and sampling times (P=0.0010), but no significant difference among treatment and time interactions (P=0.2706). There was a significant difference between algae-mix supplemented with bacteria flocs and the algae-only treatments, with comparable mean lengths of 685 µm and 607 µm, respectively (Figure 6). Due to high mortalities in the algae-mix supplemented with Culture Selco Plus and algae-mix supplemented with Protein Selco Plus, SAS did not compare these treatments in the output. The algae-mix supplemented with Culture Selco Plus treatment resulted in a mean length of 664 µm. Due to high mortality in algae-mix supplemented with Protein Selco Plus, there was not a mean length determined for this treatment (Table 5).

Survival

Survival (19.67%) was significantly higher in those juveniles fed the algae-mix supplemented with bacteria flocs treatment (Table 5). There was no significant difference in survival among the algae-only and algae-mix supplemented with Culture Selco Plus treatments for this trial, with survival rates of 4% and 1.67%, respectively (Figure 7). The algae-mix supplemented with Protein Selco Plus had 0% survival by the second sampling period of the feeding trial.

Table 1. Experimental design of culture experiment and the number of juveniles used in feeding trials.

Trial	Sump tank (or system) per treatment	Culture container per treatment	Juveniles used (per culture container, per sump, per treatment)
1	3	6	42, 64, 252
2	3	6	50, 100, 300

Table 2. Experimental design to determine a suitable diet for rearing *Epioblasma capsaeformis* juveniles in a culture system. CA = Nannochloropsis 3600 and Shellfish Diet 1800 (Reed Mariculture), commercial algae. BF = bioflocs. PRO = Clear Flo 1002 (Alken-Murray Corporation), commercial probiotic. PW = pond water (Mussel Lab man-made pond). 3 X CA = triple concentration of commercial algae. PSP = Protein Selco Plus (INVE Aquaculture), commercial rotifer supplement. CSP = Culture Selco Plus (INVE Aquaculture), commercial rotifer supplement.

Trial	Mussel Species	Host Fish Used	Treatments (replicates)	Number of Juveniles (per replicate)	System
1	*Epioblasma capsaeformis* (oyster mussel)	*Cottus carolinae* (banded sculpin)	CA + BF (3) CA + PRO (3) PW (3) 3 X CA (3)	42, 42,42 42,42,42 42,42,42 42,42,42	Recirculating aquaculture system
2	*Epioblasma capsaeformis* (oyster mussel)	*Cottus carolinae* (banded sculpin)	CA (3) CA + BF (3) CA + PSP (3) CA + CSP (3)	50,50,50 50,50,50 50,50,50 50,50,50	Recirculating aquaculture system

Table 3. List of dietary treatments and their sources in the feeding trials.

Treatment (abbreviation)	Trial	Treatment Source
Nannochloropsis 3600 (CA)	1, 2	Reed Mariculture Campbell, California
Shellfish Diet 1800 (CA)	1, 2	Reed Mariculture Campbell, California
Bioflocs (BF)	1, 2	VA Tech Aquaculture Center Blacksburg, VA
Clear Flo 1002 probiotic (PRO)	1	Alken-Murray Corporation New Hyde Park, New York
Protein Selco Plus (PSP)	2	INVE Aquaculture Salt Lake City, Utah
Culture Selco Plus (CSP)	2	INVE Aquaculture Salt Lake City, Utah
Pond water (PW)	1	Freshwater Mollusk Conservation Center man-made pond Blacksburg, VA

Table 4. Mean length and percent survival of *Epioblasma capsaeformis* juveniles fed various dietary treatments in feeding trial 1 after 51 d.

Dietary Treatments	Mean Length (µm)	Survival Rate (%)
CA + BF	748	9.92
CA + PRO	720	3.17
3 X CA	813	4.37
PW	790	5.16

Table 5. Mean length and percent survival of *Epioblasma capsaeformis* juveniles fed various dietary treatments in feeding trial 2 after 40 d. *hm = high mortality

Dietary Treatments	Mean Length (µm)	Survival Rate (%)
CA + BF	685	19.67
CA	607	4
CA + PSP	hm	0
CA + CSP	664	1.67

Table 6. Previous data of survival rates of juvenile *Epioblasma capsaeformis* at the Freshwater Mollusk Conservation Center. Survival rates were calculated by dividing the number of live juveniles placed in culture tank by the number of live juveniles removed from culture tank after a period of time. This value was then multiplied by 100 to determine the percent survival rate. In averaging data over culture years, the mean survival rate of approximately 1 mo old juveniles was 16.85% and 2 mo old juveniles was 8.82%.

Culture Year	Juvenile Age	Survival Rate (%)
2004	1 mo.	0.43
2004	1 mo.	60.2
2004	1 mo., 9 d	1.17
2004	1 mo., 9 d	7.47
2004	1 mo., 12 d	19.38
2004	1 mo., 14 d	28.21
2004	1 mo., 18 d	1.06
2003	2 mo.	4.09
2004	2 mo.	6.21
2004	2 mo.	0
2005	2 mo.	6.98
2005	2 mo., 6 d	21.31
2005	2 mo., 8 d	14.34

Figure 1. Culture container used in feeding trials to rear juveniles.

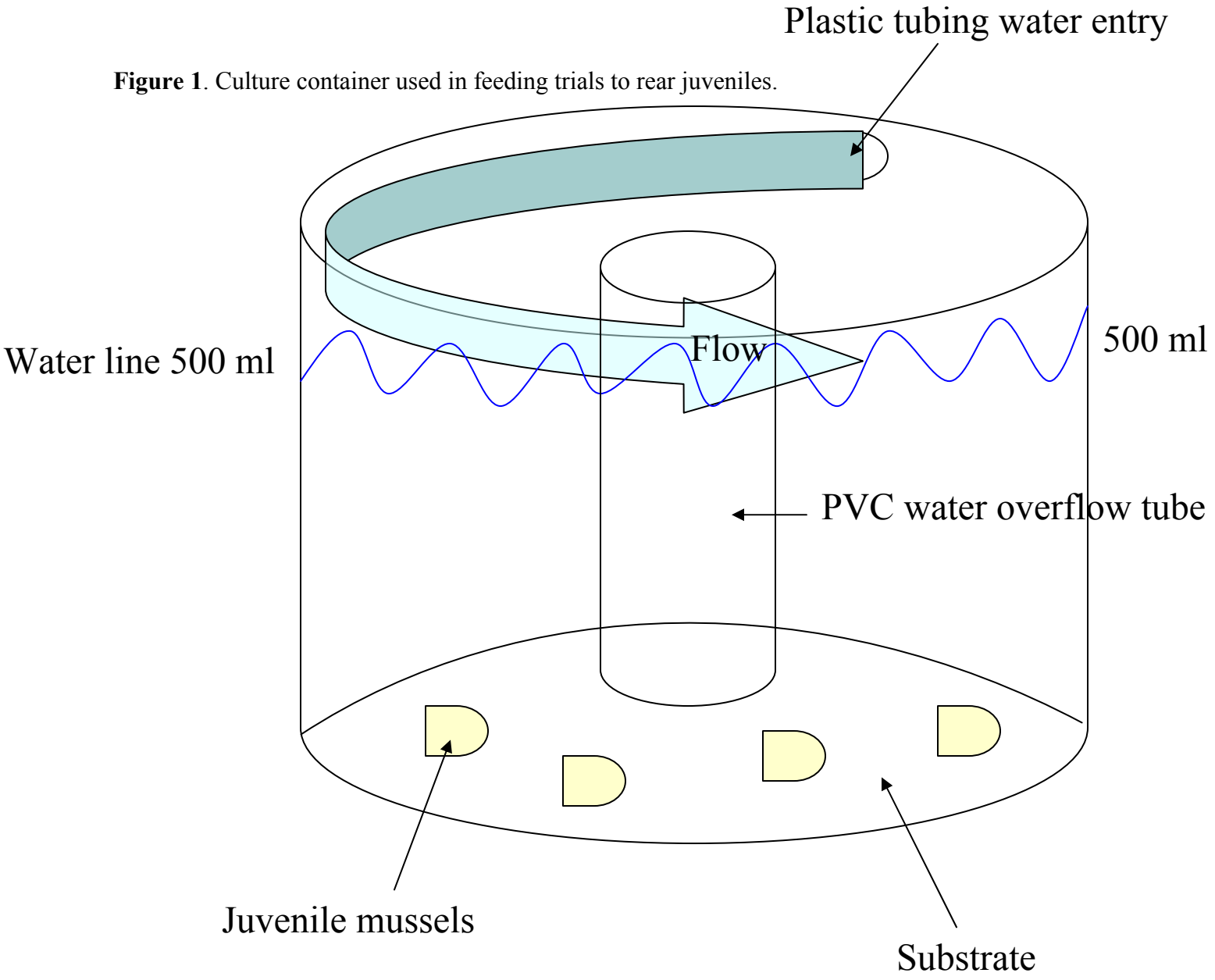


Figure 2. The feeding trial system setup; two culture containers per system (sump tank) and three individual systems per treatment. Culture containers held juveniles.

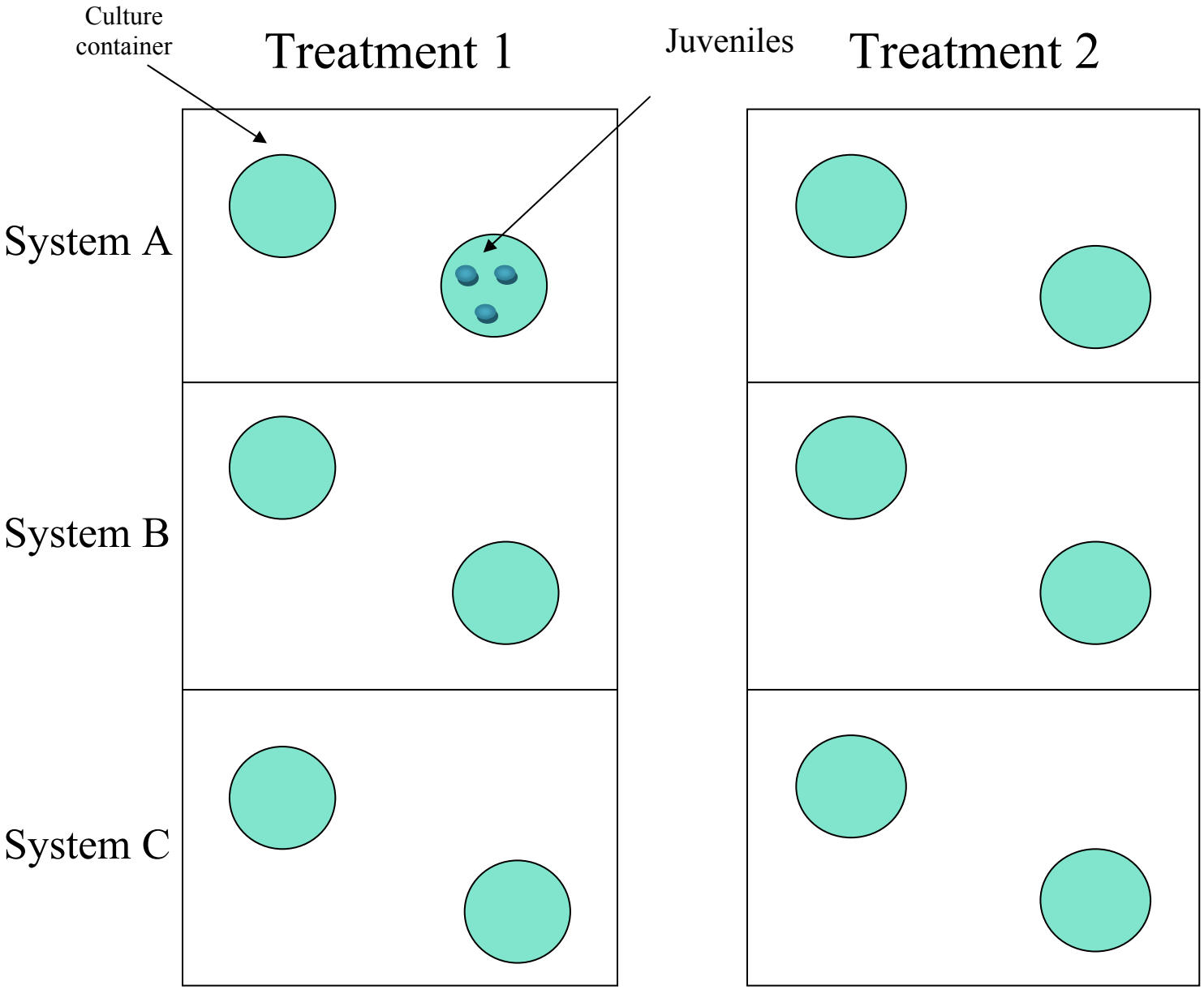


Figure 3. Juvenile dimensions; valve length was measured to evaluate growth of juveniles.

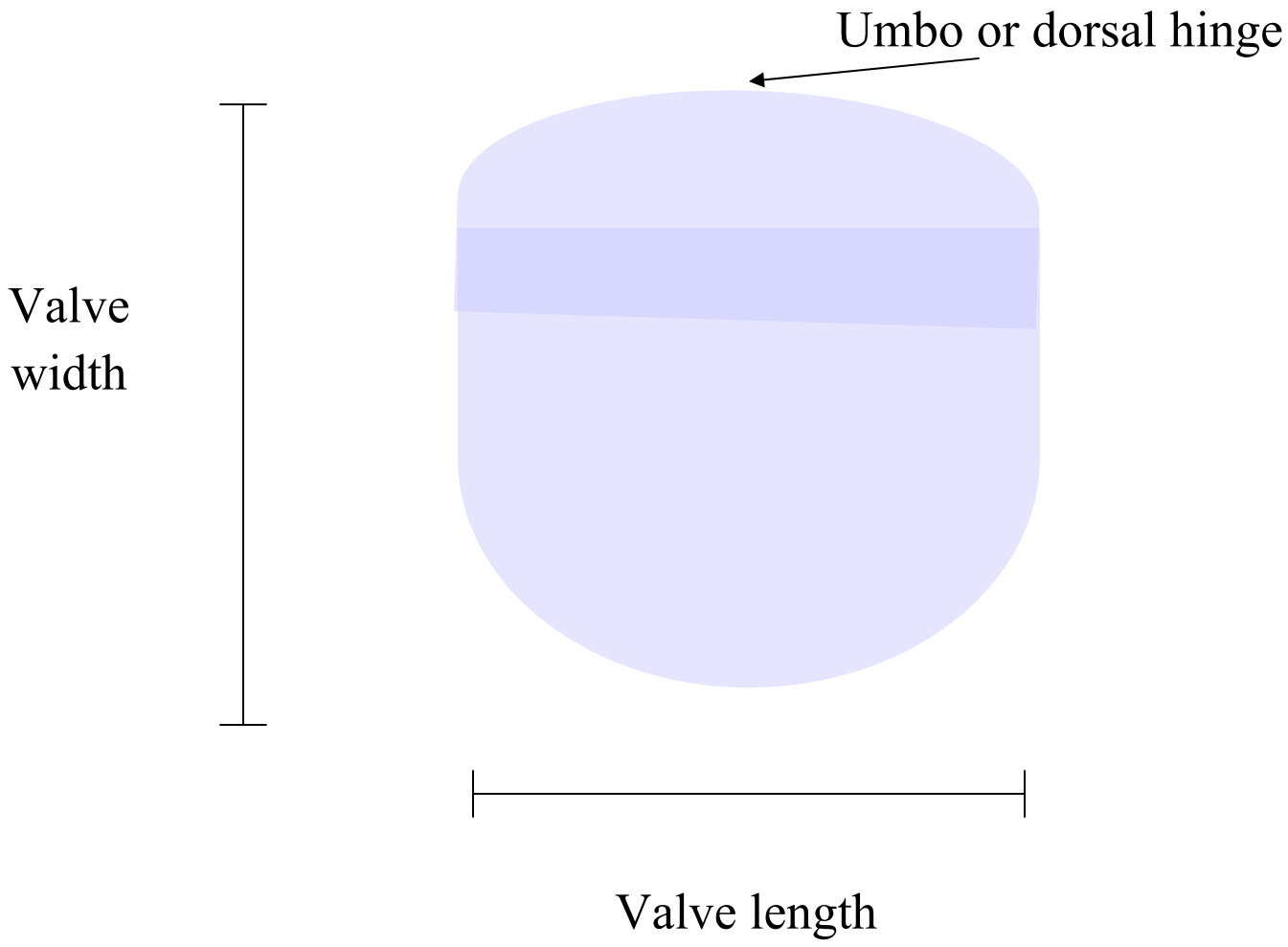


Figure 4. Mean lengths of *Epioblasma capsaeformis* juveniles fed various dietary treatments in feeding trial 1 after 51 d.

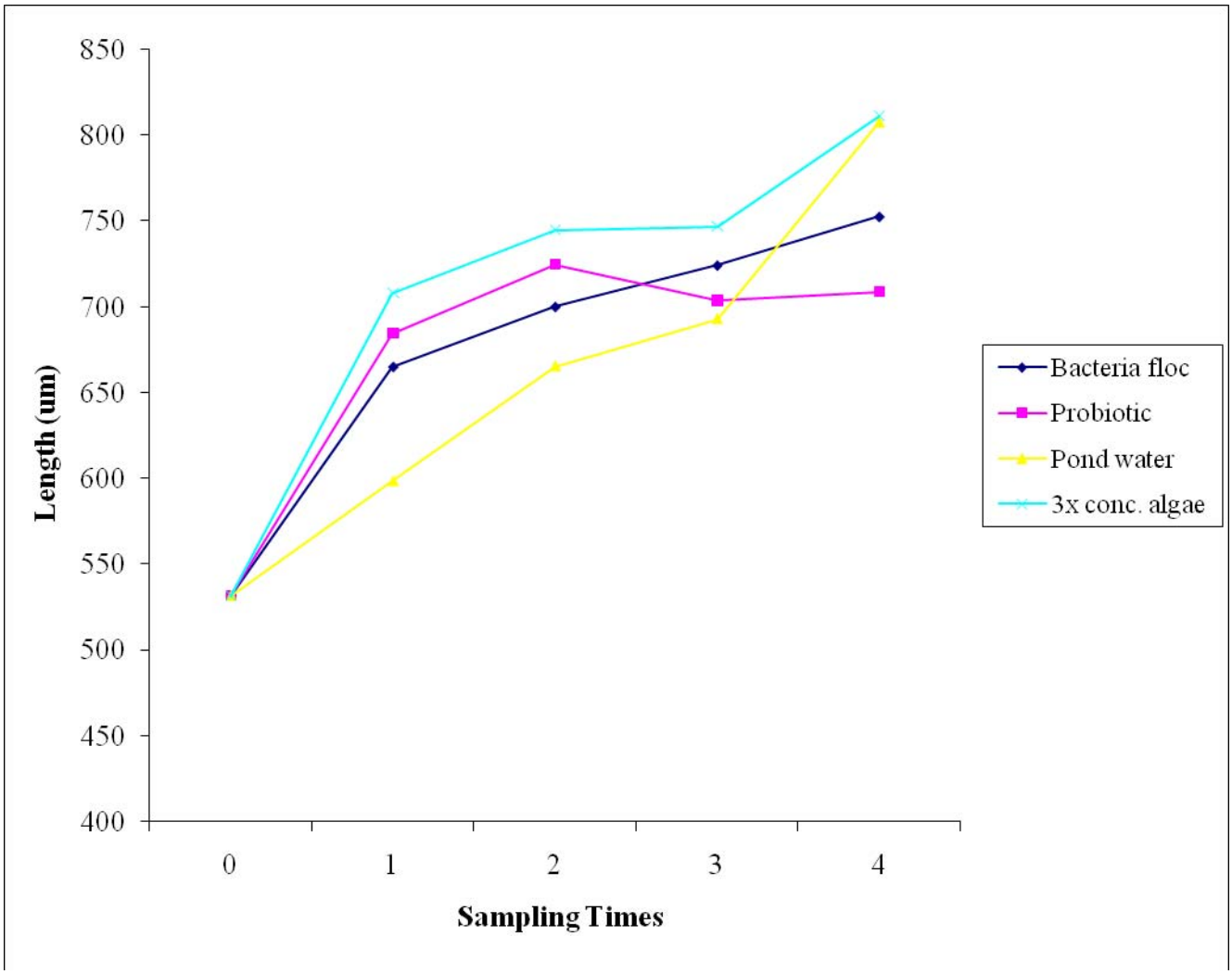


Figure 5. Percent survival of *Epioblasma capsaeformis* juveniles fed various dietary treatments in feeding trial 1 after 51 d.

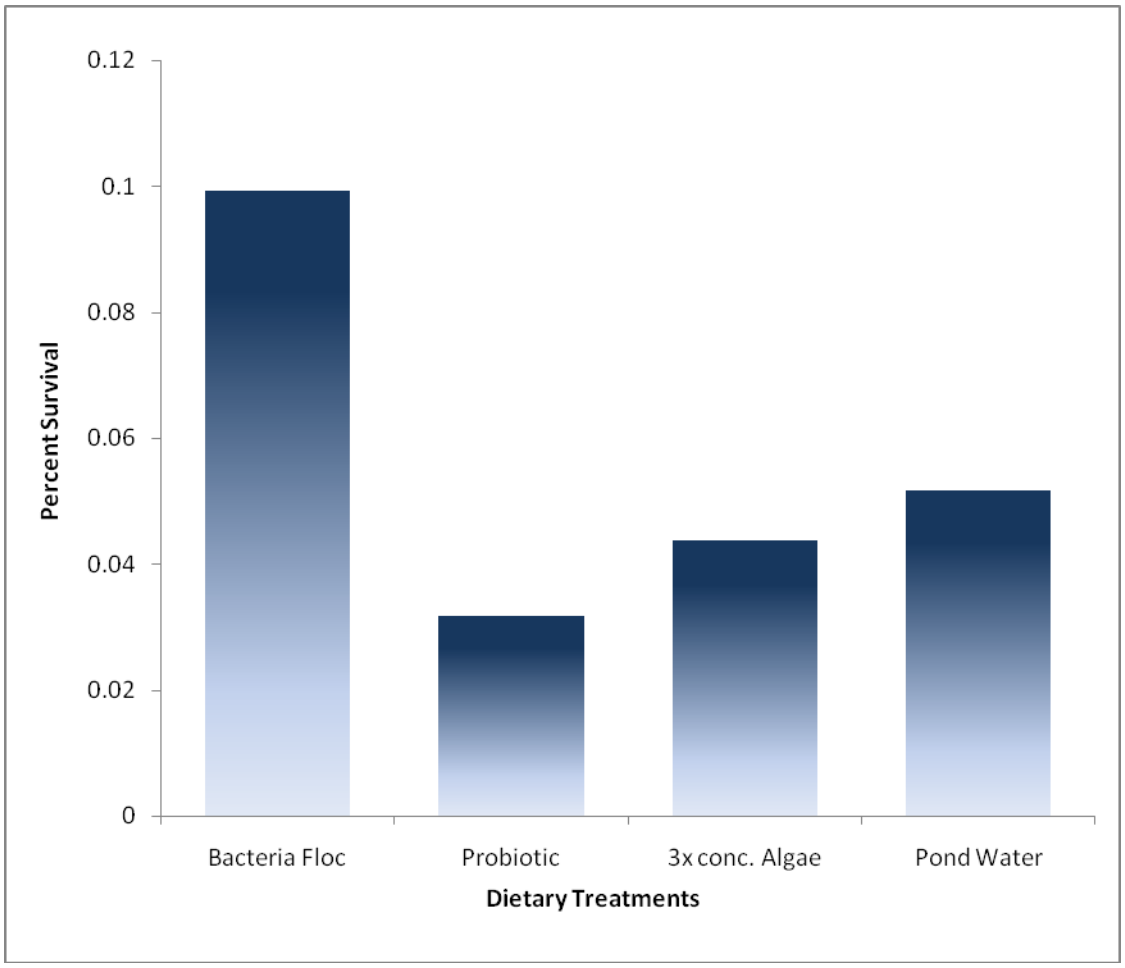


Figure 6. Mean lengths of *Epioblasma capsaeformis* juveniles fed various dietary treatments in feeding trial 2 after 40 d.

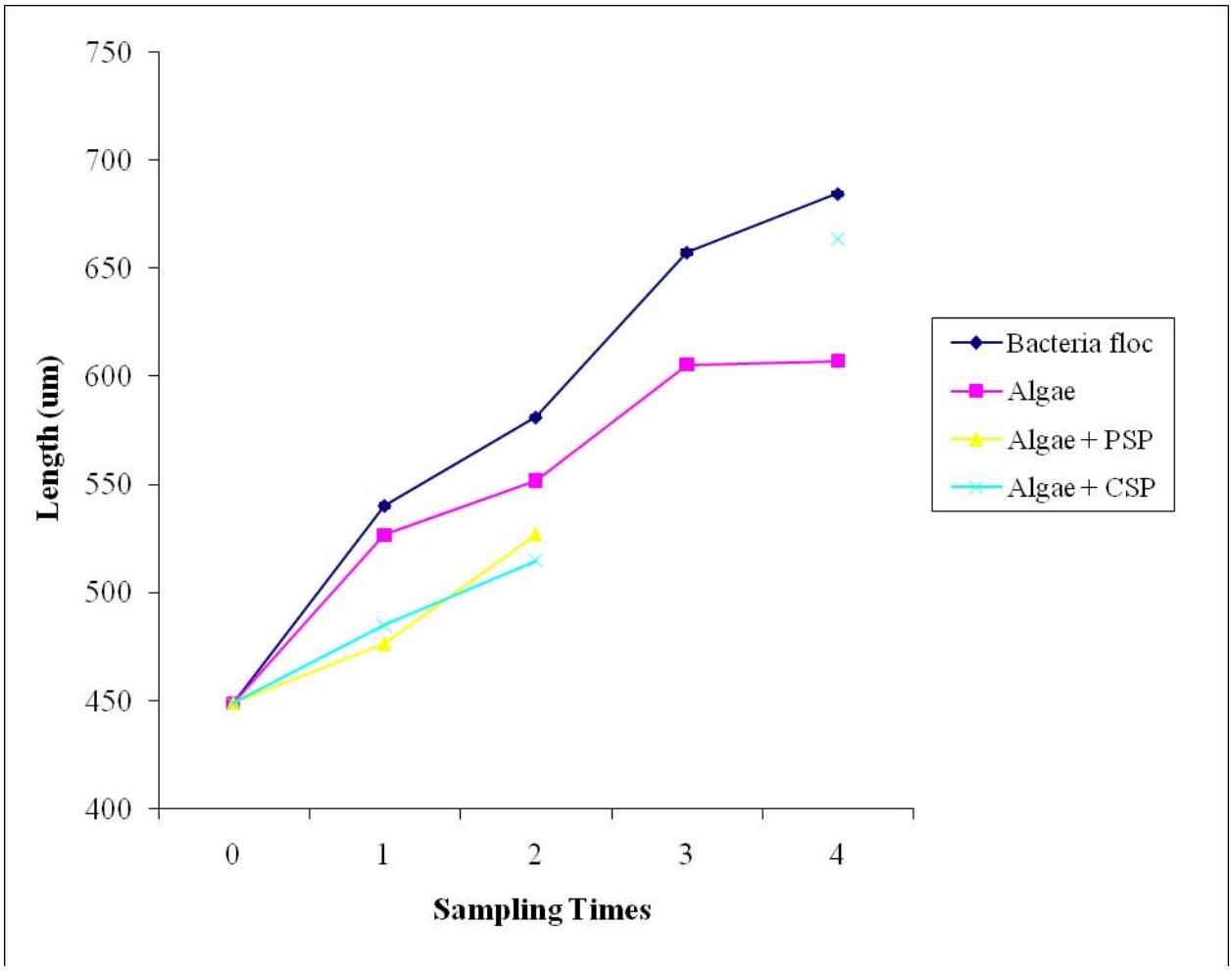
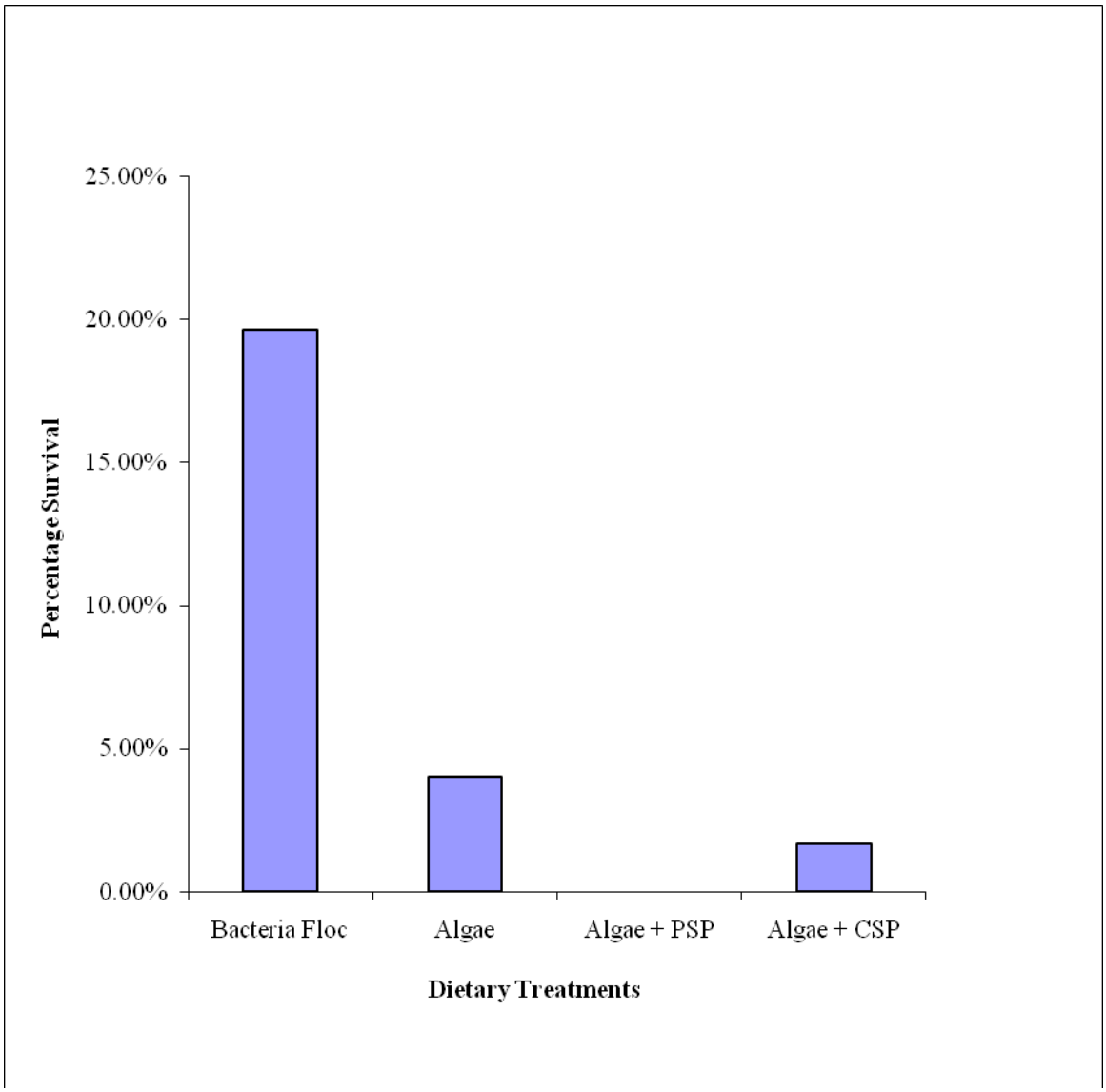


Figure 7. Percent survival of *Epioblasma capsaeformis* juveniles fed various dietary treatments in feeding trial 2 after 40 d.



Discussion

Feeding Trial 1

Growth Comparisons

In examining the results of trial 1, the triple concentration algae-mix diet resulted in juveniles with significantly greater growth, when compared to other diets. As seen from Figure 5, growth of juveniles on the pond water treatment was approaching those juveniles fed the triple concentration algae. Feeding trial duration was only 51 d; therefore, it would have been interesting to run the trial longer to see whether these two treatments converged. Although the bacteria floc supplemented algae-mix diet did not have the greatest growth at completion of the feeding trial, it did provide steadily increasing growth. Most likely there was a nutritional benefit of this diet that juveniles were utilizing, but perhaps not as quickly or as efficiently in comparison to the triple concentration or pond water treatments. Growth of juveniles fed the probiotic treatment appeared to decline (most likely due to high mortality of larger individuals) and stabilized after the second sampling time. The probiotic seemed to alter water quality by increasing nitrate level (see Appendix I), but it did not appear to have any nutritional value to juveniles.

Survival Comparisons

The bacteria floc supplemented algae-mix diet provided the highest survival at approximately 10% (9.92%), followed by the pond water treatment at 5.16%. Unfortunately survival rates from trial 1 were not high enough to be acceptable for culture, even though the bacteria floc diet provided a survival rate double that of other treatments. Although a 10% survival rate seems low, it is an improvement in survival compared to previous survival rates of *E. capsaeformis* juveniles at 2 mo of age (Table 17). In averaging the data in Table 17, mean survival rate of 2 mo-old juveniles (similar in age to juveniles used in feeding trials) would be 8.82%. Any improvement in survival rate would be helpful to successfully culture this species.

Feeding Trial 2

Growth Comparisons

In examining the length results of trial 2, the algae-mix diet supplemented with bacteria flocs resulted in the highest mean length when compared to other diets. Similar to trial 1, it seems that the diet with bacteria flocs supplied juveniles with some consistent nutritional benefit, since juveniles grew steadily over the duration of the feeding trial (Figure 6). The algae-mix diet alone provided steadily increasing growth as well, though it seems that juveniles were not utilizing nutrients as efficiently as those fed the algae-mix supplemented with bacteria flocs diet. The algae-mix diets supplemented with Culture Selco Plus and Protein Selco Plus were less suitable for juveniles, and this may have been due to non-utilizable oversupplied components of this diet. The amount of supplements used in treatments was selected from calculations determined to maintain consistency with the other treatments, so it is quite possible that overfeeding occurred. It would be beneficial to evaluate Protein Selco Plus and Culture Selco Plus again, with a lower amount of supplement added to the dietary treatments.

Survival Comparisons

The algae-mix diet supplemented with bacteria flocs resulted in the highest survival rate of nearly 20%, which is double the survival rate of trial 1 and one of the highest survival rates for juveniles of this species. Previous results of cultured 2-mo old *E. capsaeformis* juveniles have usually been less than 10% survival (Table 17). This treatment provided significantly higher survival than those juveniles fed the algae-mix only (survival of 4%). Clearly, this species needs to be fed more than an algae-mix diet alone in order to obtain greater survival of juveniles.

Jones et. al (2005) found that in the spring of the year, juvenile oyster mussels achieved a survival of 29.6%, and mean length of 664 μm at 60 d of age. In examining the highest means for both feeding trials in this study, juvenile oyster mussels for trial 1 achieved a survival of 10% and mean length of 813 μm at 65 d of age. For trial 2, survival was 20%, and mean length was 685 μm at 65 d of age.

Influence of Algae Diet on Juvenile Growth

As seen in previous studies, an algal diet is suitable for feeding freshwater mussels (Hudson and Isom 1984, Gatenby et al. 1996, Gatenby et al. 1997, Gatenby et. al 2003, Bush 2008). Most likely, the dietary demand for biochemical components in animals that feed on microalgae varies among species and life stage (Gatenby et al. 2003). In a study completed on

oyster larvae, the use of mixed algae as a diet provided more rapid growth, an enhanced proportion of metamorphosis, and more vigorous spat in comparison with a diet containing only one species of alga (Walne 1970). Studies also have been completed on various species of freshwater mussels and have confirmed the benefits of a mixed algal diet. For this reason, an algae-mix diet was selected for use in dietary treatments for feeding trials to provide the most adequate algal diet. It seems that *E. capsaeformis* juveniles can be sustained on an algae-mix diet, but supplements can improve growth and survival.

In addition to examining algal species, research has been completed on particle size selection in diets for freshwater mussels. Beck and Neves (2003) found that in developing a suitable algal diet for juvenile mussels, algae should be within the 2.8-8.5 μm particle size range. They also determined that *Villosa iris* could be fed the same size algae at all ages while in captivity, and selected for *Nannochloropsis oculata* and *Selenastrum capricornutum*. Juveniles of *E. capsaeformis* probably ingest particles in the size range of 2-8 μm (Jess Jones, pers. comm.). *Neochloris oleoabundans*, ranging in size from 2-20 μm, seems to be a suitable algal diet for adult *E. capsaeformis*, though it is important to stay within this cell size range; otherwise, mussels may be overfed (Bush 2008). Several species of *Nannochloropsis*, among other algal species, are currently fed as an algae-mix diet to juveniles at the FMCC, and this same algae-mix diet was selected for evaluation in my feeding trials. Juveniles may not have been able to feed on all algal cells present in this diet, and although the range was between 1-20 μm, much of it was < 10 μm in size.

In feeding trial 2, the algae-mix diet seemed to provide adequate nutrients for juveniles, though they did not grow to the size of those juveniles fed the triple concentration of algae. It can be concluded from these results that the algae-mix diet currently used at the FMCC seems to be suitable for feeding *E. capsaeformis* juveniles, though this diet alone does not provide the highest growth that can be obtained. From the results of feeding trial 1, it is clear that *E. capsaeformis* juveniles would benefit if fed the algae-mix diet (the current diet juveniles are fed at the FMCC) at a higher concentration. This treatment may have resulted in higher juvenile growth because increasing the concentration of algae also increased the number of algal cells of varying particle size available to juveniles. This would have allowed juveniles to feed on and utilize a greater number of algal cells. Experiments by Henley et al. (2001) suggest that a ration of approximately 30,000 cells/ml should be sufficient for the diet of juveniles. A study by Bush (2008) found that

optimum rations for adult *Epioblasma* spp. fed *N. oleoabundans* were higher than previously thought, at 40,000-80,000 cells ml^{-1} or 80,000-120,000 cells ml^{-1},depending on season. My study agrees with Bush's study in that juveniles can handle a much higher algal cell count. In feeding trials for this study, juveniles were fed a diluted mixture of Nanno 3600 (68 billion cells/ml) and Shellfish Diet 1800 (two billion cells/ml). The trial did not examine several different algal concentrations, so it is likely that juveniles could benefit from other concentrations besides a triple concentration (i.e., double or quadruple concentration). Regardless, *E. capsaeformis* juveniles seem to require a higher cell count of algae in their diet to achieve a steady growth increase under culture.

Though the triple concentration algae-mix diet was significantly better than the other diets, it should be noted that the pond water diet seemed to provide adequate juvenile growth, approaching the highest growth of juveniles fed the triple concentration algae-mix in the last sample period. This reveals that pond water may also be a suitable diet for juveniles, but does not provide juveniles with the nutrients necessary to grow as quickly as they do when fed the triple concentration of algae. For a mussel hatchery, it is important to grow mussels quickly so that they reach a size more likely to survive in the laboratory and in the wild. Perhaps pond water would be a suitable diet for *E. capsaeformis* juveniles reared in captivity, as long as this diet was supplemented with other components such as algae, bioflocs or rotifer supplements.

Although large amounts of particles are necessary to adequately feed juveniles, high food concentration will result in production of rejected material (pseudofeces), representing a loss of potentially utilizable algal cells and other components to the diet. For juveniles, it is important to develop a diet with sufficient food particles to provide adequate nutrition, but not at a level to produce significant amounts of pseudofeces. Laboratory studies also show a reduction in filtration with an increase in food for bivalves (Roper and Hickey 1995). Unfortunately it was not possible to measure pseudofeces rejected from juveniles, so one can only make assumptions about whether or not juveniles were overfed. Most likely, juveniles were not being overfed with the triple concentration algae-mix diet because treatments were added three times daily, and flow was turned off during feeding times. However, during the remainder of the time, flow was constant in the RAS culture containers with juveniles. This allowed juveniles to adequately feed, but did not allow algae to accumulate significantly at the bottom of the culture container to provide too much food. With this stated, it is important to note that when feeding any species of

juvenile mussels with a high concentration of algae, juveniles should be housed in a culture container that provides continuous flow to prevent buildup and decomposition of algae. Algae buildup in a culture unit can over-supply food for juveniles and cause poor water quality within the system.

In addition to feeding juveniles a mixed algal diet at a higher concentration, I found that feeding juveniles multiple times a day to provide more continuous nutrition is also important to achieve steady growth. Winter and Langton (1976) found that growth is not only influenced by the total amount of food presented, but also how often food is provided to bivalves. In feeding experiments where constant food concentration is maintained at adequate cell concentration, there are no periods of rest in feeding, no formation of pseudofeces, and there is steady food intake and food digestion. These characteristics provide the most suitable dietary situation for mussels reared in aquaculture systems (Winter 1978).

Influence of Bacteria on Juvenile Growth and Survival

From feeding trial 1, it is apparent that the algae-mix diet supplemented with bioflocs resulted in better survival, in comparison to the other treatments; in feeding trial 2, this dietary treatment provided the most significant growth. By evaluating the same treatment in both trials, this diet replicated its beneficial results, providing strong support for the use of bioflocs in a diet for *E. capsaeformis* juveniles. The use of bioflocs in diets for feeding aquatic organisms is a relatively recent idea and has never been tested with freshwater mussels. This is the first study examining this type of dietary treatment for freshwater mussels and can serve as a starting point for future dietary studies that could more thoroughly evaluate bacteria in their diets. Because I did not analyze the bioflocs for the strains and the abundance of the types of bacteria within the flocs, it would be difficult for other laboratories to replicate the exact recipe for this bacterial floc supplement in these feeding trials. However, other similar diets for freshwater mussels could very easily be developed to include bacteria obtained from fish wastewater.

Juvenile growth was significantly higher in trial 2 when fed the algae-mix diet supplemented with the bioflocs. In trial 1, the growth increase of juveniles fed this same treatment, although not the highest, showed steady growth during the feeding trial. In examining results of both trials, it is apparent that the bioflocs were aiding in providing nutrition, along with the algae mix. Although other treatments included an algae mix and some type of supplement, they did not provide the same level of growth as did the bacterial floc-supplemented diet.

Douillet and Langdon (1994) found that additions of bacteria as a food supplement to Pacific oyster larvae, *Crassostrea gigas*, increase larval growth, the proportion of larval set to produce spat, and the subsequent size of spat. Those larvae fed diets supplemented with bacteria also showed better growth than algae-only diets. A study completed by Burford et al. (2004) found that the natural biota of the culture system for the shrimp, *Litopenaeus vannamei*, consisting of flocculated particles, contributed significantly to their nutrition. Results of a study conducted for 60 d on giant freshwater prawns, *Macrobrachium rosenbergii*, in their post-larval stage, when fed probiotic bacteria treatments, showed significantly higher growth, percent weight gain, specific growth rate, feed efficiency ratio (FER), protein efficiency ratio (PER) and protein gain than those prawns fed the control diet (Venkat et al. 2004). Apparent from these previous studies, beneficial effects of bacteria include higher growth and feed efficiency, prevention of intestinal disorders and pre-digestion of non-nutritional factors present in a diet. My results support studies examining the nutritional benefits gained by aquatic organisms when fed bacteria.

The bioflocs most likely had a 'probiotic' effect on juveniles, which would account for the significantly higher survival rate (trial 1 and 2) and growth (trial 2) at the completion of feeding trials with flocs. A probiotic can be described as "microbial cells that are administered in such a way as to enter the gastrointestinal tract and kept alive, with the aim of improving health" (Gatesoupe 1999). Although probiotics have been used extensively in poultry and swine rearing (Lara-Flores et al. 2003), the first application of probiotics in aquaculture was on fish and was relatively recent. They can benefit aquaculture systems by changing the microbial communities in the water and sediment, reducing or eliminating harmful microbial pathogens, and improving the growth and survival of animals in the system. Water quality and control of disease are positively affected by probiotic activity (Jory 1998), and I did observe improvement in system water quality fed the probiotic supplemented algae-mix diet. Although bacterial diseases have not been examined in freshwater mussel culture, it is quite possible that diseases can contribute to high mortality of juvenile mussels. Probiotic bacteria compete with many pathogenic bacteria, such as *Aeromonas* and *Vibrio* species typically found in aquatic environments, eliminating harmful bacteria through competitive exclusion. Results from previous studies suggest that probiotics may be useful in the control of microbial disease through the stimulation of the immune system in young animals (Bachere 2003). In a study conducted by Gibson et al. (1998), they found that the Pacific oyster, *Crassostrea gigas*, was able to survive a pathogen (*Vibrio*

tubiashii) when exposed to a probiotic strain, while those oysters not exposed to the probiotic strain did not survive. Nogami et al. (1997) attempted to control the aquatic environment for the swimming crab, *Portunus trituberculatus*, with the use of microorganisms, and found that crabs had improved survival with addition of a probiotic strain; this strain repressed growth of harmful pathogens in seawater. If these bioflocs were acting as a probiotic in my study, they would have aided in disease control within the RAS culture containers, provided a nutritional source, and would also have improved juvenile feeding efficiency.

In aquaculture systems, bacteria can colonize the entire aquatic environment, including the external slime layer of freshwater and marine organisms. This benefits a system's environment, as bacteria aid in the breakdown of organic waste into carbon dioxide and water. Every time an aquatic animal is removed from its culture environment, the slime layer loses a significant amount of the natural and protective bacteria microflora. In the case of juvenile mussels, every time sampling occurs, juveniles could lose some of their protective microflora slime layer. Some species of mussels may be more sensitive to sampling events than others, and it is possible that *E. capsaeformis* and other *Epioblasma* species either do not accumulate a slime layer well under culture conditions or they are not able to quickly re-establish a protective layer, particularly after sampling events. The bioflocs were added to the culture containers three times daily, and perhaps this allowed *E. capsaeformis* juveniles to re-establish and maintain a constant healthy microflora layer. The flocs having a probiotic effect may have also prevented harmful effects of transfer stress caused by sampling. In a study completed on eastern pearlshell mussels, *Margaritifera margaritifera*, a sand substrate improved the culture conditions for juveniles by increasing the surface area to be colonized by micro-organisms (Buddensiek 1995).

In nature, aquatic organisms have microflora of the gut that may reflect the aquatic environment, as opposed to larval culture environments where the natural balance is changed (Fox 1988). By constantly using sterile water and feed within a culture system, a protective microbial community may be prevented from developing in both the aquatic system and the digestive system of the larvae. If juveniles are reared in a mostly sterile environment initially (i.e., conditioned water) and then transferred to a different water environment (i.e., pond water), they may not grow well and show poor survival when exposed to stress and potential pathogens for lack of microbial protection. On the other hand, pond water may work well as a culture medium for some species of freshwater mussels because bacteria in the water derived from

natural production are present and provide a probiotic effect. This probiotic effect may explain why juveniles had a steady growth increase when fed the pond water treatment in trial 1 and may also provide evidence as to why juvenile *V. iris* achieve such high growth and survival at the FMCC when cultured in pond water.

The bioflocs also may have provided benefits to the culture process. In aquaculture systems housing fish, solid wastes accumulate and are often discharged as sludge, leaving behind a significant amount of nutrients that are not used (Chen et al. 1997). The discharged sludge, composed of mostly organic carbon, nitrogen, and phosphorus, can result in elevated nutrients in discharge waters, causing potentially negative impacts to the environment (Naylor et al. 1998). Besides discharging the sludge or placing it in a landfill, a potential alternative could be to use heterotrophic bacteria to convert these discharged and unused nutrients into bacterial biomass. This biomass could then be used as an aquatic animal feed and reduce wastewater discharge in one step (Avnimelech et al. 1989). In a RAS, bacteria can be produced from solid fish wastes by bacteria growth reactors (Schneider et al. 2006, Sereti et al. 2003) or anaerobically in denitrification reactors (Eding, Klapwijk et al. 2003). The flocs used in this study were grown in a reactor from tilapia wastewater. The protocol for growing bacterial biomass from fish wastewater has not yet been perfected, as sometimes this system will suddenly crash (Knoesche and Tscheu 1974), but this study concluded that bacteria grown from fish waste could be used as a feed ingredient for fish diets. Many mussel propagation facilities in the United States are actually housed at a fish hatchery. By producing some kind of bioflocs from their fish wastewater, a hatchery could adequately dispose of wastewater more efficiently and cost effectively and at the same time, they could feed cultured freshwater mussels.

Some studies have found that the addition of bacteria, particularly antibiotics, to the diets of cultured bivalve larvae have resulted in variable and even adverse effects on larval survival and growth (Le Pennec and Prieur 1977); however, this does not seem to be the case in my study. At this time, a great deal is still unknown about the effects of bacteria on aquatic organisms. Perhaps the best way to determine what bacteria are suitable for use in the diets of various aquatic organisms is by conducting experiments testing various bacterial species and combinations of those species. In the case of *E. capsaeformis* juveniles, the bioflocs evaluated in this study proved to be a suitable supplement to the algae-mix diet.

Influence of Culture Factors on Juvenile Growth and Survival

Juveniles that survived the feeding trials completed during this project did not survive on the dietary treatments alone; rather, the attention to detail of every culture step achieved a healthy batch of juveniles before feeding trials were even initiated. Research on *E. capsaeformis* has been conducted for the past several years at the FMCC, and standard protocols for collection of gravid females and host fish, as well as juvenile culture have been developed. Following previous culture protocol is crucial in having success with any species of juvenile mussels, particularly endangered species. My experience with culture factors and protocols confirms those of Jones et al. (2005). Determining how to better raise this species in a culture environment is challenging because survival and growth of juveniles in captivity is affected by a multitude of factors, beginning with the earliest infestation step. In regards to *E. capsaeformis*, collection of a gravid female with viable, mature glochidia in good condition for performing a laboratory infestation is vital. In the past several years, it has been observed that *E. capsaeformis* females generally have mature glochidia in late May or early June and should be collected during this time frame to ensure best possible laboratory infestation results. Based on Jones et al. (2005), survival and growth of juvenile oyster mussels were significantly greater when propagated in the spring, when glochidia were mature and would normally be released, than in the fall. Females collected before or after spring of the year typically either do not have mature glochidia or have already emptied their gills. Glochidia extracted and used in infestations from these females will usually provide batches of juveniles that are in poor condition or have high mortality.

In addition to the condition of the female's glochidia, the condition of the host fish is also crucial to the success of the infestation and the health of the juveniles once they drop off the fish. *Cottus carolinae* (banded sculpin) are the preferred host fish of *E. capsaeformis* for laboratory infestations because they have high transformation success, and are easier to keep alive and care for in the laboratory. It is important that fish hosts for any laboratory infestation are collected from the wild when they are in good condition and not gravid, acclimated to the laboratory water temperature, and provided with consistent flow, temperature and feed. Host fish should be acclimated to laboratory conditions for 1-2 wks prior to infestation.

For trial 1, gravid females were collected in late May to ensure mature glochidia, but due to an unsuccessful feeding trial that delayed the project during the summer, females had to be collected in November for trial 2. Surprisingly, these females did have mature glochidia, despite

the time of year. This may have been due to the severe drought during the summer causing low water levels in the Clinch River, as well as warmer temperatures lasting into the fall. These river conditions may have caused females to remain gravid longer than what is usually observed, due to the unusual spring/summer environmental conditions.

Host fish for all infestations were collected in good condition and acclimated to the laboratory before being utilized in infestations to prevent stress. With fish in healthy condition, the chance of the infestation providing a healthy batch of juveniles was much more likely. By ensuring the best conditions in the early steps of juvenile culture, the batches of juveniles used in this project theoretically were in excellent condition for feeding trials.

In reference to the feeding trials, there are many variables that can affect growth and survival of any mussel juveniles, and these should either be controlled or tested. In this case, dietary treatments were being tested in trials to determine what type of diet should be fed, specifically to *E. capsaeformis* juveniles, to provide better growth and survival in laboratory conditions than what has been previously recorded. Variables that affect juvenile growth and survival include system type, flow, temperature, substrate type, diet (both quantity and quality), and water quality. A recirculating aquaculture system (RAS) was selected for use in this study based on previous culture success by Jones et al. (2005). This RAS provided adequate flow in the culture containers, but not so much as to flush juveniles out of the container. In feeding trials for this project, good water quality for mussels was monitored and maintained by frequent water changes. Ammonia levels were closely monitored due to known toxic levels to freshwater mussels (Augspurger et al. 2003) Temperature was maintained at approximately 21° C in the RAS with chiller units, and fluctuations throughout the day were minor. Based on studies with *V. iris* by Gatenby et al. (1996), substrate has been determined to aid in juvenile pedal-feeding as well as providing some potential nutrients. In a later study, Jones et al. (2005) found more specifically that survival and growth of *E. capsaeformis* and *V. iris* juveniles were significantly greater when cultured in a sediment substrate rather than sand or no substrate. Based on the results of these previous studies, sediment was included in culture containers to enable *E. capsaeformis* juveniles to adequately pedal-feed on dietary treatments, which was the feeding technique observed by these juveniles.

Conclusions and Management Recommendations

Few studies have been conducted on the feed requirements of juvenile mussels, and *Epioblasma capsaeformis* in particular. As a result, this study provides useful but preliminary knowledge on diet requirements of these juveniles. The results from this study can assist the Freshwater Mollusk Conservation Center (FMCC) Blacksburg, VA, as well as other mussel hatcheries, in determining a more suitable feeding protocol that can be applied to propagation of *Epioblasma* species. Below are several management recommendations that have been determined from the research completed in this study.

1) Bioflocs can be included as part of the diet for *E. capsaeformis* juveniles to enhance growth and survival. The use of bioflocs at particularly fish hatcheries may aid in disposing of potential fish waste in an environmentally beneficial way.
2) The current juvenile algae mix diet used at the FMCC can be increased to triple the concentration of what is currently fed to *Epioblasma capsaeformis* juveniles in order to enhance growth and survival. The algae-mix diet itself seems to be suitable, as it provides several species of algae of varying cell size within the range juveniles can feed on. Juveniles would greatly benefit by being fed multiple times per day to provide consistent and continuous nutrients.
3) If pond water is used for juvenile culture at the FMCC, it should be analyzed periodically for various water quality components to monitor possible changes throughout the year, which could affect the health and survival of juveniles. In particular, pond water should be tested for pH, ammonia and chloride levels. A record should be kept of how often well water is used to fill the pond as this changes aspects of its water quality and this type of fluctuation could have adverse effects on juveniles.
4) Frequent water changes within recirculating aquaculture systems holding juveniles are necessary for promoting good water quality and controlling possible pathogens. If water is changed daily, water quality can be monitored less frequently.
5) The current culture protocol, determined by Jones et al. 2005 for *E. capsaeformis*, should be followed in order to ensure the healthiest juveniles for propagation purposes.

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APPENDIX A. Product information on Nannochloropsis 3600 (CA) (Reed Mariculture, Campbell, CA).

Characteristics

Cell count: 68 billion/ml

Live algae equivalent: 3600 L @ 19 million cells/ml

Algal type: green algae

Size: 1-2 µm

Dry weight: 18.40%

Species typically fed this product: Fish, rotifers

Storage
Store in refrigerator. Shelf life is up to three months.

Nannochloropsis 3600 Proximate Analysis/Nutritional Profile

Dry weight	18.4 %
Calories from 10 ml dry weight	44.4
Protein	52.11
Carbohydrate	16
Lipid (total)	27.64
Vitamin C	0.85
Chlorophyll A	0.89

APPENDIX B. Product information on Shellfish Diet 1800 (CA) (Reed Mariculture, Campbell, CA).

Characteristics

Cell count: 2 billion/ml

Algal type: green algae

Size: 5-20 µm

Dry weight: varies based on algal species

Storage
Store in refrigerator. Shelf life is up to three months.

Algal Species (size range from 5-20 µm)	**Percent of total algal amount (%)**
Nannochloropsis	5
Tetraselmis	20
Pavlova	20
Isochrysis	25
Thalassiosira weissflogii	30

Shellfish Diet 1800 Proximate Analysis/Nutritional Profile

Algal Species in Shellfish Diet 1800	**Nannochloropsis**	**Tetraselmis**	**Pavlova**	**Isochrysis**	**Thalassiosira weissflogii**
Dry weight	18.4%	9%	9%	9%	9%
Calories from 10 ml dry weight	44.4	48.2	~45	45.5	~22
Protein	52.11%	54.66%	51.60%	46.69%	~50%
Carbohydrate	16%	18.31%	22-24%	24.15%	n/a
Lipid (total)	27.64%	14.27%	19.56%	17.07%	n/a
Vitamin C	0.85%	0.25%	n/a	0.40%	n/a
Chlorophyll A	0.89%	1.42%	n/a	0.98%	n/a

APPENDIX C. Total suspended solids (TSS) data of harvested bioflocs (BF) grown at the Virginia Tech Aquaculture Center.

Sample	Initial Weight (g)	Volume of H_2O (ml)	Final weight (g)	TSS (mg/L)	Average TSS/ sample	Mean ± SD (95% confidence intervals)
1a	1.1228	10	1.1337	1090.0000	1067.6190	1062 ± 113 (+1287, -836)
1b	1.1299	10	1.1396	970.0000		
1c	1.1208	7	1.1288	1142.8571		
2a	1.1185	7	1.1257	1028.5714	945.8333	
2b	1.1463	8	1.1530	837.5000		
2c	1.1200	7	1.1268	971.4286		
3a	1.1250	7	1.1337	1242.8571	1171.4286	
3b	1.1253	7	1.1333	1142.8571		
3c	1.1089	7	1.1168	1128.5714		

APPENDIX D. Composition of bioflocs. Analyses provided by A & L Eastern Laboratories, Inc., Richmond, VA.

Parameter	Unit	Mean Wet Basis	Mean Dry Basis
Moisture	%	96.87	
Dry Matter	%	3.14	
Nitrogen	%	0.24 ± 0.007	7.63 ± 0.01
Crude Protein	%	1.43	47.69 ± 0.11
Crude Fat	%	0.02	0.61 ± 0.09
Crude Fiber	%	0.69	22.01 ± 0.65
Ash	%	0.61 ± 0.02	19.33 ± 0.16
Calcium	%	0.16 ± 0.01	5.05 ± 0.14
Potassium	%	0.01	0.21 ± 0.007
Magnesium	%	0.03	0.87 ± 0.007
Sodium	%	0.02	0.59 ± 0.007
Phosphorus	%	0.06 ± 0.007	1.72 ± 0.07
Sulfur	%	0.01	0.44
Copper	ppm	1	32
Iron	ppm	2	74 ± 1.41
Manganese	ppm	6	198.5 ± 4.95
Zinc	ppm	8.5 ± 0.71	273.5 ± 6.36
Aluminum	ppm	2	59 ± 4.24

APPENDIX E. Product information on Clear Flo 1002 probiotic (PRO) (Alken-Murray Corporation, New Hyde Park, NY).

Product Name

Clear-Flo 1002 probiotic

Bacterial Composition

11 natural, non-pathogenic, spore-forming, gram-positive *Bacillus*

Characteristics

Bacteria count and size: 2 X 10^{10} cfu per gram 1 μ in size

Appearance: free flowing, light tan powder

Odor: yeast-like

pH: neutral

This product is a soluble, super-concentrated probiotic for use in aquariums and garden ponds. The bacteria in this product benefit aquatic organisms in a captive environment by promoting better digestion and stronger immune systems in fish and crustaceans, reducing or eliminating turbidity in the water and sludge buildup, and promoting a healthy aquatic environment by reducing nutrient loads and producing many enzymes. Aerobic and facultative anaerobic strains in this product oxidize deadly hydrogen sulfide and reduce buildup of nitrates. The probiotic is safe and requires only small doses added directly to the water column to become effective.

System Requirements

Dissolved oxygen level: above 2 ppm (mg/L)

Temperature range: 5° to 35° C (41° to 95° F)

pH range: 4.5 to 8.5.

All system requirements were met by the system used in the feeding trial.

Dosage Instructions and Application

Can either apply dry powder directly to the water column of the system or slowly add water to form a smooth paste. Different dosages of the probiotic were recommended based on the size of the system and how contaminated the system was. In regards to the system size used in feeding trials (378-3,780 L) for each 378 L, the initial dose is 0.5 g, followed by 0.2 g once per week for 3 doses, then maintain with 0.3 g twice each month.

Storage

Store in a cool, dry place and avoid contact with eyes, skin or open wounds and wash hands with warm, soapy water after handling.

APPENDIX F. Product information on Protein Selco Plus (PSP) (INVE Aquaculture, Salt Lake City, UT).

Ingredients

Oils and fats, vitamins (including Vitamin C), marine algae, minerals, emulsifiers, antioxidants, marine DHA, immunostimulants, phospholipids

Characteristics

Protein Selco Plus is a concentrated rotifer enrichment paste. Immunostimulants present in this product enhance stress resistance in rotifers and larval fish.

Sum (n-3) HUFA: minimum 90 mg/g dry weight

DHA/EPA: minimum 2.5

System Requirements

Dissolved oxygen level: minimum 5 ppm

Temperature range: 25° to 27°C

Dosage Instructions and Application

Cut off required amount of Protein Selco Plus and blend for 3-5 minutes in water with a kitchen blender. Use a maximum 100 g of product/ L water.

Storage

For prolonged storage, refrigeration is advised. Protein Selco Plus was stored in the refrigerator during the duration of the feeding trial.

APPENDIX G. Product information on Culture Selco Plus (CSP) (INVE Aquaculture, Salt Lake City, UT).

Ingredients

Oils and fats, vitamins, marine algae, minerals, emulsifiers, antioxidants

Characteristics

Culture Selco Plus is a concentrated rotifer enrichment paste. Immunostimulants present in this product enhance stress resistance in rotifers and larval fish. This product lowers bacterial development and floccule formation within culture tank water, while enriching it. It also provides for a more dynamic rotifer population
Sum (n-3) HUFA: minimum 17 mg/g dry weight

System Requirements

Dissolved oxygen level: minimum 4 ppm

Temperature range: 15 º C maximum

Dosage Instructions and Application

Blend Culture Selco Plus for 3-5 minutes in water with a kitchen blender. Use a maximum 100 g of product/ L water.

Storage

Store in cool, dry place. For prolonged storage, refrigeration is advised. Once opened, the product should be used within 1 month, kept well closed and stored in refrigerator. This product expires after one year. Culture Selco Plus was stored in the refrigerator during the duration of the feeding trial.

APPENDIX H. Mean water quality data from feeding trial 1. Data were used in determining a protocol for water changes during a feeding trial.

Treatments	**Ammonia nitrogen (mg/L N)**	**Nitrate (mg/L NO_3-N)**	**Nitrite (mg/L NO_2-N)**	**pH**	**Dissolved oxygen (DO)**
Bacteria flocs (BF)	0.05	0.98	0.01	8.18	7.77
Probiotic (PRO)	0.07	1.88	0.01	8.14	7.99
Pond water (PW)	0.05	0.78	0.01	8.08	7.82
Triple conc. Algae (3 X CA)	0.10	1.55	0.02	7.94	7.81

APPENDIX I. Pond water quality analyses from feeding trial 1. Sample 1 was taken at 0 d and sample 2 was taken at 20 d. Analyses were provided by the Virginia Tech Water Quality Laboratory, Seitz Hall.

Sample	pH	Total hardness as CaCO3 (mg/L)	Chloride (mg/L)	Ammonia-N (mg/L)	Nitrate-N (mg/L)	Nitrite (mg/L)	Orthophosphate (mg/L)
1	8.92	220	25	0.10	0.40	0.008	0.02
2	8.90	220	23	0.11	0.40	0.007	0.04

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APPENDIX J. Percent organic matter analyses of sediment used in feeding trial 1 and 2. Analyses were provided by the Virginia Tech Soil Testing Laboratory, Smyth Hall.

Sample	Sediment weight (g)	Sediment ash weight (g)	Percent organic matter
Trial 1 Sediment	3.833	3.672	4.2
Trial 2 Sediment	3.824	3.644	4.7