BIOLOGY, CAPTIVE PROPAGATION, AND FEASIBILITY OF PEARL CULTURE IN THE PINK HEELSPLITTER (*POTAMILUS ALATUS*) (SAY, 1817) (BIVALVIA: UNIONIDAE)

by

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ABSTRACT

Pink heelsplitter (*Potamilus alatus*) mussels collected from Kentucky Lake, TN were held at two bottom locations (0.6 m, 2.5 m) and suspended in pocket nets (at depth about 1.0 - 1.5 m) in a pond at the Freshwater Mollusk Conservation Center (FMCC), Virginia Tech, for 1 yr. Survival of mussels after 1 yr was significantly different, with poorest survival (30 %) in the bottom of the deep end; and no difference between the shallow end (83.3 %) and the suspended pocket nets (63.3 %). Survival of mussels was inversely related to water temperature (r = - 0.72); lowest monthly survival occurred in summer, resulting in a significant difference among the three locations with a similar trend after 1 yr. The glycogen reserves of mussels in captivity for 1 yr differed by pond location, higher in mussels at the shallow end than those in suspended pocket nets and at the deep end. Therefore, the shallow end of pond was more suitable for holding mussels long-term, while the suspended pocket nets are an alternative site for holding captive mussels. Additionally, dissolved oxygen was very low at the deep end (1.9 mg/L) in summer, while it was adequate (range from 5.7 - 6.4 mg/L) at the location of suspended pocket nets, and 5.0 mg/L at the shallow end (24.7 ℃).

Data for 40 specimens indicated that sexual dimorphism in valve shape occurred in *P. alatus*. Female mussels had a significantly ($p < 0.0001$) greater ratio of height (H) to length (L) (52.3 %) and width (W) to length (31.8 %) than males (H/L: 48.4 %; W/L: 28.8 %), respectively. The posterior ends were somewhat round to oval in males and bluntly squared or truncated in females. Female mussels were more inflated than males. These morphological differences can be used to distinguish females from males during field collections.

The red drum (*Sciaenops ocellatus*) was identified as a new fish host for *P. alatus*, as 48 active juveniles were transformed by this species, which is not a natural host. Four glochidia were observed on the fins versus 2,307 on the gills of five red drum. Freshwater drum also was verified as a suitable host fish, but black crappie (*Pomoxis nigromaculatus*), banded sculpin (*Cottus carolinae*), yellow perch (*Perca flavescens*) and nile tilapia (*Oreochromis nilotica*) did not support transformation of glochidia to juveniles.

Survival and growth of propagated juveniles of *P. alatus* were assessed regarding the effects of algal diets (*Nannochloropsis oculata* and *Neochloris oleoabundans*) and substrate type (fine sediment and sand). Overall, survival of juveniles after 17 d ranged from 23.8 to 66.8 %, with mean of 48.5 %; however, survival dramatically declined during the next 2 wk period to only 5.8 % (range of 1.8 to 7.8 %). Survival rate of juveniles was significantly different ($p = 0.027$) between substrates, but not in diets ($p = 0.520$), with the lowest survival rate of 23.8 % in sand substrate and fed *N. oculata*. Juveniles grew faster in fine sediment (23.0 % increase in shell length) than in sand substrates (10.5 % increase) ($p = 0.002$). Moreover, mean growth rate of juveniles was 4.9μm/d during the first 2 wk, but decreased to 0.2 μm/d in the remaining 2 wk. Therefore, fine sediments seemed more appropriate for juvenile

culture compared to sands. Both species of algae, *N. oculata* and *N. oleoabundans*, can be used to feed juveniles in the laboratory.

Adult pink heelsplitters were used to study feasibility of pearl production by using two surgical implants (non-nucleated implant = NNI, and image pearl implant = IPI) in two ponds of different nutrient levels (FMCC pond and Duck pond). NNI and IPI pearls with purple or purplish luster were successfully produced in *P. alatus*. Pearl weight was not significantly different ($p = 0.562$) between two ponds. No differences in monthly survival rates of mussels were observed in either pond ($p = 0.051$), or among mussels with surgical implants and the no-surgery control mussels ($p = 0.881$). Consequently, *P. alatus* can be considered a potential species for producing purple pearls in pearl culture. Additionally, mussels in the Duck pond had higher ($p < 0.0001$) glycogen levels, similar to those in wild collected mussels, than those in the FMCC pond, indicating that this pond environment may be more suited for holding implanted mussels in captivity.

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INTRODUCTION

Diversity of freshwater mussels in North America

Freshwater mussels (families: Unionidae and Margaritiferidae) attain their greatest diversity in North America, with 297 recognized taxa (281 species and 16 subspecies, Williams et al. 1993). Of these, only 70 taxa (23.6 %) are considered stable, while a majority of the species (213 taxa, 71.7 %) require some form of federal or state protection and are designated as endangered, threatened, extinct, or of special concern (Neves and Williams 1994). The decline of this fauna has been attributed to habitat degradation and destruction due to impoundment, sedimentation, water pollution, and nonindigenous species invasion. Because mussels have a complex life history, declines and extinction of host fish populations also have had profoundly negative effects on mussel recruitment (Neves et al. 1997, Neves 1999, Hallac and Marsden 2001). Jones and Neves (2002) recommend artificial propagation as an effective strategy to restore and recover imperiled species.

Freshwater mussels are ecologically and economically important. As filter feeders, they clean the water by removing suspended inorganic particulates, detritus, and plankton. Allen (1914) reported that an average-sized mussel could filter over 30 L of water within a 24-hr period. They are also an important source of food for wildlife, and their shells provide habitat for other aquatic organisms. Freshwater mussels embed themselves into the substrate and have little capacity to move long distances; therefore, they are recognized as useful indicators of water quality. Freshwater mussel shells are economically valuable as the raw material for beads used to produce pearl nuclei (Williams et al. 1993), jewelry and crafts (shell carving), and as an animal food additive (Hua 2001, Hua et al. 2003). The importance of some pearly mussels to the

pearl culture industry is evidenced by the great numbers of pearls produced annually (Hua 2001).

Life cycle and reproduction

Most freshwater mussels are dioecious and have a parasitic life stage during their complex life cycle. Sperm are released into the water column by mature male mussels and filtered by female mussels via their inhalant aperture. Fertilization is internal. Probability of fertilization is enhanced by water velocity, since the sperm have limited dispersal under lentic conditions, such as in lakes and ponds (Fuller 1974). Embryos develop into larvae, called glochidia, in the female's gill marsupium, and then are expelled to the water via pores or by rupture of the marsupium margin. Glochidia must attach to the gills or fins of fishes to metamorphose into juveniles. Otherwise, they will fall to the bottom of streams and quickly perish (Neves and Widlak 1987). The capability to parasitize is affected by the glochidial morphology, which can be hooked, axe-head, or hookless (Howells et al. 1996). If glochidia attach to a suitable fish host, they develop further following encystment and metamorphosis, to the free-living stage as juveniles, and grow into adults.

One gravid female mussel releases large numbers of glochidia annually; however, only those glochidia that successfully attach to the appropriate fish hosts continue further development. Moreover, only those juveniles that reach suitable habitat are able to survive and grow (Howard 1922). Therefore, the linchpin of reproductive success for freshwater mussels depends on the abundance of suitable host fish and appropriate habitat (Neves et al. 1985).

The Freshwater Mollusk Conservation Society has listed the identification of fish hosts as a top priority in the National Strategy for the Conservation of Freshwater

Mussels (Biggins et al. 1995), because the propagation and restoration efforts require appropriate host species in the laboratory and in the fish assemblage at sites. O' Dee and Watters (1998) indicated that hosts are known for only about one-third of the North American unionid species. Although many hosts have been identified via in-situ observation, hosts of only one-third of the nearly 300 North American species have been validated by artificial infestation (Watters 1994a).

Freshwater mussels have two reproductive strategies, long-term (bradytictic) brooders and short-term (tachytictic) brooders. Long-term brooders spawn and fertilize eggs in the late spring or summer, house glochidia in their gills throughout the winter, and release them the following spring and summer. Short-term brooders spawn and fertilize eggs in spring and discharge glochidia in summer of the same year (Neves and Widlak 1988).

Lefevre and Curtis (1912) produced the first juvenile mussl from artificial propagation. Subsequently, other techniques of artificial propagation have been evaluated, including floating crates, tanks, and cement-lined or earthen ponds (Howard 1922). Early success in artificial production of juveniles was reported by Hudson and Isom (1984), and juveniles were successfully raised in a laboratory system. Thereafter, additional biologists succeeded in artificial propagation and juvenile culture with better survival rates (Gatenby et al. 1997, O' Beirn et al. 1998, Beaty 1999, Henley et al. 2001). Researchers at FMCC at Virginia Polytechnic Institute and State University in Blacksburg, Virginia have conducted propagation of 18 species of freshwater mussels. Both laboratory and hatchery culture techniques have been developed, and 11 endangered species have been successfully propagated at the FMCC. More than 500,000 juvenile mussels of 10 species have been released into the Tennessee River and its tributaries from 1998 to 2004. Feasibility of long-

term captivity of mussels for conservation and propagation also has been studied at other sites in the United States (Newton et al. 2001, Hallac and Marsden 2001, Boyles 2004).

Status of the pink heelsplitter

The pink heelsplitter, *Potamilus alatus* (Say, 1817), of the family Unionidae, has six congeneric species: fat pocketbook *Potamilus capax* (Green, 1832), pink papershell *Potamilus ohiensis* (Rafinesque, 1820), bluefer *Potamilus purpuratus* (Lamarck, 1819), Texas heelsplitter *Potamilus amphichaenus* (Frierson, 1898), inflated heelsplitter *Potamilus inflatus* (I. Lea, 1831) and purple heelsplitter *Potamilus poulsoni*. Freshwater drum (*Aplodinotus grunniens*) is reported as the sole host fish for the glochidia of *P. alatus* (Parmalee and Bogan 1998). Other host fishes may occur for other species within *Potamilus*, but none have been confirmed.

The pink heelsplitter is a large species, with a maximum shell length about 185 mm. The nacre color is dark purple with iridescence at the posterior end. It is considered a common species and is widespread throughout the Mississippi River drainage, ranging south to Arkansas, west to Kansas and Nebraska, and north to Minnesota. It also occurs in the St. Lawrence River system from Lake Huron to Lake Champlain, with range extending to the Canadian Interior Basin in parts of the Red River of the North and the Winnipeg River. However, Perkins and Backlund (2000) reported that it had become less common downstream in South Dakota because of dams, which obstruct the migration of the freshwater drum.

Freshwater pearl culture background

The first American freshwater pearl was found accidentally in a mussel in the Notch Brook River near Paterson, New Jersey by David Howell in 1857 (Fassler

1991). According to Austin (1995), John Latendresse, known as the father of U.S. cultured freshwater pearls, initiated freshwater pearl culture experiments on mussels in Tennessee using Japanese technology in 1963, but the venture failed (Sweany and Latendresse 1982, Fassler 1991). In the late 1970s, Mr. Latendresse established five freshwater pearl farms based on availability of new technology for culturing freshwater pearls using the washboard (*Megalonaias nervosa*). Although achieving some success, the pearl farms were not economically viable.

In China, the technology of freshwater pearl culture was developed about 2,000 years ago. However, commercial freshwater pearl culture dates back only to the late 1960s and early 1970s (Hua et al. 2002). Native freshwater mussels, such as the triangle sail mussel (*Hyriopsis cumingii*) and the wrinkle comb mussel (*Cristaria plicata*), are widely used for pearl culture in China. The annual production of freshwater pearls increased exponentially from 500 kg in the late 1960s to 20 metric tons in 1972, and exceeded 2000 metric tons in 1995 and 1996. Today, China produces 95 % of all freshwater pearls sold in the world market (Hua et al. 2003).

Research goals

The objectives of my study were as follows:

1. Evaluate the survival rate and conditions of adult pink heelsplitters (*Potamilus alatus* Say, 1817) (Bivalvia: Unionidae) held in a pond for one year (Chapter 1).
2. Examine shell morphology and sexual dimorphism in the pink heelsplitter (Chapter 2).
3. Test the suitability of alternative host fish species for transforming glochidia of the pink heelsplitter (Chapter 3).
4. Determine effects of diet and substrate type on culture of juveniles of the pink

heelsplitter (Chapter 4).

5. Test the feasibility of pearl production using the pink heelsplitter (Chapter 5).

CHAPTER 1: SURVIVAL RATE AND CONDITION OF ADULT PINK HEELSPLITTERS (*POTAMILUS ALATUS*) (SAY, 1817) (BIVALVIA: UNIONIDAE) HELD IN A POND FOR ONE YEAR

INTRODUCTION

Holding freshwater mussels in captivity can be necessary for conducting effective ecological, physiological, and economic studies, as well as propagation and conservation. However, collection of mussels is often stressful, and can cause mortality to the mussels. Previous projects on the relocation of mussels have not been very successful; mean mortality rate of mussels reached 50 % of 37 projects completed (Cope and Waller 1995). More recently, a number of systems have been developed and tested for species' ability to adapt to captivity. Burress and Neves (1995) evaluated survival of fifteen species of freshwater mussels in captivity in four separate pond sites in Virginia, and reported that survival rates varied by species, captivity period, and habitat conditions. Gatenby (2000) also studied holding conditions for the captive care of four species of unionid mussels in ponds. Survival rates varied among species, reached 73 % after 1 yr, but later declined significantly. Boyles (2004) successfully held four species of mussels after 1 yr with a mean of survival rate over 77 %, and a high of 96 % at White Sulphur Springs National Fish Hatchery. Liberty (2004) obtained a similar survival rate in a raceway at the Aquatic Wildlife Conservation Center, Marion, Virginia.

Although survival rates of captive mussels were related to species and captivity period, they also are influenced by habitat suitability and captive methods. Dunn and Layzer (1997) concluded that survival rate varied by species and captive facilities, when comparing 20 species of mussels relocated in various types of holding facilities.

Evaluating suspended nylon pocket nets and benthic dish racks, Gatenby (2000) reported that mussels held in pocket nets generally had higher survival rate than those in dish racks. Culture methods in commercial aquaculture include hanging culture (suspended nets in water) and bottom culture for marine bivalve production (Wang 1993, Wang and Tian 1998). Bottom culture is widely used for edible clams and quahogs, while scallops are suspended in metal or plastic mesh frames and nets, or are hung individually from strings (often referred to as ear hanging). Although bottom culture is popularly employed for oyster culture, off-bottom culture produces regularly shaped shells preferred by consumers. Freshwater mussels used for pearl culture are usually held in net bags, net cages or pocket nets; and suspended in ponds, reservoirs or lakes for pearl production (Hua et al. 2002). The advantage of hanging culture is that it maximizes the 'cubic' water column by using the total water body instead of the 'square' area in bottom culture. Moreover, hanging mussels are free of substrate, such that occurrence of disease caused by the organisms living in the substrate is minimal.

In natural habitats, the pink heelsplitter inhabits sandy bottoms in shallow lakes, soft sandy river overbanks, and coarse gravel in rivers, usually in areas up to 1 m in depth (Parmalee and Bogan 1998). However, pink heelsplitter mussels in reservoirs also are found in submerged sloughs and creek channels as deep as 5 to 7 m, and in the main submerged river channel at depths ranging from 5 to 20 m (Don Hubbs, Mussel Program Coordinator, Tennessee Wildlife Resources Agency, personal communications).

To successfully hold mussels in a pond, pond management is a direct and effective way to increase survival of captive mussels. For instance, pond fertilization is commonly used to promote algal production, so that pond water is more productive

to satisfy the food demands of bivalves and filter-feeding fishes (NACA 1985, Hua et al. 2001).

Biochemical composition of mussels is commonly used to quantify the health condition in mussels (Haag et al.1993, Naimo et al. 1998, Gatenby 2000, Newton et al. 2001, Liberty 2004). Glycogen is a primary energy reserve in adult bivalves (De Zwaan and Zandee 1972, Gabbott 1983, Hummel et al. 1989, Leavitt et al. 1990), and is considered an indicator of condition of freshwater mussels (Naimo and Monroe 1999, Patterson et al.1999, Gatenby 2000, Boyles 2004, Liberty 2004). Environmental changes can stress mussels, evident by reduction in glycogen content (Hummel et al. 1989, Naimo and Monroe 1999). Gabbott and Walker (1971) reported that *Ostrea edulis* L. had to use body-reserve glycogen, resulting in a decline of energy reserves, due to high temperature and no food. While many studies of glycogen dynamics have been previously conducted, glycogen reserves in the pink heelsplitter have not been studied.

The objective of this study was to evaluate survival rates of the pink heelsplitter in captivity for 1 yr at 2 bottom depths and suspended in net pockets in a pond at the Freshwater Mollusk Conservation Center (FMCC). Body condition was examined by comparing glycogen reserves in captive mussels to those from the wild, parent population in Kentucky Lake. Water temperature and dissolved oxygen were monitored at each location in the pond.

METHODS AND MATERIALS

Collection and sites

Mussels were collected from a submerged creek channel in the Kentucky Lake portion of the Tennessee River (TRM 103.0), Humphreys County, TN by Don Hubbs (Mussel Program Coordinator, Tennessee Wildlife Resources Agency). Mussels were found in sand, gravel, and clay substrates at water depths less than 5 m. They were shipped overnight to FMCC in a cooler with ice to maintain cool temperatures and reduce metabolism during transport. Mussels were quarantined in a 1000 L isolated recirculating system for 1 mo to verify that mussels were not infested with zebra mussels. After quarantine, each mussel either was tagged by gluing a plastic tag on the periostracum, or marked directly with a unique number.

Additional mussels, originally collected in Kentucky Lake, TN, were held in a recirculating system for 1 mo for behavioral studies (mid-October to November, 2003). These mussels were used to replace dead mussels during the 1-yr experimental period. To compare glycogen levels between captive and wild mussels, wild mussels were collected from the source location at the same sampling times.

The experiment was conducted at the FMCC, Virginia Polytechnic Institute and State University, Blacksburg, Virginia. This facility has one 185 m^2 building for propagation of freshwater mussels, two laboratories for feeding studies, one storage building and a 1000 m^2 pond. This pond holds 985 m^3 of water. It is 1 m deep at the shallow end and 3 m at the deep end. Since pink heelsplitters usually inhabit areas with water current (Parmalee and Bogan 1998), a paddle-wheel (Little John Aerators, Model: 571, 0.5 horsepower) was installed in the pond to create flow and aeration.

Fertilization of pond

Ammonium nitrate was applied at roughly 0.5 kg every 2 wk to the FMCC pond, but pond water remained mostly unproductive according to 1 yr of water quality analysis (details in Chapter 5).

Experiment and sampling

Ninety mussels were randomly assigned to three captivity treatments in the FMCC pond (Figure 1-1). In two bottom treatments, individuals were confined in containers (bottom culture) at two different depths with three replicates for each ($t = 2 \times 3$, $n = 10$/replicate). In a third treatment, mussels were suspended in the water column in 3 pocket nets (hanging culture, $t = 3$, $n = 10$/replicate).

Treatment 1: shallow end

Thirty mussels were placed at 0.60 m water depth in a sandy-clay substrate. The mussels were randomly assigned to three 0.50 m× 0.50 m× 0.20 m bottomless plastic containers (10 mussels per container).

Treatment 2: deep end

Thirty mussels were placed on the pond bottom at a water depth of 2.5 m. Three plastic buckets (0.60 m × 0.35 m × 0.20 m) with a mixed substrate of sand, gravel, and fine sediment were used to hold experimental mussels. Mussels were randomly assigned to the plastic buckets (10 mussels per container). Containers were tied with a rope and submerged to the pond bottom.

Treatment 3: nets in suspension

Plastic nets (0.60 m × 0.45 m) with rows of pockets were used for holding mussels. Three plastic pocket nets were hung from a surface line spanning the width

of the pond. Two pulleys were fixed at each end of the line to retrieve and return nets for sampling. Each plastic net contained 10 mussels; two rows of pockets held 3 mussels and one row held 4 mussels. Pocket nets were suspended between 1 and 1.5 m from the water surface, and 3 nets were used as replicates.

Survival was monitored monthly for 1 yr, from October 2003 to October 2004. Mussels were defined as dead when valves remained open and did not close when stimulated. They were considered alive when valves closed in response to a stimulus. Since sample size was limited to 10 mussels per replicate, dead mussels were replaced with additional live mussels during the 1 yr period.

Glycogen determination

Glycogen levels were compared between captive and wild mussels at 6 mo and at 1 yr. Three mussels per treatment and 3 wild mussels were sampled on each date. Mantle tissue was removed and placed in a vial, and frozen at -60 ℃ for glycogen analysis. Vials of mantle tissue were dried in a freeze-drier (Labconco, model 79480, Kansas, MO) for 3 d until the weight of mantle tissues became constant. Vials were placed in the freeze drier covered by tying cheesecloth on the top after removing their caps to evaporate moisture. Samples were treated at -40℃ and a vacuum pressure of 110×10^{-3} torr. The dried mantle tissues were chopped into small pieces, and ground into powder using a mortar and pestle, and stored frozen at -60 ℃ for glycogen analyses.

Glycogen content was determined using a phenol-sulphuric method (Dubois et al. 1956, Naimo et al. 1998). A standard glycogen solution was prepared with glycogen powder from *Mytilus edulis* (Type VII, Sigma Chemical Company, St. Louis, MO). A stock solution of glycogen was made by dissolving 250 mg of glycogen powder in a

50 mL flask, filled with deionized water to reach the concentration of 5000 mg/L. A series of standard concentrations of glycogen were obtained by diluting the glycogen stock solution into 0, 250, 500, 1000, 2000, 2500, and 5000 mg/L. A sub-sample of 250 µL from each concentration was added in a labeled cryovial, ready for the standard curves. Samples of freeze-dried mantle tissue powder (2.5 - 5.0 mg) were placed in 2 mL labeled cryovials with 500 µL of 30 % potassium hydroxide (KOH). The same amount of KOH was added to the cryovials with standard solution. All of them were covered tightly, and placed in a 100 ℃ water bath for 20 min. All cryovials (standards and samples) were shaken for 30 sec after removal from the water bath, and then placed on ice for 5 min. Then 750 µL of EtOH (ethyl alcohol) was added to the cryovials and returned in the 100 ℃ water bath for 15 min. Contents of cryovials were transferred to labeled test tubes and rinsed with 5 mL of deionized water. To the test tubes was added 350 µL of deionized water, while 100 µL deionized water was added to the standard test tubes. Sample and standard tubes were completely mixed by vortexing, and a 250 µL aliquot of each tube was transferred in labeled test tubes with two replicates. To each test tube was then added 100 µL of 80 % phenol and 5 mL of concentrated sulfuric acid and held for 30 min at room temperature. Meanwhile, a spectrophotometer (Genesis 8; Thermo Spectronic, Rochester, NY) was allowed to warm up for 30 min, and later used to measure the absorbance of test samples at an wavelength of 490 nm. One standard curve was prepared from the levels of standard concentration, and one average value of two replicates was used to calculate the glycogen content for each sample from the equation: mg glycogen/g = (mg glycogen/L/1000 mL)/g dry tissue.

Because Gatenby (2000) reported that survival of captive mussels was related to water temperature during the captive period, water temperature was monitored

continually at 1 hr intervals using Hobo temperature loggers (Onset Computer Corporation, Pocasset, MA). Dissolved oxygen (DO) also was measured.

Data analysis

Survival rates and glycogen levels among treatments and wild controls were analyzed using the statistics software JMP (SAS Institute Inc. Cary, NC, 2001) (Sall et al. 2001). Survival rates were calculated by month; survival included replacements, at the end of each month, and survival of the initial group (3 × 30), excluding mussel replacements.

Survival rate and glycogen content were analyzed for normality using the Kolmogorov - Smirnov test (Sall et al. 2001) due to small sample size. They were then analyzed with the Analysis of Variance (ANOVA) test, if data were normally distributed. Eventually, data were analyzed by Tukey - Kramer HSD (Honest Significant Difference) to compare differences in mean survival rate of mussels among the three holding locations; and Least Significant Difference (the LSD) in mean glycogen content of mussels among three holding locations and wild mussels (collected from Kentucky Lake). Data were analyzed with the rank-based nonparametric Kruskal - Wallis test, if they were non-normally distributed.

RESULTS

Final survival rate of *P. alatus* after 1 yr differed significantly among treatments ($p = 0.001$, $n = 3$). Survival was lowest in the deep bottom treatment (30.0 %), and survival rates between shallow end (83.3 %) and suspended net pockets (63.3 %) did not differ significantly (Table 1-1 and Figure 1-2).

Mean monthly survival rates with replacement at the deep end, shallow end, and

in suspension were 92.8 %, 97.0 %, and 95.9 %, respectively, from 24 October 2003 to 25 October 2004. There was no significant difference among the three captive locations for monthly survival rates (Table 1-2, Figure 1-3), except during August 2004 ($p = 0.006$, $n = 3$), when survival was lowest in the deep end treatment. There was no significant difference in mean survival rate between shallow and suspended holding methods (Table 1-3).

Glycogen analysis was not performed after 6 mo because the sampled mussel tissue was inadequate for glycogen determination. Because of small sample size ($n = 10$), I snipped a small piece of tissue from live mussels instead of sacrificing specimens. Two of these sampled mussels at the shallow bottom were found dead at the monthly sampling on 23 July 2004. Data on glycogen content of dry weight (DWT) were from captive mussels held at three locations in the pond after 1 yr in captivity. Mean glycogen content of mussels among the three captive locations, compared to wild mussels was significantly different ($p = 0.001$, $n = 3$). Glycogen content of mussels held in the suspended nets and wild reference specimens was lower than those at the shallow end; higher than those at the deep end. There was no significant difference in mean glycogen content between wild specimens and mussels in the suspended nets (Table 1-4, Figure 1-4). Glycogen content had positive correlation ($r = 0.77$) with survival of mussels (Figure 1-8), in which the highest values (glycogen and survival) were at the shallow end and the lowest at the deep end.

Glycogen content differed by sex ($p = 0.001$, $n = 5, 8$) in the wild population, being higher in males than females (Figure 1-5). Mean glycogen content in male mussels reached 690.9 mg/g, while it was as low as 308.5 mg/g in female mussels on 20 April 2005. Glycogen was higher in males (916.3 mg/g) than in females (748.7 mg/g) on 7 June 2004. In addition, glycogen levels in *P. alatus* exhibited seasonal

variation; high in later summer and low thereafter.

Water temperatures (WT) at the two bottom locations in the pond and air temperature were recorded hourly throughout the year and reported as mean daily temperature (Figure 1-6A, 6B, 6C). Overall, temperatures at the two pond bottom locations were similar, with a maximum of 28.1 ℃ and 28.4 ℃ on 3 August 2004, respectively, and a minimum of 2.3 ℃ and 2.1 ℃, respectively, on 15 December 2004. Air temperature was highest at 25.7 ℃ on 1 August 2004 and lowest at -12.2 ℃ on 20 December 2004. Water temperatures at the deep end location from 24 August to 24 October 2004 were lost due to malfunction of the Hobo temperature logger; therefore, I applied a trendline (dashed line in Figure 1-6B) to connect the discontinuous data. Mortality of mussels was positively related to water temperature ($r = 0.72$) at both ends of the pond (Figure 1-7A, 7B).

Dissolved oxygen level was lowest at the bottom of the deep end (1.9 mg/L), while it was 5.8-6.4 mg/L at 0.2 m above the bottom of deep end, and 5.0 mg/L at the shallow end of the pond, when water temperature was about 24.7 ℃ (Appendix 1-1).

DISCUSSION

Mussels of *P. alatus* had a high survival after 1 yr in the FMCC pond, particularly at the shallow end and in pocket nets, 83.3 % and 63.3 %, respectively. Gatenby (2000) studied the captive survival of *P. alatus* in a pond in West Virginia and reported only 23 % after 1 yr, comparable to survival rate of mussels at the deep end of my pond (30.0 %). Similar results were exhibited in summer (August 2004) when water temperature was high (Table 1-1, Table 1-2). This result corroborates that of the previous study; namely, that 6 species of mussels held in pocket nets and suspended in

a pond had higher survival than those held in racks on the pond bottom (Gatenby 2000).

Fuller (1974) indicated that low dissolved oxygen concentration could affect respiration, growth, and glycogen storage of freshwater mussels. Four mussel species (*Elliptio crassidens*, *Lampsilis subangulata*, *Medionidus penicillatus* and *Pleurobema pyriforme*) experienced high mortality when dissolved oxygen concentrations were below 5 mg/L (Johnson et al. 2001). Chen et al. (2001) reported that mussels were unable to maintain normal oxygen consumption under low DO levels, and suggested that a critical DO concentration should be above 2 - 3 mg/L for *Amblema plicata, Quadrula pustulosa, Elliptio complanata*; 3.5 - 4.0 mg/L for *Pleurobema cordatum*, and 6 mg/L for *Villosa iris*. Otherwise, mussels may become stressed if exposed to low DO for hours or days (Davis 1975). DO was only 1.9 mg/L at the sediment surface in the deep end, and increased to 6.0 mg/L from 0.20 m above the bottom up to the water surface at a water temperature of 24.7 ℃. DO was adequate (5.0 mg /L) at 25.4℃ at the shallow end (Appendix 1-1). Oxygen consumption of the Chinese pearl mussel, *Hyriopsis cumingii* was 0.06 mg O_2/g h at 20 to 22 ℃, and was positively correlated with WT (Hua 2003 a). Likewise, Bartsch et al. (2000) reported that high WT increased oxygen consumption in mussels. Therefore, low DO at the deep end at the FMCC pond may have been present for 2 mo, because the measurement was taken at the end of June, and WT was high (over 25℃) through August. Low DO may have stressed captive mussels and caused high mortalities at this bottom location. Conversely, mussels placed at the shallow end and in suspended pocket nets had high survival, likely unaffected by DO level.

The paddle-wheel (and aerator) facilitated water circulation throughout the pond and increased dissolved oxygen and available phytoplankton. It moved water more

effectively at the shallow end, which may have positively affected survival. However, the paddle-wheel was incapable of providing water flow to the bottom of the deep end. Gatenby (2000) also reported that flow rate was negligible at the bottom of her ponds with a paddle-wheel in operation. Johnson et al. (2001) reported that mortality of unionid mussels increased when velocity at the substrate surface dropped below 0.01 m/s. Therefore, mussels at the deep end may have experienced higher mortality due to lower velocity and DO than those at the shallow end and in suspended pocket nets. Although some freshwater mussels, such as *Elliptio complanata*, *Utterbackia imbecilis*, and *Pyganodon grandis*, can temporarily lower metabolic activity to meet reduced dissolved oxygen (Burky 1983, Sheldon and Walker 1989, McMahon 1991), *P. alatus* may not be capable of doing so since those at the deep end exhibited high mortality when DO was low.

During summer, I observed that the surface of substrate at the deep end was black with a H_2S smell, although I did not test for H_2S. In addition to possible anaerobic conditions at the bottom, hydrogen sulfide (H_2S) is highly toxic to cellular respiration by disrupting oxygen transport by aerobic organisms (Berzofsky et al. 1971). Kraus et al. (1996) reported that *Solemya reidi* (Bivalvia: Solemyidae) showed a reduction in rate of deoxygenation of the cytoplasmic hemoglobin due to sulfides. However, I did not observe similar conditions at the shallow end. Therefore, lower survival of mussels at the deep end at the FMCC pond also may be related to hydrogen sulfide production from organic matters decomposition.

To assess high variation in survival, it is essential to consider body condition of mussels. Glycogen reserve was lower in captive mussels at the deep end, when compared to wild mussels from Kentucky Lake collected and measured concurrently (Table 1-4, Figure 1-4). In previous research, glycogen level declined when wild

bivalves were brought into captivity (Gabbott and Walker 1971, Lomte and Jadhav 1982, Gatenby 2000, Monroe and Newton 2001), indicating that mussels can be stressed after relocation. Patterson et al. (1999) reported that glycogen levels of adult *Amblema plicata* (Say, 1817) and *Quadrula pustulosa* (I. Lea, 1831) were extremely low in starved mussels compared to fed mussels. Likewise, Gatenby (2000) also reported that all mussels in both racks on the bottom and pocket nets appeared to terminate feeding, and gradually starved to death. I did not record gut contents and fullness of stomach in the pink heelsplitter, but I did observe mussels with empty or somewhat empty stomachs. Algal density was insufficient, since water transparency reached about 2 m in the pond. A suitable secchi depth in a productive pond used for mussel culture is 25 - 30 cm (Hua 2001). Mussels at the shallow end and suspend nets may have had more food available because of the paddle-wheel, resulting in relatively higher glycogen reserve (458.6 mg/g and 323.1 mg/g DWT), compared to those at the deep end (215.5 mg/g DWT) of the pond (Table 1-4). Mussels at the deep end had low glycogen, indicative of their poor condition and survival, perhaps due to starvation. Obviously, glycogen content at three locations exhibited a positive correlation (r = 0.77) with survival of mussels (Figure 1-8), in which the highest peaks (glycogen and survival) occurred at the shallow end and the lowest at the deep end. From these data, there was no significant difference in suitability of locations between shallow and suspended pocket nets, based on mean survival rate. Therefore, suspended nets were used to hold mussels in the subsequent pearl culture experiment (Chapter 5).

Based on monthly samples, survival remained high throughout winter 2003, declined as WT increased in summer, and was again high in winter 2004 (Figure 1-2). Results supported the inference that temperature was an important environmental factor affecting metabolic rates, survival, and growth in mollusks (Bayne 1976,

Newell 1979, Newell and Branch 1980). Comparing the correlation of water temperature and survival of mussels at both bottom locations (Figure 1-7A, Figure 1-7B), mortality increase was associated with increasing temperature ($r = 0.72$). Gatenby (2000) reported that the high mortality of mussels in summer in her experiment could be attributed to the lethal effect of high temperature (which exceeded 28 ℃ for 4-5 d), and enhanced by poor health in mussels with low glycogen level (mean glycogen = 10.2 ± 4.2 mg/g, dry ETOH-preserved tissue). Lethal temperature was documented at 29 ℃ for *Anodontoides ferussacianus* (Salbenblatt and Edgar 1964). In my study, the temperature at the pond bottom occasionally exceeded 29℃ in July and August; it reached 28.7℃ for 4 consecutive days in July and early August. Water temperatures exceeded 29 ℃ for 8 consecutive days in July and 4 consecutive days in August. Water temperature was not recorded in the water column at the suspended nets due to paddle-wheel mixing. Therefore, high mortality in summer was likely influenced by high WT.

Haag et al. (1993) reported that females of *Lampsilis radiata* had lower glycogen content (0.08 mg/g in tissue wet weight) than males (0.13 mg/g). Likewise in my study, glycogen content differed ($p = 0.001$) by sex in wild *P. alatus* collected from Kentucky Lake; higher in male mussels (Figure 1-5). This may be explained by the reproductive traits of *P. alatus* since this is a long-term brooder in the subfamily *Lampsilinae* (Ortmann 1919). Female mussels often brood glochidia in gills for 9 - 12 mo (Clarke 1981), so that they demand more energy (e.g., glycogen) to sustain brooding glochidia (Mackie 1984); consequently, glycogen levels decline over time.

Seasonal differences in glycogen content seemingly occurred in *P. alatus*; glycogen levels were highest in early summer, declined throughout the year, and reached their lowest levels in spring (Figure 1-5). The seasonal patterns were unclear

due to limited data, but they may be related to the reproductive characteristics of female and male spawning in late August and early September (Haggerty 2000). Mussel condition should increase after spawning, but subsequently decline after fertilization and glochidia development (Gatenby and Neves 2002).

CONCLUSIONS AND COMMENTS

Overall, survival of *P. alatus* was lowest in the deep end of the pond, compared to the other two locations after 1 yr; however, there was no significant difference in survival rates of mussels from the shallow end and suspended pocket nets. Monthly survival rates did not differ significantly among the three captive locations throughout the year, except in August 2004 (mussels at deep end with poorest survival). Therefore, mussels can be relocated to sites with high DO, adequate velocity, sufficient food, and in shallow locations in ponds for long-term holding. The hanging method using suspended pocket nets could be an alternative option to hold mussels if a shallow site is not available. Moreover, I recommend using the hanging method for pearl culture, because it is convenient to adjust mussel locations to use the most suitable environment and food supply seasonally and facilitate production of lustrous pearls.

Mean glycogen content of mussels was higher at the shallow end than at the other two locations (deep end and suspended pocket net) after 1 yr. Limited food supply at the other locations may have been sub-optimal for growth. However, mussels can recover from environmental stress if suitable conditions are provided to them. For example, Chinese pearl-producing mussels *H. cumingii* and *Cristaria plicata,* when collected from the field, are usually held in a fish pond for over 1 yr for nucleated pearl culture, and survival rate can be nearly 95 % in ponds with suitable water

quality and adequate food. Therefore, management of the FMCC pond should be intensified to increase the survival of mussels in captivity. Fertilization is a direct and effective way to increase productivity. I recommend using multiple components with nitrogenous fertilizer at 0.7g (N)/ m^2, phosphorus fertilizer at 0.4g (PO_4)/ m^2, and potassium fertilizer 0.15g (K)/ m^2. Calcium carbonate is usually applied at 30g/m^2 to supplement and maintain calcium concentration at 20 - 30 ppm (Hua et al. 2001). In addition to fertilization, feeding mussels by supplemental food (such as soybean milk) can enhance mussel health directly and fertilize water indirectly due to dissolved input (Hua et al. 2001). Moreover, culture of zooplanktivorous and herbivorous fish can promote algal growth by food chain effects, and promote more productive water via remaining fish food. Therefore, a new protocol of pond water management needs to be emphasized in further use of this pond.

Glycogen levels differed by sex, with males possessing higher glycogen content than females. Furthermore, glycogen in *P. alatus* exhibited seasonal variation; high in later summer and low thereafter. A more detailed understanding of the energy reserves of this species may be advantageous to their long-term holding in captivity. I recommend further study of glycogen dynamics using the protocol described but increasing frequency of samples.

High water temperatures are stressful and can be a lethal factor to survival of mussels, and DO less than 2 mg/L can cause the mortality of pink heelsplitter. Low DO at the deep end of the pond likely contributed to high mortality there. Hydrogen sulfide was documented indirectly at the deep end of pond, and may be another factor contributing to high mortality at the deep end. More measurements of DO and quantitative analysis of H_2S should be conducted in the future. Ultimately, to increase the survival of captive mussels, determination of a baseline of suitable water quality

parameters such as minimum flow and DO, maximum temperature, un-ionized ammonia and H_2S should be established in the future studies.

Table 1-1. Comparison of mean (± 1 SE) survival rates of mussels (excluding replacements, $n = 3$) at the deep, shallow and suspended pocket net locations in the FMCC pond after 1 yr (24 October 2003 to 25 October 2004). *P*-value was determined by ANOVA test, with $\alpha = 0.05$ significant level. Means followed by the same letter are not significantly different at $\alpha = 0.05$ level using Tukey's HSD.

Treatment	$\overline{X} \pm$ SE (%)
Deep	30.0 ± 0.0^{a}
Shallow	83.3 ± 3.3^{b}
Suspended	63.3 ± 8.8^{b}

Table 1-2. Mean survival rates (± 1 SE) of mussels held in three pond locations in the FMCC pond from 24 October 2003 to 25 October 2004. Survival rates were determined monthly as the percentage of live mussels with replacement. *P*-values were determined by ANOVA test, if data were normally distributed, and they were determined by Kruskal - Wallis tests, if data were non-normally distributed, with $\alpha = 0.05$ significant level. Significant difference is represented by * in *p* value column.

Sample	Deep	Shallow	Suspended	
Date	$\overline{X} \pm$ SE ($n = 3$)	$\overline{X} \pm$ SE ($n = 3$)	$\overline{X} \pm$ SE ($n = 3$)	*P* value
10/24	100 ± 0	100 ± 0	100 ± 0.0	1.000
11/20	100 ± 0	100 ± 0	96.7 ± 3.3	0.368
12/19	100 ± 0	100 ± 0	100 ± 0.0	1.000
01/16	100 ± 0	100 ± 0	100 ± 0.0	1.000
02/20	100 ± 0	100 ± 0	96.7± 3.3	0.368
03/23	100 ± 0	96.7 ± 3.3	96.7 ± 3.3	0.565
04/23	96.7 ± 3.3	100 ± 0	100 ± 0.0	0.368
05/19	82.6 ± 3.8	96.7 ± 3.3	93.3 ± 6.7	0.179
06/23	83.1 ± 8.7	93.3 ± 6.7	92.5 ± 3.8	0.522
07/23	95.2 ± 4.8	88.0 ± 7.2	93.0 ± 3.5	0.644
08/21	63.1 ± 2.0	96.7 ± 3.3	88.0 ± 7.3	0.006 *
09/20	78.3 ± 11.7	90.7 ± 4.9	95.2 ± 4.8	0.350
10/25	100 ± 0	100 ± 0	94.4 ± 5.6	0.368

Table 1-3. Comparison of mean (± 1 SE) survival rates of mussels (with replacements, $n = 3$) at three locations in the FMCC pond during August 2004. *P*-value was determined by ANOVA test, with $\alpha = 0.05$ significant level. Means followed by the same letter are not significantly different at $\alpha = 0.05$ level using Tukey's HSD.

Treatment	$\overline{X} \pm$ SE (%)
Deep	63.1 ± 2.0^{a}
Shallow	96.7 ± 3.3^{b}
Suspended	88.0 ± 7.3^{b}

Table 1-4. Mean (± 1 SE) glycogen content (mg/g dry weight) of *P. alatus* at the deep, shallow and suspended pocket net locations in the FMCC pond from 24 October 2003 to 25 October 2004. *P*-value was determined by ANOVA test, with $\alpha = 0.05$ significant level. Means followed by the same letter are not significantly different at $\alpha = 0.05$ level using LSD.

Treatment	*N*	$\overline{X} \pm$ SE (mg/g)
Deep	3	215.5 ± 4.8^{c}
Shallow	3	458.6 ± 27.0^{a}
Suspended	3	323.1 ± 35.4^{b}
Wild *	2	330.7 ± 5.2^{b}

* Wild = Kentucky Lake

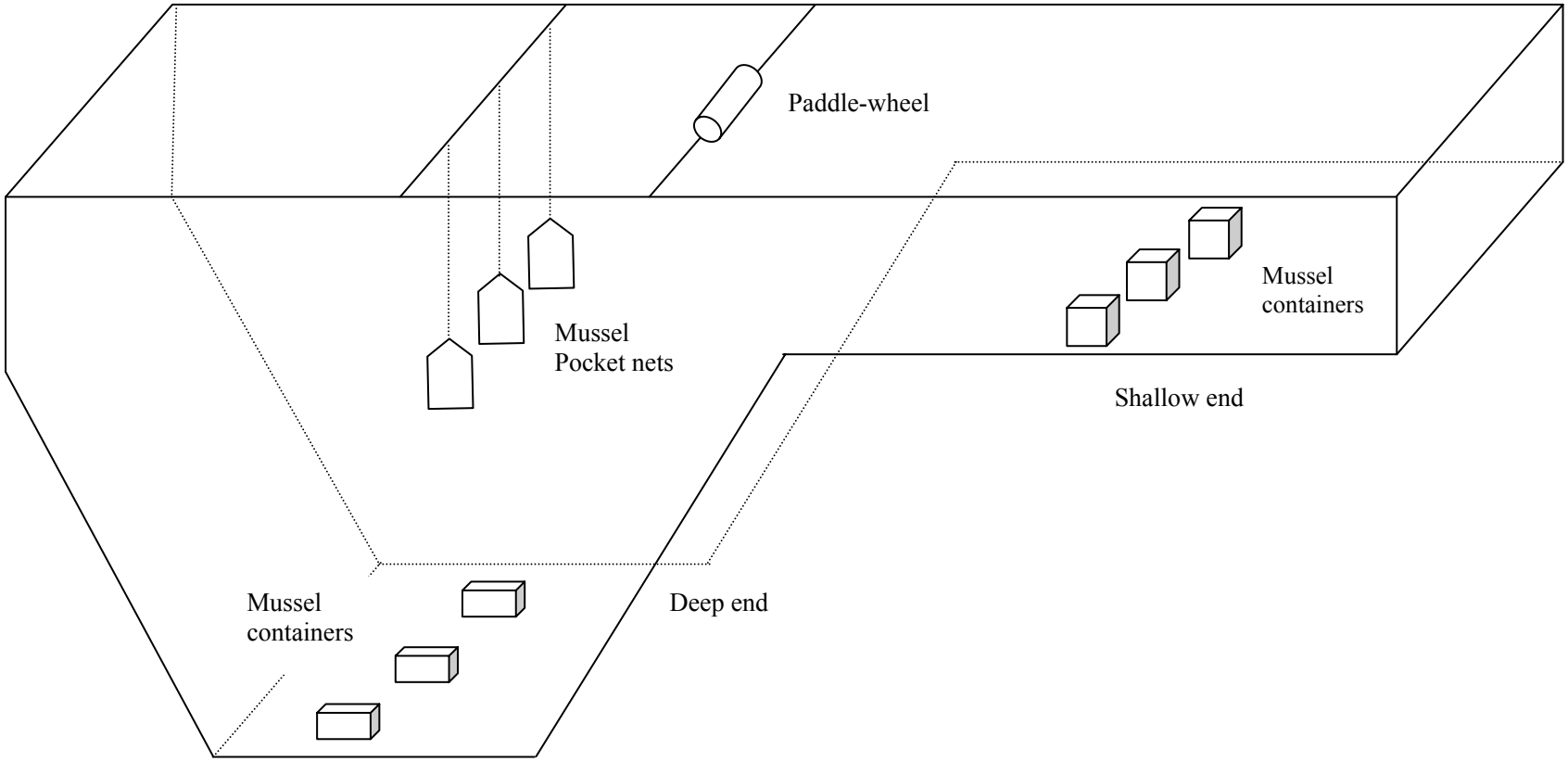


Fig. 1-1. Pond profile showing placement of bottom and hanging culture treatments in a pond at Freshwater Mollusk Conservation Center with three treatments at shallow end, deep end and in suspension, and three replicates per treatment. The pond holds 985 m^3 water in 1000 m^2 of area; deep end is 2.5 m, shallow end is 0.6 m.

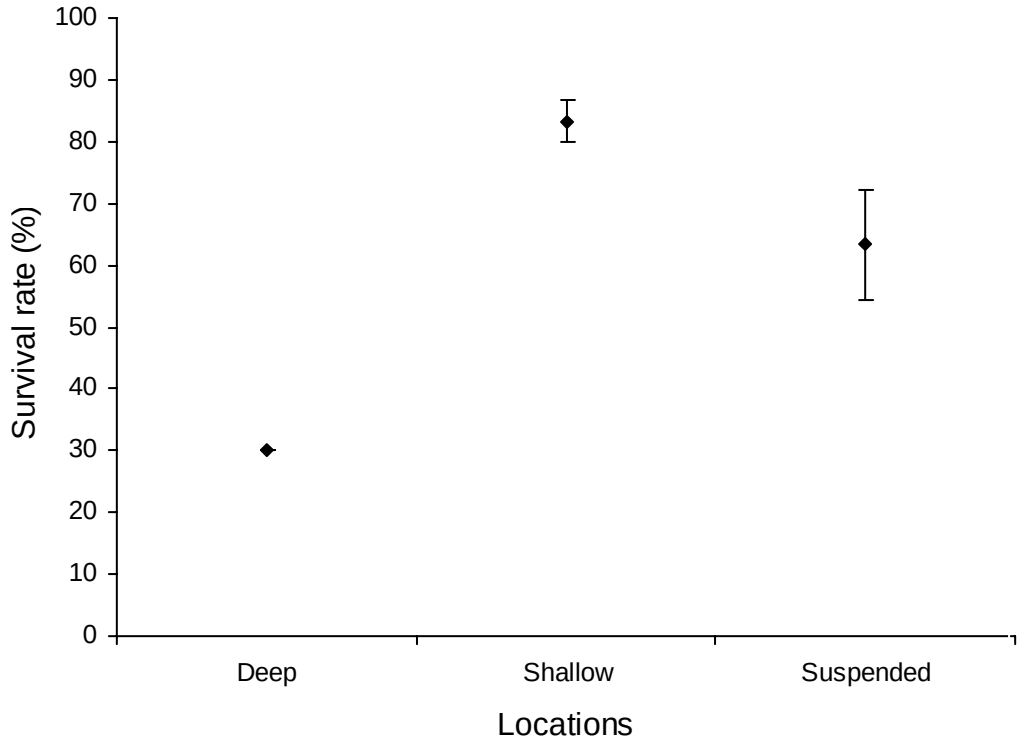


Fig. 1-2. Mean survival rate (± 1 SE) of *P. alatus* after 1 yr in captivity at three locations in the FMCC pond ($p = 0.001$, $n = 3$).

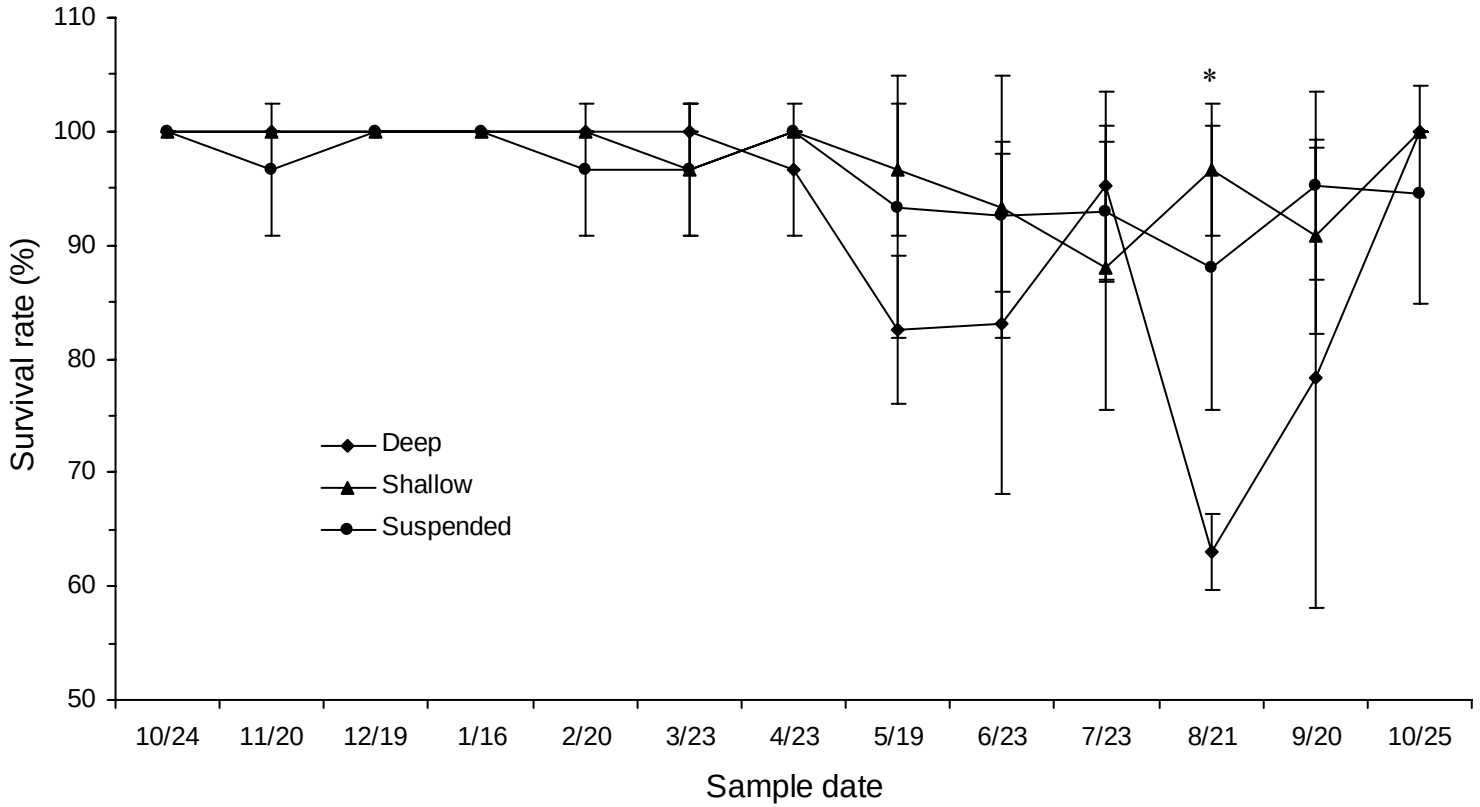


Fig. 1-3. Mean monthly survival rates of *P. alatus* (with replacement) in three holding locations in the pond from 24 October 2003 to 25 October 2004. Significant difference is represented by * among three locations.

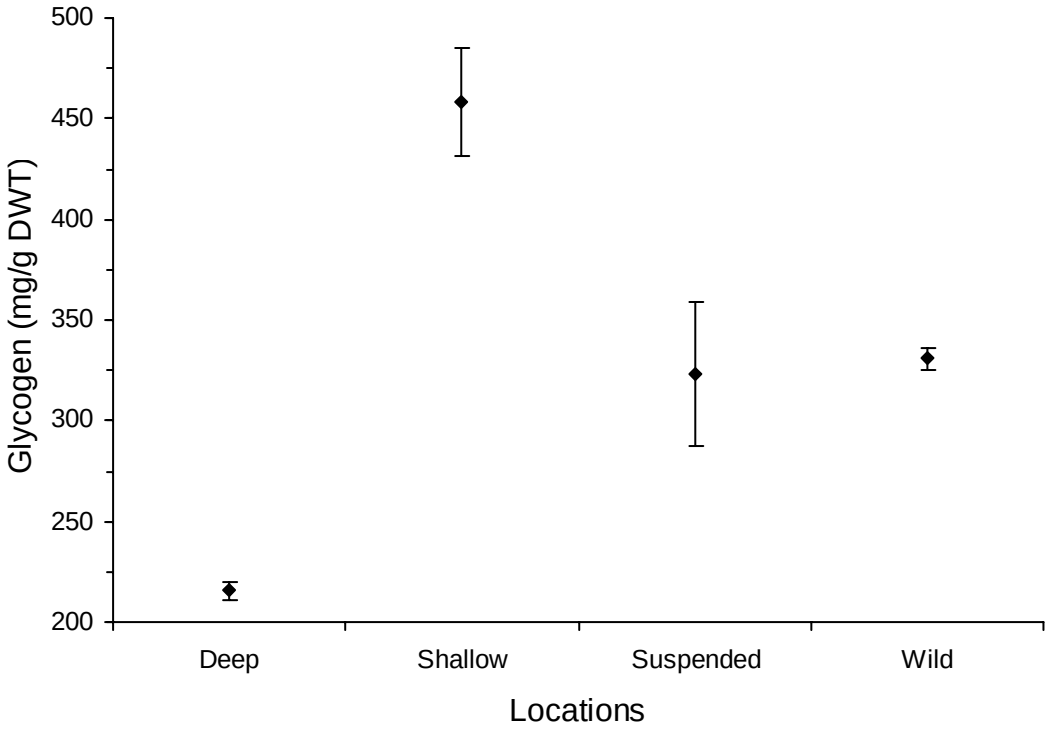


Fig. 1-4. Mean (± 1 SE) glycogen content (mg/g dry weight) of captive *P. alatus* at the deep end, shallow end and suspended pocket nets locations after 1 yr (24 October 2003 to 25 October 2004), compared to wild-caught specimens ($p = 0.001$, $n = 3$).

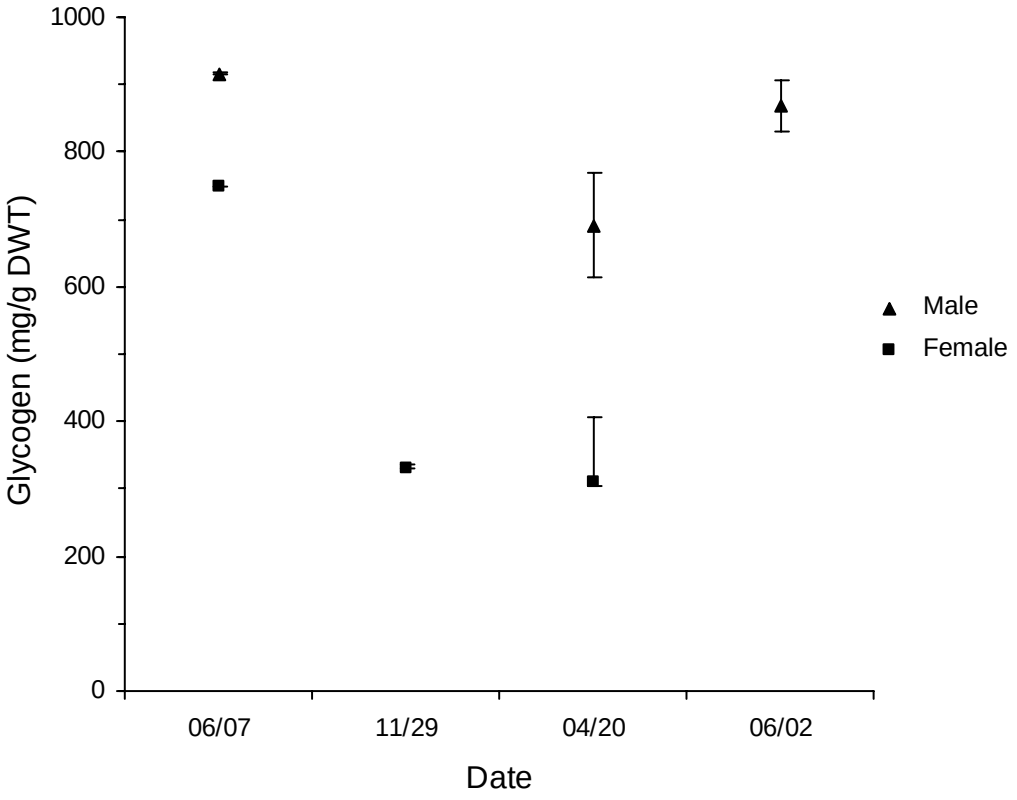


Fig. 1-5. Mean (± 1 SE) glycogen reserves (mg/g DWT) in female and male *P. alatus* collected June 2004 - June 2005 in Kentucky Lake portion of the Tennessee River (TRM 103.0), Humphreys County, TN. ($p = 0.001$, $n = 5, 8$)

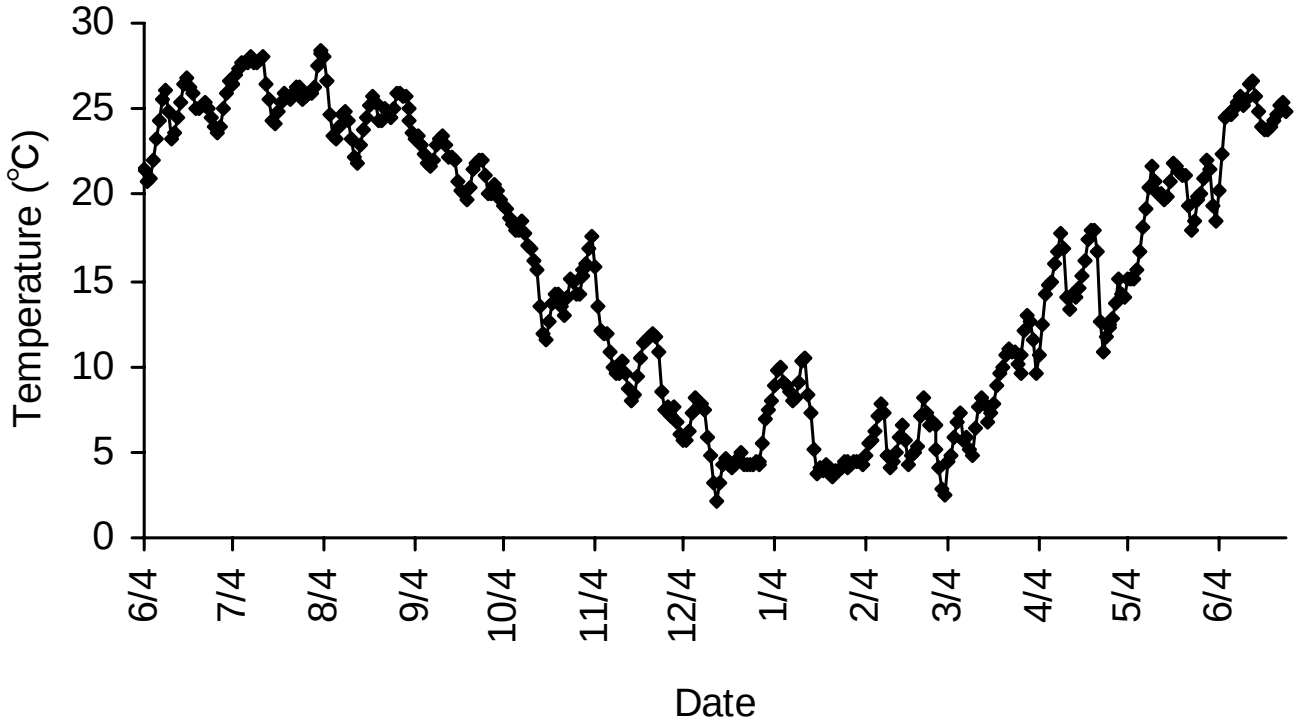


Fig. 1-6A. Water temperature at shallow end of FMCC pond throughout the year.

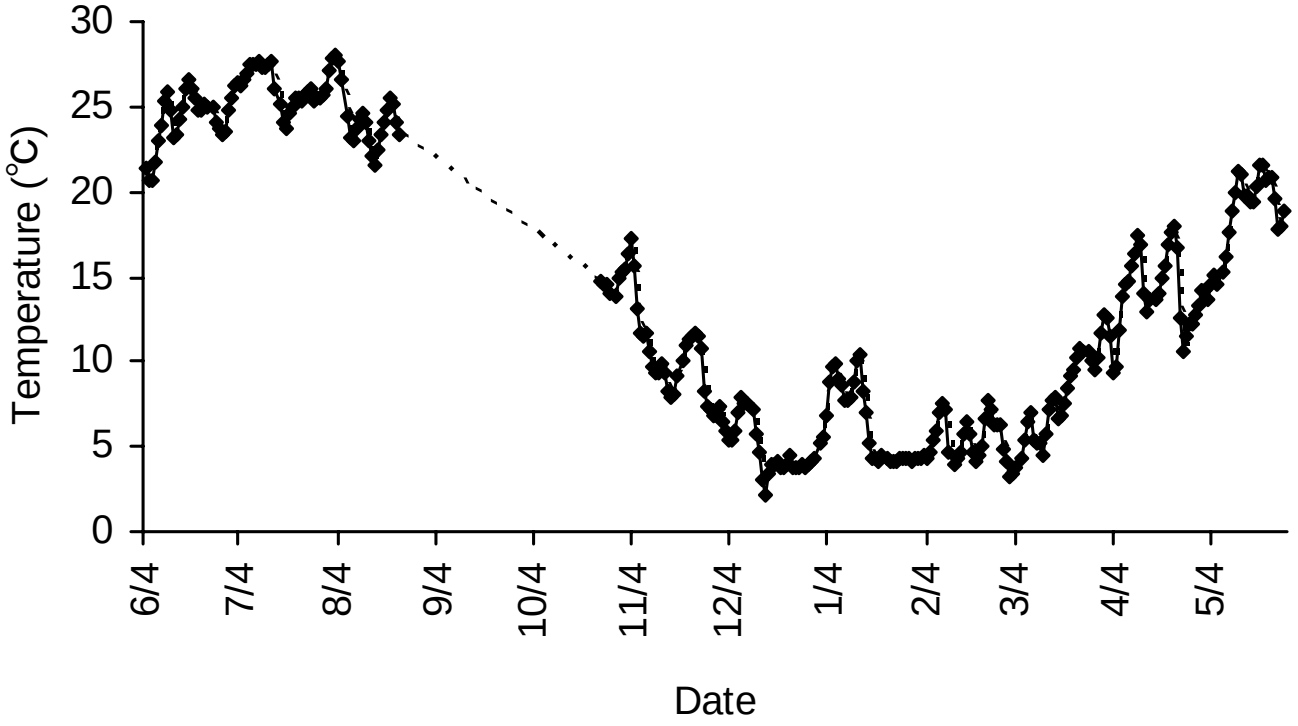


Fig. 1-6B. Water temperature at deep end of FMCC pond throughout the year. Dashed line connects missing data for 24 August to 24 October due to malfunction of temperature logger.

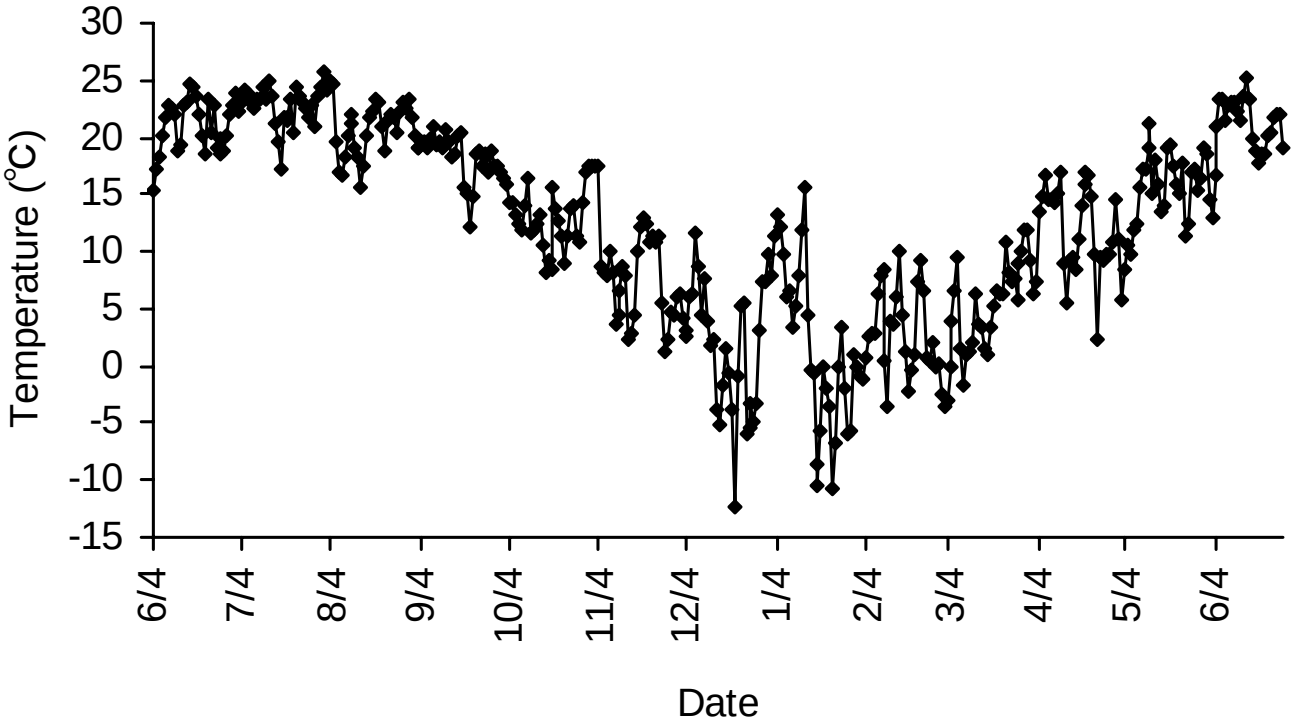


Fig. 1-6C. Air temperature at the FMCC pond throughout the year.

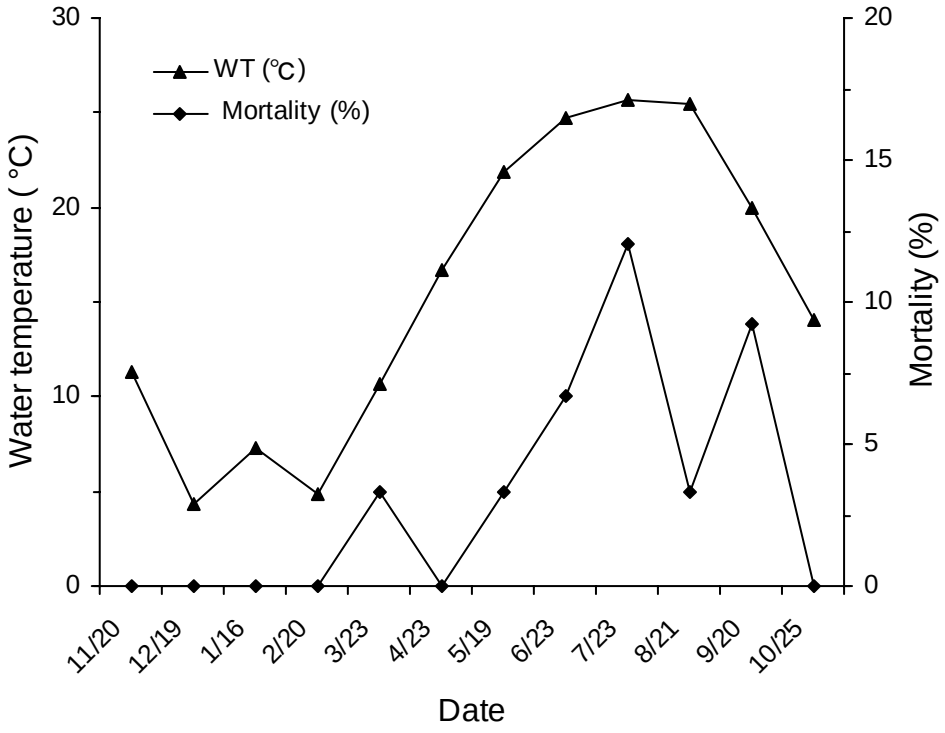


Fig. 1-7A. Monthly mortality of *P. alatus* and water temperatures (WT) at the shallow end of FMCC pond, November 2003 - October 2004 (r = 0.72).

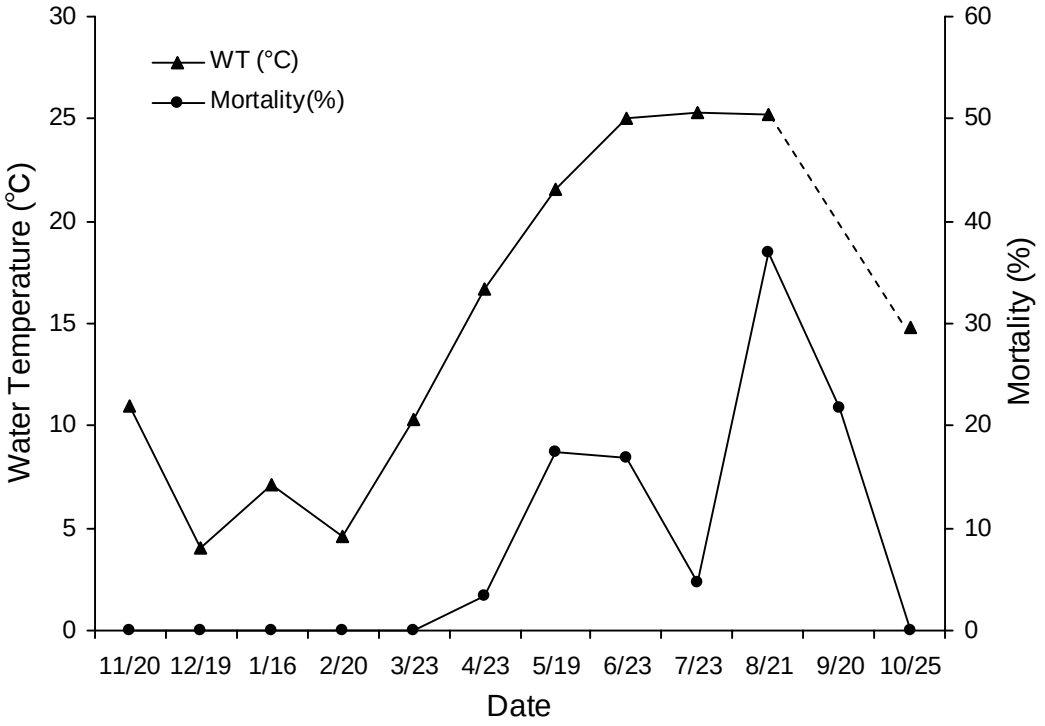


Fig. 1-7B. Monthly mortality of *P. alatus* and water temperatures at the deep end of FMCC pond from November 2003 to October 2004. Data of temperature on 20 September 2004 was lost due to malfunction of temperature logger (r = 0.72).

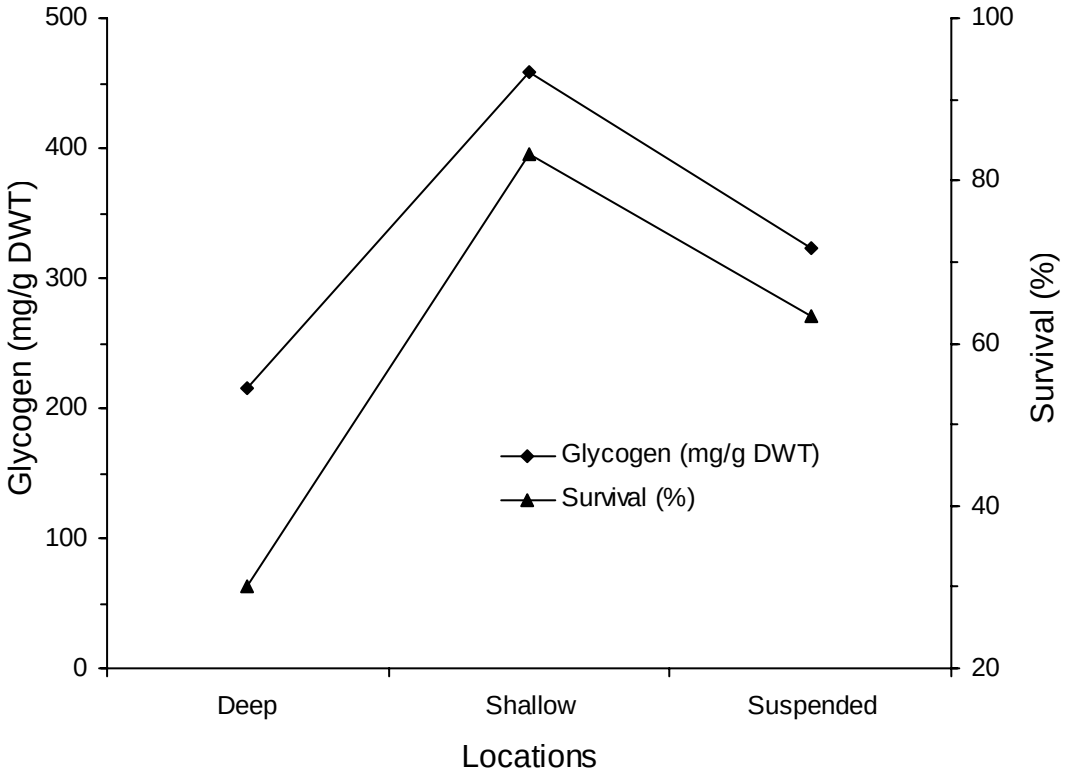


Fig. 1-8. Mean glycogen level and survival rate of *P. alatus* held in the deep end, shallow end and suspended pocket nets in FMCC pond after 1 yr (r = 0.77).

Appendix 1-1. DO levels and water temperature (WT) at the deep end, shallow end and water column (from water surface to the bottom of deep end) in FMCC pond in June 2004.

Water column (m)	WT (℃)	DO (mg/L)
0	25.6	6
0.5	24.9	6.4
0.9	24.8	6
1.3	24.8	5.8
1.5	24.7	6.1
1.7	24.7	5.9
1.9	24.7	6.1
2.1	24.7	5.9
2.3	24.6	6.1
2.5 (Deep end)	24.6	1.9
Shallow end	25.4	5.0

CHAPTER 2: SHELL MORPHOLOGY AND SEXUAL DIMORPHISM IN THE PINK HEELSPLITTER (*POTAMILUS ALATUS*) (SAY, 1817) (BIVALVIA: UNIONIDAE) IN KENTUCKY LAKE

INTRODUCTION

The pink heelsplitter (*P. alatus*) is a large species with two elongate and somewhat rectangular valves. Both valves have a smooth surface and are usually thin in young individuals, becoming thick with age. External color varies from dark green to brown, with light tan to dark green rays in young shells, becoming black without rays in older ones. The umbo is flattened and slightly elevated above the hinge line. The ventral margin is straight with a rounded anterior end (Figure 2-1). A triangular wing extending from the dorsal posterior to the umbo is a key character, which is well-developed and complete in young individuals, tending to break and frazzle with age (Cummings and Mayer 1992). The wing structure becomes more brittle when dried. Except for general external morphology, traits of sexual dimorphism in *P. alatus* have not yet been determined quantitatively.

Shell morphology and growth of freshwater bivalves have been widely studied in relation to local habitat characteristics (Ansell et al. 1978, Lutz and Jablonski 1978, Kautsky et al. 1990, Norberg and Tedengren 1995, Ryan et al. 1999). However, sexual dimorphism has been reported only in some species of freshwater mussels, in genera such as *Lampsilis*, *Truncilla*, *Unio*, *Astarte*, *Castalia, Epioblasma* and *Villosa* (Ortman 1921, Coe 1943, Avelar et al. 1991, West and Metcalfe-Smith 2000). Kirtland (1834) first reported sexual dimorphism in the Unionidae. He observed that females of 8 species of the Lampsilini and 1 species of Pleurobemini (*Plethobasus cyphyus*) had more truncated

posterior ends and inflated ventro-posterior regions than did males. Consequently, he speculated that sexual dimorphism might occur in all unionaceans, since female mussels have marsupial demibranchs for glochidial development. However, later studies reported external sexual dimorphism only in the tribes Lampsilini and Amblemini (Burch 1975, Davis and Fuller 1981). Sastry (1979) agreed that sexual dimorphism in bivalves was uncommon since the reproductive habits of bivalves were not specialized.

Degree of sexual dimorphism in bivalves varies by species. The relative dimensions of shell length, height, and width (obesity, inflation) were reported to describe sexual dimorphism in some European species of bivalves since the early 1880s (Hazy 1881, Israel 1910). Later, Grier (1920a) measured 7 dimensions (relative degree of inflation, height, posterior length of shell, anterior length of shell, length of posterior hinge line, length of anterior hinge line, and thickness) of 12 mussel species to compare with the earlier studies of Utterback (1916) and Walker (1918) to verify sexual dimorphism in bivalves. He found inconsistent sexual dimorphism in some species, which disagreed with Utterback (1915) and Walker (1918). Ball (1922) confirmed Grier's conclusion that morphology of shells differed between females and males, but found that it varied among species in particular measurements, such as obesity and ratio of relative height to length. However, none of these species were statistically analyzed to determine whether there were significant differences among the measurements. Later studies on sexual dimorphism within species were more rigorous and used statistical analysis. Kotrla and James (1987) indicated that adults of *Villosa villosa* exhibited sexual dimorphism in shape, but not in size dimorphism. However, Blay (1989) studied a Nigerian freshwater bivalve *Aspatharia sinuate* (Unionacea: Mutelidae) and did not find sexual dimorphism

at shell morphology.

Shell shape differences in freshwater mussels have implications for propagation, as females of some species can be more readily selected at sites of collection. For example, females in the genus *Lampsilis* have obviously inflated posterior valves, which provide a region for enlarged and expanded gills for glochidia (Burch 1975), although sexual dimorphism in most species is subtle. Those subtle differences were often ignored. Parmalee and Bogan (1998) noted that sexual dimorphism in *P. alatus* is slight and inconsistent, although they generalized that the valve of female *P. alatus* is slightly more inflated and broadly rounded at the posterior ventral margin. The purpose of this study was to test this generalization by analyzing shell measurement data for morphological dimensions of *P. alatus* with adequate samples of males and females.

METHODS AND MATERIALS

Collection and sites

Mussels were hand-collected from Kentucky Lake, the Tennessee River (TRM 103.0), Humphreys County, TN, and quarantined for 1 mo. The specimens were collected from the same site to minimize ecophenotypic variation for assessment of sexual dimorphism.

Sex determination

The sex of the mussel was determined from the gill marsupium based on breeding condition. Gravid females only fill and expand the outer gills with glochidia, while they remain flat in males during the period of gravidity. However, gills of female mussels are also flat when they are not gravid. Therefore, gonads of mussels were sampled by biopsy,

squashing them on a microscope slide and examining them under a microscope to verify sex based on detection of ova or sperm.

Morphological measurements

Sex-validated mussels were those selected for dimension measurements. Forty sexually mature males and 40 females were examined to compare shell characteristics. Three general dimensions of length, height and width were measured to the nearest 0.01mm with a digital vernier caliper. Length (L) is the maximum distance between anterior and posterior ends of the shell, height (H) is the maximum distance between umbo and the ventral margin of the shell perpendicular to the shell length, and width (W) is the maximum transverse distance perpendicular to the length and height metrics (Ball 1922, Eagar et al. 1984) (Figure 2-3). As judged by previous studies, I focused on these dimensions that affected shell shape.

Data analysis

The ratios of height to length and width to length were used to compare morphological differences among female and male mussels since these parameters were previously used to describe shape variation in other species (Ball 1922, Eagar et al. 1984, Blay 1989). Data were analyzed using JMP software (Sall et al. 2001, Version 4.0). An analysis of variance was used to test for morphological differences in valves between sexes, and two-way ANOVA was used to compare differences in relative dimensions and sex. A significance level of $\alpha = 0.05$ was used in all statistical testing.

RESULTS

Morphological observations

Sizes of sampled female mussels ranged from 74.6 to 129.1 mm (n = 40) in length; male mussels ranged from 85.2 to 150.2 mm (n = 40) (Table 2-1). Sexual dimorphism was typically evident at the posterior end of valves. The posterior end was somewhat round to oval in male mussels, but bluntly squared or truncated in female mussels. Anterior ends of both sexes are smoothly rounded. Female mussels are more obese than males (Figure 2-2).

Statistical comparison

Descriptive statistics of shell dimensions in male and female mussels are presented in Table 2-1. Mean ratio (percentage) of height to length (H/L) and width to length (W/L) in female valves (H/L: 52.3 ± 2.8; W/L: 31.8 ± 2.3, n = 40) are significantly greater than those in males (H/L: 48.4 ± 2.8; W/L: 28.8 ± 1.6, n = 40) ($p < 0.0001$), as shown in Figure 2-4. I attempted to determine which dimension, H/L or W/L, is the best metric for differentiating sex. Analyses indicated no significant difference among the interactions of sex and type of ratios ($p = 0.270$), although there are highly significant differences between sex ($p < 0.0001$) and various ratios ($p < 0.0001$). Therefore, both dimensions, H/L and W/L, are necessary for differentiation of sexes in *P. alatus*. These data indicate that sexual dimorphism can be discerned by shell shape.

DISCUSSION

Gravid female mussels obviously can be identified by gill inflation with glochidia, but sex can be difficult to distinguish when females are not gravid. Recognition of

females based on external shell morphology then becomes important. In this study, females were found to be more inflated than males and have a bluntly squared or truncated posterior end versus the sharply round posterior end in males (Figure 2-2). The difference was quantified by statistical analysis of shell dimensions (length, height, and width), showing that female and male *P. alatus* differed significantly in height to length and width to length rations (Table 2-1, Figure 2-4).

Variation of shell morphology seems to be ecophenotypic. Previous studies indicated that shell morphology changes with ecological conditions such as water velocity, substrate, temperature, food, oxygen, and other factors (Isely 1911, Coker 1914, Grier 1920b). Ortmann (1920) noted that shells of the same species vary in shape depending on location in stream, resulting in more obese mussels inhabiting downstream reaches. Ball (1922) concurred that shells of the same species were more swollen in larger rivers than those found in small streams. Watters (1994b) found that streamlined mussels readily burrowed in more variable substrates due to their advantageous shapes with umbos, deeper sulcus, and an obese, posteriorly truncated shell. The morphological differences in sexes of *P. alatus* were readily visible based on specimens in this study, both from external observations (Figure 2-2) and from comparison of dimensions (Table 2-1); differences in relative shell length, height, and width were highly significant between females and males. These results did not agree with Parmalee and Bogan's (1998) statement that "sexual dimorphism is slight and not always obvious" in *P. alatus*. Genetically-based differences may be modified by environmental factors such as temperature, photoperiod, and food source, resulting in dissimilarity in shape and size of mussels (Lutz and Jablonski 1978, Seed 1980, Blay 1989, Seed and Suchanek 1992).

However, environmental factors alone did not obscure sexual dimorphism in *P. alatus* in this study, since all specimens were collected from the same site.

In addition to environmental effects, morphological variations in mussels also may be caused by genetic factors caused by natural selection. Seed (1980) stated that "perhaps one of the most striking features concerning the evolution of such a diverse group as the bivalves has been the repeated appearance of a comparatively restricted number of very successful shell morphologies." Ortmann (1920) mentioned that the vagility of host fish would affect mussel populations. A host fish with limited vagility would result in limited gene flow and reduction in diversity of demes. Graf (1997) suggested that host changes might influence genetic diversity within a mussel population. Therefore, the morphological differences by sex seem related to mussel life cycle and reproductive strategies, because fecundity and egg development are experienced only in female mussels. Most relevant to my results, Mills and Cote (2003) favored the explanation that morphological differences in sex may be related to reproductive characteristics. Female mussels modify shape to meet the requirements of high fecundity and egg development, resulting in inflated valves. However, male mussels likely do not need to reshape shells for gamete production. This may explain why female mussels of *P. alatus* have more obese valves than males, due to the requirement of greater volume to accommodate gravid marsupial demibranchs. Entire outer demibranchs in female mussels were observed full of glochidia, indicating that all of the water tubes in the outer demibranchs are marsupial. These full demibranchs readily differentiate females from male mussels, as opposed to species with few water tubes in the demibranchs carrying glochidia (Heard and Guckert 1970, Kotrla and James 1987). The difference in obesity among sexes was

evident statistically; females have a greater ratio of relative width to length than males (Figure 2-4). Advantages of unionacean shell morphology were described by Watters (1994b); an obese, posteriorally truncated shell helps the mussel to settle itself into the substrate. Morphological differences in female and male mussels might also be caused by the preference of location in natural reaches to increase fertilization rate (Mills and Cote 2003), resulting in selection of shapes. In this study, mature female mussels have a greater mean ratio of relative height to length than mature males (Figure 2-4). It appears that a wider shape in females may help stabilize themselves as they bury in soft substrates.

CONCLUSIONS AND COMMENTS

Specimens of *P. alatus* were collected from the same locality in the Tennessee River, TN (TRM 103.0), and shell dimensions exhibited sexual dimorphism. Female mussels had a bluntly squared or truncated posterior margin from the base, and a relative obese and broad shell shape. Male mussels had a somewhat round to oval posterior end with somewhat compressed valves. There were highly significant differences in the ratio of relative height to length and width to length between the sexes. Female mussels have a greater height and width compared to length. H/L and W/L are both important factors for the differentiation of separating males from females.

Table 2-1. The mean length (L) (± SE), height (H) (± SE), and width (W) (± SE), mean ratio of height to length (H/L) (± SE), mean ratios of width to length (W/L) (± SE), and range of dimensions in female and male valves of *P. alatus.* Significant difference is represented by * (n = 40).

Sex	L (mm)	H (mm)	W (mm)	H/L (%) *	W/L (%) *
	$\overline{X}$ ± SE	$\overline{X}$ ± SE	$\overline{X}$ ± SE	$\overline{X}$ ± SE	$\overline{X}$ ± SE
	(Min - Max)	(Min - Max)	(Min - Max)	(Min - Max)	(Min - Max)
Female	105.1 ± 1.9	55.0 ± 1.2	33.5 ± 0.8	52.3 ± 0.4	31.8 ± 0.4
	(74.6 - 129.1)	(39.2 - 73.7)	(22.9 - 44.2)	(47.0 - 60.0)	(28.2 - 40.1)
Male	126.4 ± 2.3	61.0 ± 1.1	36.3 ± 0.7	48.4 ± 0.4	28.8 ± 0.3
	(85.2 - 150.2)	(44.0 - 76.7)	(22.8 - 42.8)	(42.7 - 55.9)	(25.6 - 33.4)

Fig. 2-1. External morphology of *Potamilus alatus* in young specimen (top) and old specimen (bottom).

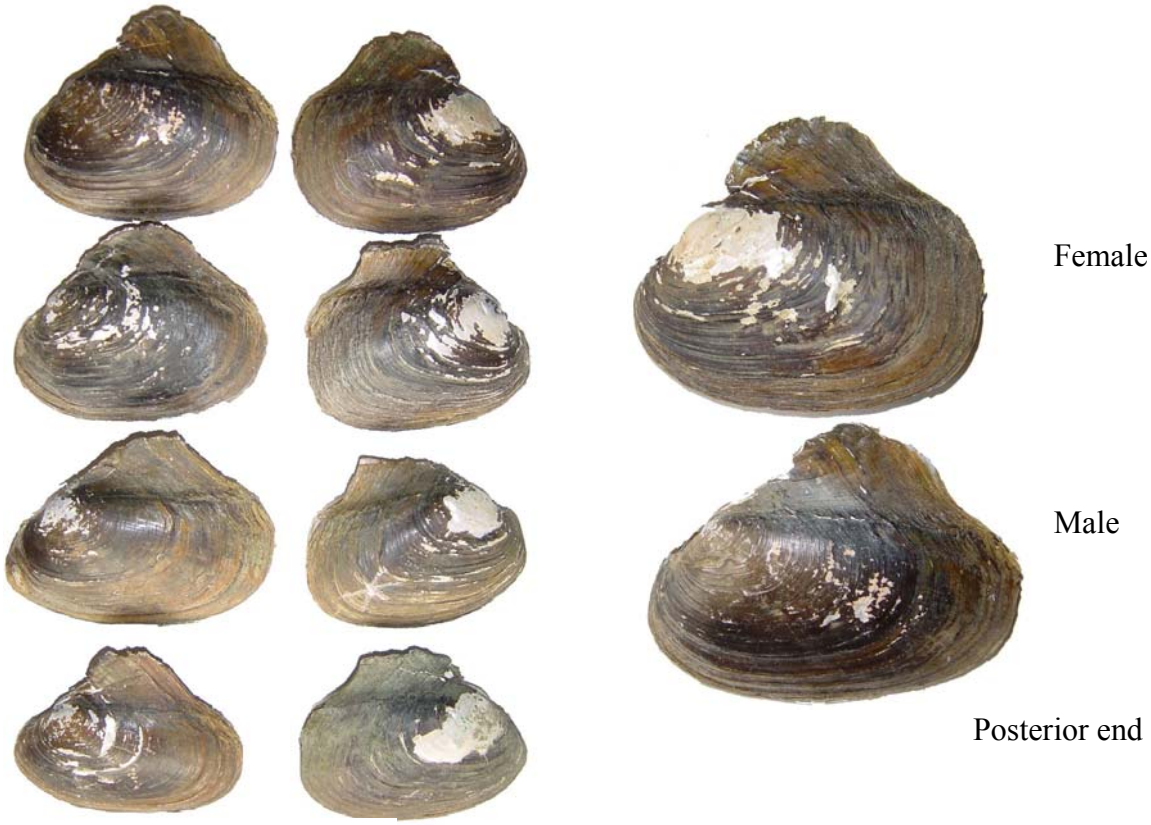


Fig. 2-2. Morphological differences in adult female and male *P. alatus.* Sizes of mussels ranged from74.6 mm to 150.2 mm.

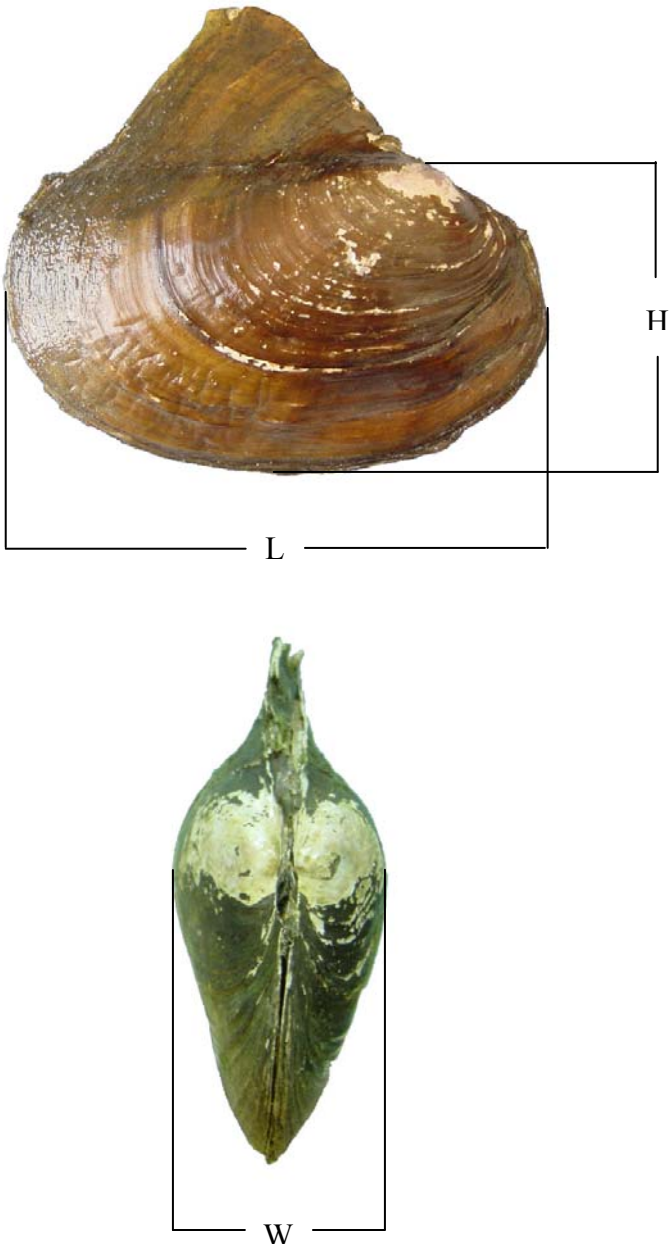


Fig. 2-3. External measurements of *P. alatus*. Length (L) is the maximum distance between anterior and posterior ends of the shell, height (H) is the maximum distance between umbo and the ventral margin of the shell perpendicular to the shell length, and width (W) is the maximum transverse distance perpendicular to the length and height metrics.

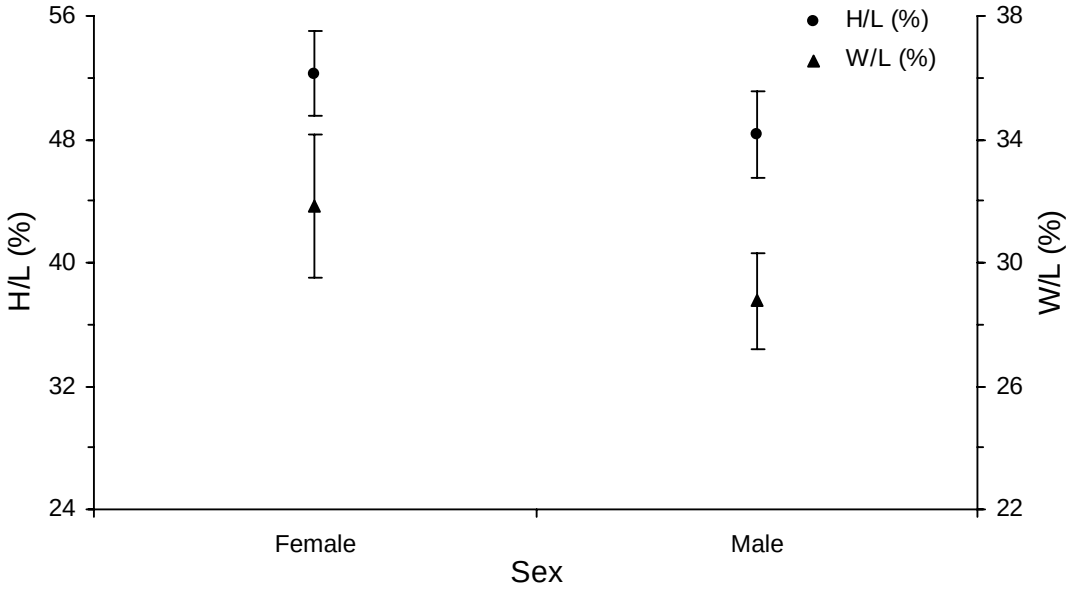


Fig. 2-4. Comparison of mean ratios (± 1SE) of height to length (H/L) and width to length (W/L) in valves of female and male *P. alatus* ($p < 0.0001$, $n = 40$).

Appendix 2-1. Measurements of length (L), height (H) and width (W), and ratios of height to length (H/L) and width to length (W/L) and of female and male valves of *P. alatus.*

No.	Male					No.	Female				
	L (mm)	H(mm)	W (mm)	H/L (%)	W/L (%)		L (mm)	H (mm)	W(mm)	H/L (%)	W/L (%)
71	140.0	70.4	39.4	50.3	28.2	A109	98.2	50.6	27.7	54.4	28.2
A017	139.3	68.7	35.7	49.3	25.6	90	117.3	63.8	35.9	52.0	30.6
23	136.5	62.5	38.1	45.8	27.9	*	118.8	61.8	35.4	57.0	29.8
*	141.4	72.4	40.2	51.2	28.4	59	102.0	58.2	30.7	54.0	30.1
L288	130.5	61.4	39.9	47.1	30.6	A011	110.6	59.8	32.9	57.1	29.8
93	121.6	64.6	37.1	53.1	30.5	*	129.1	73.7	42.5	53.9	32.9
*	150.2	68.2	42.4	45.4	28.2	*	117.3	63.2	37.9	47.6	32.3
99	131.6	68.1	38.5	51.8	29.2	*	125.0	59.5	37.8	59.8	30.3
A097	133.5	65.3	35.7	48.9	26.8	*	121.3	72.5	44.2	52.4	36.4
*	137.2	76.7	42.3	55.9	30.8	*	113.1	59.2	35.6	48.9	31.4
*	140.6	66.9	42.8	47.6	30.4	L283	107.9	52.8	31.7	55.2	29.4
*	131.7	61.2	37.1	46.5	28.1	L299	100.9	55.7	38.0	53.0	37.7
A018	119.5	57.5	35.9	48.1	30.0	L296	126.3	67.0	42.8	51.8	33.9
L294	135.4	62.3	39.5	46.0	29.1	*	106.0	55.0	30.9	47.0	29.1
*	123.9	64.4	41.4	52.0	33.4	A098	114.7	53.9	35.7	53.6	31.1

Appendix 2-1 (continued). Measurements of length (L), height (H) and width (W), and ratios of height to length (H/L) and width to length (W/L) and of female and male valves of *P. alatus*.

No.	Male					No.	Female				
	L (mm)	H(mm)	W (mm)	H/L (%)	W/L (%)		L (mm)	H (mm)	W(mm)	H/L (%)	W/L (%)
A051	148.4	68.5	40.7	46.2	27.4	*	108.8	58.3	36.3	53.5	33.4
*	139.9	64.9	39.7	46.4	28.4	*	109.7	58.7	44.0	52.6	40.1
A050	113.1	54.2	32.5	48.0	28.7	A005	108.6	57.2	35.8	47.0	33.0
A020	106.9	55.0	32.3	51.5	30.2	*	103.9	48.8	30.9	48.9	29.7
*	108.9	52.0	32.4	47.8	29.8	98	100.1	48.9	30.9	50.6	30.9
*	127.7	63.1	37.3	49.4	29.2	A000	97.0	49.1	32.4	51.1	33.4
A010	101.4	48.2	29.4	47.5	29.0	A003	98.7	50.5	30.4	52.6	30.7
N2	129.5	58.9	36.8	45.5	28.4	A166	106.8	56.2	32.9	51.2	30.8
*	134.4	62.1	35.6	46.2	26.5	*	104.0	53.3	35.0	51.6	33.7
A100	128.2	55.5	36.5	43.3	28.4	*	87.4	45.1	25.7	49.8	29.4
*	98.3	51.5	28.9	52.3	29.4	*	113.5	56.6	36.4	49.1	32.1
112	121.6	58.1	33.7	47.8	27.8	*	101.6	49.9	31.8	53.4	31.3
*	85.2	44.0	22.8	51.7	26.8	*	108.4	57.8	33.9	51.9	31.3
A107	139.5	64.8	39.8	46.4	28.5	A008	112.8	58.5	34.4	53.5	30.5
*	138.1	63.5	37.1	45.9	26.9	*	74.6	39.9	25.1	52.2	33.7

Appendix 2-1 (continued). Measurements of length (L), height (H) and width (W), and ratios of height to length (H/L) and width to length (W/L) and of female and male valves of *P. alatus.*

No.	Male					No.	Female				
	L (mm)	H(mm)	W (mm)	H/L (%)	W/L (%)		L (mm)	H (mm)	W(mm)	H/L (%)	W/L (%)
*	125.9	57.8	35.5	45.9	28.2	61	102.0	53.3	32.7	52.3	32.0
*	112.9	57.1	34.1	50.6	30.2	*	109.3	57.2	34.9	55.7	31.9
*	122.9	62.5	37.2	50.8	30.3	A009	101.6	56.6	32.2	50.5	31.7
*	138.1	69.2	37.6	50.1	27.2	*	96.2	48.6	30.0	54.3	31.2
A040	113.0	54.0	30.7	47.8	27.2	*	104.1	56.5	32.7	56.2	31.5
*	132.7	62.9	41.4	47.4	31.2	*	94.8	53.3	31.3	51.8	33.0
*	104.0	52.0	28.3	50.0	27.2	*	92.5	47.9	29.0	49.4	31.3
A026	128.0	54.6	39.3	42.7	30.7	A006	100.9	49.9	31.5	51.9	31.2
A029	135.7	66.2	36.5	48.8	26.9	*	80.5	41.7	25.9	50.7	32.1
*	110.7	50.1	31.2	45.3	28.2	A004	77.4	39.2	22.9	50.7	29.6

Note: Male mussels with * symbols were sampled for germ cell examination.
Female mussels with * symbols were used for propagation.
Others with numbers were used for studies of captive mussels in ponds and pearl production.

CHAPTER 3: SUITABILITY OF ALTERNATIVE HOST FISH SPECIES FOR TRANSFORMING GLOCHIDIA OF THE PINK HEELSPLITTER (*POTAMILUS ALATUS*) (SAY, 1817) (BIVALVIA: UNIONIDAE)

INTRODUCTION

Freshwater mussel species vary in host specificity, with some species being host generalists and while others are host specialists. Glochidia of host generalists are able to parasitize a wide variety of fish species, while host specialists are only able to parasitize specific host species (Zale and Neves 1982, Haag and Warren 1997, Layzer et al. 2003). The pink heelsplitter is a long-term brooder (Ortmann 1919). In Wisconsin, Baker (1928) reported that females brood glochidia from August to the following June, retaining them over winter for release the following June or July. Holland-Bartels (1989) reported that *P. alatus* releases its glochidia from late May to early July in Navigation Pool 7, near river mile 709 of the upper Mississippi River. However, Haggerty and Garner (2000) observed *P. alatus* releasing glochidia from April or early May until September in Alabama. Based on these studies, it seems that glochidial release varies with latitude. Released glochidia are obligate parasites that must encyst on a suitable host fish to metamorphose to juveniles. Glochidia that attach to incompatible species will drop from fish within a few days after attachment and die. Incompatibility is considered to be immunological, but the mechanisms involved are unknown (Meyers and Millemann 1977; Young and Williams 1984; O'Connell and Neves 1999).

The freshwater drum *(Aplodinotus grunniens)* is the only reported host fish for the pink heelsplitter (Fuller 1974, Watters 1994a); however, other host species could be present within the wide range of *P. alatus.*

Freshwater drum belong to Sciaenidae, a large family of about 270 species with most living in marine or brackish water (Chao 1986). Freshwater drum inhabit freshwater rivers and lakes throughout North America. “Except for color, freshwater drum resembles its marine relative the red drum (*S. ocellatus)*. The fish is deep-bodied and equipped with a long dorsal fin divided into two sections. The dorsal fin usually has 10 spines and 29 - 32 rays.” (Texas Parks and Wildlife 2005). Red drum is a euryhaline species, distributed in coastal waters of the Gulf of Mexico and from Florida to Massachusetts on the Atlantic Coast (Davis 1990), which can survive in fresh water. It was reported that some fish species of similar phylogeny (i. e., same genus or family) could be natural hosts for a mussel species (Neves et al. 1985). Therefore, I tested *S. ocellatus* as an alternative host fish for *P. alatus* because of its ability to live in freshwater and its relatedness to the only known host, *A. grunniens*. Other potential host fishes tested included black crappie (*Pomoxis nigromaculatus*), yellow perch (*Perca flavescens*), banded sculpin (*Cottus carolinae*), and Nile tilapia (*Oreochromis nilotica*) although this latter species does not co-occur with the pink heelsplitter.

This study attempted to identify additional host fish species for the pink heelsplitter using induced infestations in the laboratory. Suitability of hosts was determined by artificially infesting designated fish species with glochidia. Fish were considered to be suitable hosts if parasitized glochidia successfully metamorphosed to the juvenile stage. Morphology of glochidia and transformed juveniles also were observed.

METHODS AND MATERIALS

My study was conducted using recirculating water systems at the FMCC propagation laboratory, which have been used for holding and conditioning adult mussels for spawning, and for juvenile rearing (Henley et al. 2001). Living Stream (380 L) recirculating water systems, and other round-tanks (1000 L), aquaria (200 L, 150 L and 75 L) associated with water recirculating systems, biomedia, and chillers were used for holding infested fish.

Fish preparation

Adult freshwater drum were collected using electrofishing techniques from the French Broad River, TN by biologists with the Tennessee Valley Authority on 29 April and 21 May 2004. A total of 33 freshwater drum (mostly 18 - 30 cm), 3 black crappie and one yellow perch was collected. They were held in a 230 L cooler filled with cool water and transported to the laboratory. We used electrofishing techniques on 2 November, 2004 to harvest 21 *A. grunniens* (about 20 - 30 cm) from the Caney Fork River, Dekalb, TN, approximately 0.8 km downstream of the Center Hill Dam in a backwater area. Fish were held in cool, salt-treated water (0.05 %) with aeration during transport to the laboratory.

According to Johnson and Metcalf (1982), wild-caught freshwater drum have high mortality rates during transport into captivity. Therefore, hatchery-raised freshwater drum also were used in my study; 50 small freshwater drum (7.5 - 8.5 cm in total length) were received from the Langston University Aquaculture Research and Demonstration Facility, Logan County, OK on 3 November 2004. Fish had been raised in a 0.2-ha pond before delivery. To increase the survival probability, fish were starved for 1 d prior to

shipment to reduce ammonia problems in the water. Drum were shipped in a sealed plastic package, and two packages were used for the shipment.

Red drum were provided by Sea Center Texas, a restocking facility in Freeport, TX. operated by the Texas Parks and Wildlife Department in conjunction with Dow Chemical Corporation. These fish were tank-spawned from captive broodstock using photothermal manipulation. Larvae were pond-reared in brackish water ponds and transitioned to dry commercial feed at approximately 40 d post-hatch. These fish were held in recirculating systems at the Virginia Tech Aquaculture Center for approximately 4 mo, maintained on Meilck Aquafeed (45 % crude protein, and 12 % total lipid), fed to satiation twice daily.

Nile tilapia were provided by Virginia Tech Aquaculture Center. banded sculpin were provided by the FMCC laboratory.

Potential host fish were held in recirculating aquaculture systems (RAS). Crayfish and night crawlers were fed to the adult host fish. Small freshwater drum were fed black worms and chopped night crawlers. Red drum and tilapia were fed pelleted fedd. Fish were fed once a day during the parasitic period and every other day while juveniles were dropping from the hosts. For small drum shipped from Oklahoma, fish were placed in a holding system to reduce delayed mortality, and food was supplied immediately upon arrival. Collected fish were quarantined in a 0.5 % salt treatment and fed a quality diet for 3 - 19 d prior to infestation to verify that they were disease- and glochidia-free prior to infestation.

Host fish tests

Fish hosts were tested using a standard protocol described by Neves et al. (1985) and

Weaver et al. (1991). Glochidia were removed from the gill marsupia of female mussels by the injection of water with a syringe and hypodermic needle. To determine the viability of glochidia, samples were tested with saline solution at 0.1 - 1 % concentration; valves of matured glochidia shut immediately. Glochidia were used for host fish tests if at least 70 % closed their valves. Fish and numerous mature glochidia were placed in 1 - 2 L of water under aeration for infestation. Host fish were transferred to an aquarium when 300 - 1000 glochidia had attached to the host, determined by inspection of gills.

Tanks were siphoned every other day, and the water was run through 125 and 800 micron sieves to collect juveniles that had ex-cysted from hosts. Siphoned materials were observed with a microscope (× 40), and tanks were sampled every other day. Siphoning continued for 3 d after the last juvenile was collected. The metamorphosed juveniles were placed in a 75 L aquarium recirculating system with fine sediment on the bottom, fed alga (*Nannochloropsis oculata)* once a day at 20 ℃, to attempt culture for several weeks.

The first infestation was performed on 3 May 2004 using 3 freshwater drum and 11 tilapia. Fish were infested with glochidia from 12 gravid *P. alatus*. The infestation time lasted 60 min. Freshwater drum were placed in a Living Stream recirculating system (around 380 L) with a chiller set at 17℃. Tilapia were held in two aquaria (150 L) with the temperature maintained at 20℃. Siphoning of tanks for juveniles started on 10 May 2004.

The second infestation was performed on 24 May 2004 using 28 freshwater drum, 3 black crappie and one yellow perch with an infestation time of 10 min. Infested fish were placed in a Living Stream recirculating system with a chiller at 19℃. Fifteen tilapia also were tested, with an infestation time of 20 min; then they were placed in two round-tank

recirculating systems (around 1000 L) at 20℃. A total of 5 gravid *P. alatus* were used for this infestation.

The third infestation was conducted at the Aquatic Wildlife Conservation Center, operated by the Virginia Department of Game and Inland Fisheries on 31 May 2004 using 2 gravid *P. alatus* and 4 freshwater drum (about 25 cm) infested for 15 min. Fish were kept in a round tank (around 1000 L).

The fourth infestation was performed on 15 November 2004 using 5 large freshwater drum, 25 small freshwater drum, and one gravid *P. alatus*. The infestation lasted for 5 min. Infested large drum were held in two tanks (200L) at 19 ℃ and 17 ℃; small drum were held in 6 tanks (75 L) at 19℃. Temperatures were increased to 20 ℃ on 28 November 2004.

The fifth infestation was conducted on 21 November 2004 using 11 large freshwater drum, 19 small freshwater drum, 4 sculpin, and 5 red drum, and 7 gravid *P. alatus*. Infestation lasted for 5 min. Infested large drum were placed in a round tank at 19 ℃, and small drum and sculpin were held in 5 tanks (75 L) at 19℃. Temperatures were increased to 20 ℃ on 28 November 2004. Red drum were held in a round tank (1000 L) filled with fresh water at room temperature (22 - 27℃).

The sixth infestation was performed on 8 January 2005 using 10 red drum and 3 gravid *P. alatus.* Infestation times lasted for 2, 3, 4, and 5 min, respectively, to test infestation rate. Five infested fish were kept in one round tank (1000 L) with fresh water at 22℃, and the remaining 5 fish were placed in a round tank (1000 L) with 0.05 % salt treatment at room temperature (22 - 24℃).

The seventh infestation was performed on 24 February 2005 using 5 red drum and one gravid *P. alatus*. Infestation times lasted 6, 7, 8, 9, and 10 min, respectively. Fish were kept in a round tank (1000 L) filled with fresh water at room temperature (20 - 23℃).

The eighth infestation was performed on 20 April 2005 using 21 red drum, 9 large freshwater drum, and 7 small freshwater drum used in the fall infestations in 2004 and overwintered in the FMCC laboratory. The infestation used 3 gravid *P. alatus*. Large freshwater drum were infested for 6 min, and small ones for 7 min at a water temperature of 22 - 23℃ in tanks. Eight red drum were infested for 5 min, and the other 13 were infested for 10 min. All of them were held in a round tank (1000 L) at room temperature (25 - 28℃). Red drum were conditioned to fresh water for 10 d prior to infestation by gradually reducing the salinity.

Measurements and data analyses

Juveniles were siphoned from each tank and counted. The morphology and dimensions of glochidia and juveniles were examined by microscope. Length and height of glochidia and juveniles were measured with a stereo microscope (Model Wild M3Z, Heerbrugg, Switzerland) at 40x with an ocular micrometer and converted to microns (µm). Total ammonia content was measured to monitor the environment in tanks during host testing.

Numbers of juvenile mussels transformed among freshwater drum at different sizes were compared using JMP (SAS Institute Inc. Cary, NC, 2001, Sall et al. 2001). The normality of these data was tested using Kolmogorov - Smirnov test (Sall et al. 2001) due to the small sample size. Then I used the rank-based nonparametric method of the

Wilcoxon test to assess the probability of significant differences.

RESULTS

Description of glochidia and juveniles of *P. alatus*

Length of glochidia of *P. alatus* (Figure 3-1) ranged from 205.1 to 230.8 μm, with a mean of 221.8 ± 12.6 μm (n = 20). Height ranged from 384.6 to 410.3 μm, with a mean of 392.3 ± 12.01 μm (n = 20) (Appendix 3-1). Glochidia have an ax-head external shape with a greater height of about 43.5 %, when compared to length. The dorsal margin is almost straight, while ventral margin is slightly curved, and anterior and posterior margins are equivalent. Each glochidium has two obvious lateral valve gapes and two lanciform hooks at the lateral margins of the ventral flange of each valve (Figure 3-2).

Length of juveniles varied from 359.0 to 410.3 μm, with a mean of 378.2 ±16.4 μm (n = 20); height ranged from 359.0 to 410.3 μm, with a mean of 383.3 ± 15.5 μm (n = 20) (Appendix 3-1). Length was slightly less than height as 1-d-old juveniles dropped from host fish. The exterior shape of juveniles was fan-like (Figure 3-3A), and great morphological changes had occurred in the glochidial stage. Four thin wings developed on the sides of the anterior and posterior margins until the two lateral valve gapes were closed, which made the shell longer. Hooks were absent, with one muscular transparent foot, which allowed movement on the bottom. Juveniles became longer with age, as they increased more in length than height (Figure 3-3 B-E).

Results of infestation

In the first infestation, no juveniles were collected from the tilapia tanks, but only

valves of glochidia. Two freshwater drum died on the first and second day after infestation. One fish was observed with about 1,000 encysted glochidia on its gills, 90 on the pectoral fins, 27 on the dorsal fin, 60 on the pelvic fin, 11 on the anal fin, and 18 on the caudal fin. Juveniles transformed on a single large freshwater drum (about 50 cm in length) after 31d at 17 ℃. A total of 715 juveniles were obtained from this fish over a collection period of 29 d (Table 3-1). A peak abundance of 203 juveniles was collected on June 12, and 211 juveniles on June 14 (Table 3-1), after 40 and 42 d of metamorphosis (Figure 3-4).

In the second infestation, 12 freshwater drum died the day after infestation due to over-infestation. Four fish were examined to determine the number of encysted glochidia on the gills and fins (Table 3-2). Glochidia were mostly on the gills of host fish. Four juveniles were collected on 12 July from the remaining fish. No juveniles transformed on the black crappie or yellow perch; consequently, they were not suitable host fishes for *P. alatus*.

For the third infestation, only five juveniles were collected from freshwater drum at the AWCC.

In the fourth and fifth infestation, glochidia encysted on the gills and fins of freshwater drum were examined on 6 December (from the infestation on 15 November 2004) and 10 December (from the infestation on 21 November 2004). The level of infestation was 318 to 385 individuals/fish.

Numbers of juveniles collected from the two infestations were analyzed separately. Average transformed juveniles from the large freshwater drum in tank 2 (LT2) was 34 individuals/fish; small freshwater drum in tanks 5, 6, and 7 (ST5 - 7) transformed an

average of 129 juveniles/fish, based on the fourth infestation on 15 November 2004. Data were collected until 18 December 2004, since collected juveniles were combined with tank 3 (large drum infested on 21 November 2004). However data are still reliable, as most juveniles (232) were collected from tank 2 and tank 3 by 18 December 2004, versus 14 juveniles thereafter. Likewise, only 15 juveniles were collected from small freshwater drum in tank 3 - 5 after that date, while 1546 juveniles had been collected before 18 December 2004. Similar results were found from the fifth infestation (21 November 2004), that small freshwater drum in tanks 10 - 13 transformed more juveniles (average rate of 260 juveniles/fish) compared to large drum in tank 4 (85 juveniles/fish) (Table 3-3) (Appendix 3-1). Flatworms were found in tank 1 and tank 3; therefore, data from these tanks were not used for analyses. However, numbers of juveniles transformed were not significantly different based on host fish size ($p = 0.127$).

Five infested red drum stopped eating after infestation, and died within 1 wk. Numbers of attached glochidia observed are shown in Table 3-4. No juveniles were collected from the sculpin.

For the six and seventh infestation, red drum in salt water started eating on the second day after infestation, but those in fresh water did not eat during the post-infestation period and gradually died. No juvenile mussels were collected from the red drum in fresh water or salt water.

On the eighth infestation, red drum transformed a total of 48 juveniles after 13 d (6 May 2004) before all of fish died. The metamorphosed juveniles from red drum appeared active, although pedal-feeding seemed slower than those transformed from freshwater drum. Fifty-four juveniles were collected from large freshwater drum, and 358 juveniles

from small freshwater drum beginning on 9 May 2005 (Table 3-5).

Of the potential host fish tested (freshwater drum, red drum, black crappie, yellow perch, sculpin, and tilapia), only freshwater drum and red drum were suitable hosts for *P. alatus* (Appendix 3-2). The red drum is obviously not a natural host fish, but can be used for transformation of glochidia.

Total ammonia content was around 0.03 mg/L in tanks 1 - 3, and 0.02 in tanks 5 - 14. tank 4 held 8 large freshwater drum, and was a big round tank with a total ammonia concentration ranging from 0.01 to 1.29 mg/L.

DISCUSSION

The dimension of glochidia from my measurements, length of 221.8 ± 2.8 µm (n = 20) and height of 392.3 ± 2.7 µm (n = 20), were slightly greater than those reported by Hoggarth (1999) and smaller than those of Lefevre and Curtis (1912). They were comparable to Surber's (1912) measurements in length but differed in height (380 µm) and somewhat different from Ortmann's (1912) results of 200 µm in length and 380 µm in height (Table 3-6). Differences among measurements may be caused by small sample sizes, accuracy of equipment, or possible differences among populations. My gravid females of *P. alatus* were collected from Kentucky Reservoir on the Tennessee River, while Hoggarth (1999) received gravid mussels from the Muskingum River, north of Luke Chute Lock and Dam, OH. Furthermore, environmental factors, and maternal size and condition may influence embryonic development, resulting in population differences, which might explain some of the variability in glochidia sizes. In general, the morphologic features of *P. alatus* in this study were similar to those previously described;

glochidia were large in size, axe-shaped, with lanciform hooks and lateral valve gapes.

Freshwater mussels typically use specific host fish, and some mussels only have one or a few species of host fish (Hoggarth 1992, Watters 1996). From my data, freshwater drum and red drum were confirmed as suitable hosts for *P. alatus.* That red drum can be an alternative host for *P. alatus* agrees with Neves et al. (1985), who concluded that phylogenetically similar fish species often are suitable hosts for mussel species. However, compatibility or incompatibility of the mussel and host fish relationship is not fully understood. Neves et al. (1985) indicated that this might be related to physiological mechanisms, where fish may produce anti-glochidial factors in their serum when they are infested by glochidia (Meyers et al. 1980, O'Connell and Neves 1999). Glochidia may carry antigens incompatible with host fish proteins, resulting in an antibody reaction by non-host fish, such as black crappie, banded sculpin, yellow perch and tilapia in this study.

Although red drum is not a natural host fish, 48 active juveniles were transformed by this species (Appendix 3-2). According to observations on infestation rate, glochidia seemed to prefer the gills of red drum rather than fins. Only 4 glochidia were observed on the fins versus 2,307 on the gills of 5 red drum (Table 3-4). Fish fins are more separated from the immune system than gills, resulting in reduced host response (Kennedy 1975). The underlying mechanisms of resistance of fins to glochidia are unknown; further studies on related biochemical or other factors are needed.

Salinity is thought to be a lethal factor for most glochidia of freshwater mussels (Liquori and Insler 1985, Anders and Wiese 1993). According to the second host fish test, red drum in the saltwater (0.05 %) tank started to eat on the second day after infestation,

but fish in totally fresh water did not eat after infestation. It may be that salt water of 0.05 % salinity killed the glochidia, resulted in sloughing from the fish gills, allowing red drum to feed again. These glochidia were observed on red drum reared in fresh water, but were absent on fish reared in salt water.

Generally, red drum lived longer when fewer glochidia were attached (Table 3-4), therefore red drum can be a host for *P. alatus* but only at a low infestation rate. Miranda and Sonski (1985) indicated stocking of red drum fingerlings in fresh water should be successful at temperatures above 9℃ and chloride concentrations exceeding 130 mg/L. Pursley and Wolters (1994) reported that juvenile red drum grew fast at 100 mg/L total hardness, and higher chloride and hardness levels increased growth, survival and feed conversion. In my study, chloride concentration was very low, close to 0, since well water was used to supply the red drum culture system with total hardness of 245-277 mg/L. Total ammonia nitrogen concentrations were maintained around 0.22 to 0.54 mg/L. Red drum lived as long as 25 d prior to infestation without mortality, indicating that this system may meet the requirements of red drum.

Freshwater drum was re-confirmed as a suitable host fish for *P. alatus,* as evidenced by the number of glochidia that metamorphosed into juveniles. Seven hundred fifteen juveniles were produced by one large fish on the first infestation; 9,266 juveniles (54 fish) were collected from the fourth and fifth infestations, and 412 juveniles from 16 fish at the eighth infestation. Khym and Layzer (2000) stated that infestation intensity differed by fish size as well as species; i. e., big fish transformed more glochidia due to greater gill surface area. In this study, there was no significant difference ($p = 0.127$) in transformed juveniles between two sizes of freshwater drum used for host testing, although data

showed that (Table 3-3) large drum transformed fewer glochidia and small drum transformed more. These results disagreed with the conclusion of Khym and Layzer (2000). The difference in number of metamorphosed juveniles varied not only by fish size, but perhaps also by their conditions. Large freshwater drum collected from the wild might have been infested by glochidia since they had been exposed in river where presented with freshwater mussels, that might relevantly reject glochidia of *P. alatus* due to the anti-glochidial factors. However, small freshwater drum were glochidia-free since they were produced from a hatchery. Furthermore, larger freshwater drum were stressed by electrofishing, and by food and environmental changes, perhaps resulting in poor health. However, small freshwater drum, delivered from the hatchery to our laboratory holding system, resulting in less stressful conditions and greater transformation of glochidia.

Temperature is a primary factor in the duration of glochidial metamorphosis (Zale and Neves 1982). Yeager and Saylor (1995) stated that the time of complete transformation depended on water temperature; 24 - 29 d for metamorphosis of *Quadrula intermedia* when temperature was 19.2 ℃ and up to 42 - 47 d when temperature decreased to 15 ℃. In Weaver et al. (1991), glochidia of *Pleurobema oviforme* metamorphosed in 13 - 16 d at 20.5 - 24 ℃, and in 9 - 12 d when the temperature was increased to 23 - 26.5 ℃. In my study, glochidia of *P. alatus* metamorphosed on freshwater drum in 31 - 59 d at 17 ℃; in 22 - 46 d and 19 - 40 d at 19 - 20 ℃; and 20 - 23 d at 22 - 23 ℃, respectively. The duration was very short on red drum when temperature was high; only 17 -20 d at 25 - 28 ℃.

The glochidia mainly attached to the gills rather than fins of freshwater drum (Table

3-2), with over 84 % on the gills, similar to red drum. Results seem contrary to that of Kennedy's (1975) explanation of physical isolation of fins with reduced host response. Glochidia attached to pelvic fins more than other fins, possibly due to high density of glochidia on the bottom of the infestation containers.

Over-infestation could be a major factor causing mortality of host fish. From this study, large freshwater drum (20 - 30 cm in length) can host at least 350 glochidia per fish, and small freshwater drum (7.5 - 8.5 cm) about 250. Therefore, I suggest 250 - 350 glochidia per freshwater drum may be a safe infestation level for *P. alatus*.

CONCLUSION AND COMMENTS

Red drum was identified as a host for *P. alatus* the first time, although this species is not a natural host. Freshwater drum was verified as a suitable host fish for *P. alatus*. Moreover, hatchery-produced freshwater drum were seemingly more suitable as hosts when compared to the wild fish, since more juveniles were produced from hatchery fish. Black crappie, banded sculpin, yellow perch and tilapia were tested and found unsuitable host fishes.

The finding of red drum as an alternative host provides potentially important information for *P. alatus* conservation and population restoration. The acclimation of a brackish water species, red drum, in fresh water was successful, as a total of 5 fish were held in fresh water over 2 mo by gradually reducing the salinity. Fish lived comfortably in fresh water, and fed normally without mortality during the holding period. Protocols to maintain fish feeding after infestation are an important complement to their use as host

fish for propagation of the pink heelsplitter.

Other natural host fish species may occur within the range of the pink heelsplitter. Therefore, further research on the identification of additional hosts is needed for possible use in captive propagation. The level of infestation for freshwater drum is suggested to be around 250-350 glochidia per fish to prevent over-infestation and mortality.

Table 3-1. Number of juveniles transformed on freshwater drum, infested on 3 May 2004 at 17 ℃.

Date	Days Post-infestation	No. of Juveniles
Jun 03	31	1
Jun 06	34	3
Jun 10	38	79
Jun 12	40	203
Jun 14	42	211
Jun 16	44	39
Jun 18	46	69
Jun 20	48	34
Jun 22	50	12
Jun 23	51	6
Jun 25	53	17
Jun 28	56	17
Jun 30	58	15
Jul 01	59	9
Total	59	715

Table 3-2. Number of glochidia encysted on gills and fins of the freshwater drum, infested on 24 May 2004.

Encystment locations		Fish 1	Fish 2	Fish 3	Fish 4
Gills		4,292	5,189	2,474	1,200
Fins	Pectoral	58	30	64	93
	Pelvic	120	166	262	66
	Anal	25	13	43	11
	Dorsal	78	16	31	27
	Caudal	389	12	68	18
Subtotal on fins		562	237	468	215
Total		4,854	5,426	2,942	1,415

Table 3-3. Comparison of mean number of juveniles transformed by large and small freshwater drum from infestations on given dates.

Infested Date	Number of juveniles/fish	
	Large drum	Small drum
11/15/2004	34	129
11/21/2004	85	260
4/20/2005	6	51

Table 3-4. Number of glochidia encysted on gills and fins of dead red drum, infested on 21 November 2004.

Date of death	Number of dead fish	Gills	Fins
11/23	1	547	0
11/24	1	416	1
11/25	1	418	0
11/26	1	658	0
11/28	1	268	3

Table 3-5. Number of juveniles collected from freshwater drum and red drum infested on 20 April 2005.

Date	Freshwater drum		Red drum
	Large fish (n = 9)	Small fish (n = 7)	
05/06/05			36
05/07/05			10
05/09/05	9	118	2
05/12/05	45	240	
Total	54	358	48

Table 3-6. Comparison of glochidial dimensions (mean length and height) among studies.

	Length (μm)	Height (μm)
This study	221.80 ± 2.81 (*n* = 20)	392.31 ± 2.70 (*n* = 20)
Hoggarth (1999)	216 ± 9.5 (*n* = 5)	378 ± 7.66 (*n* = 5)
Lefevre and Curtis (1910,1912)	230	410
Surber (1912)	220	380
Ortmann (1912)	200	380

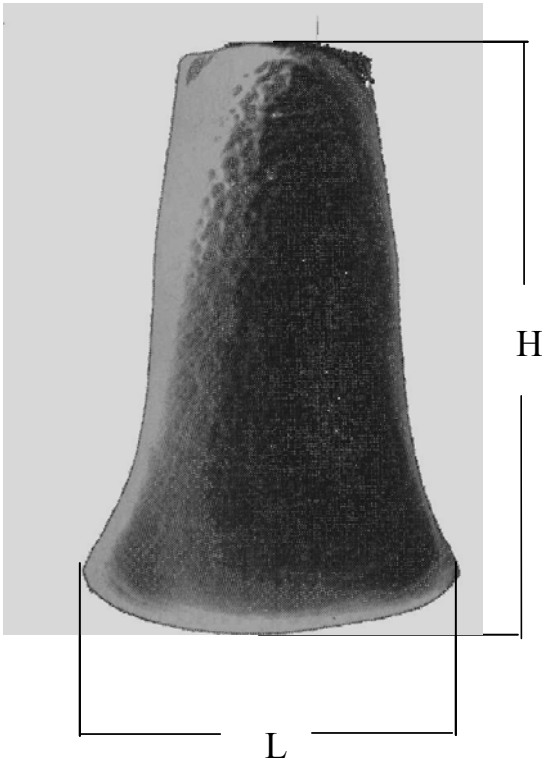
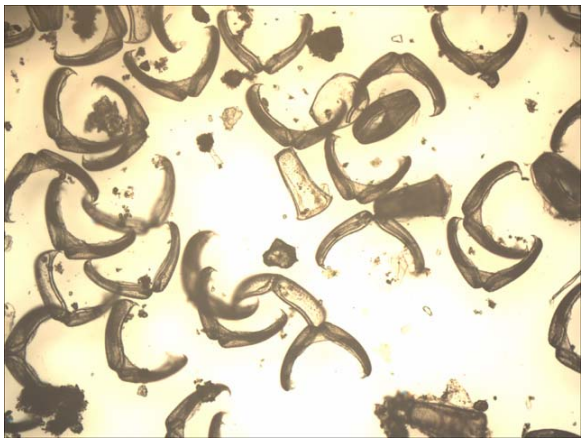
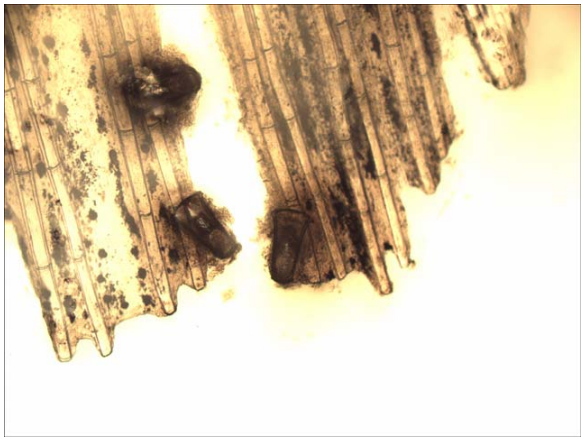


Fig. 3-1. Dimensions of glochidium of *Potamilus alatus*. length (L), height (H). Length was defined as the greatest distance from anterior to posterior margins. Height was defined as the greatest distance from dorsal to ventral margins, perpendicular to length.



Glochidia of *P. alatus*



Glochidia attached to fins of *A. grunniens*

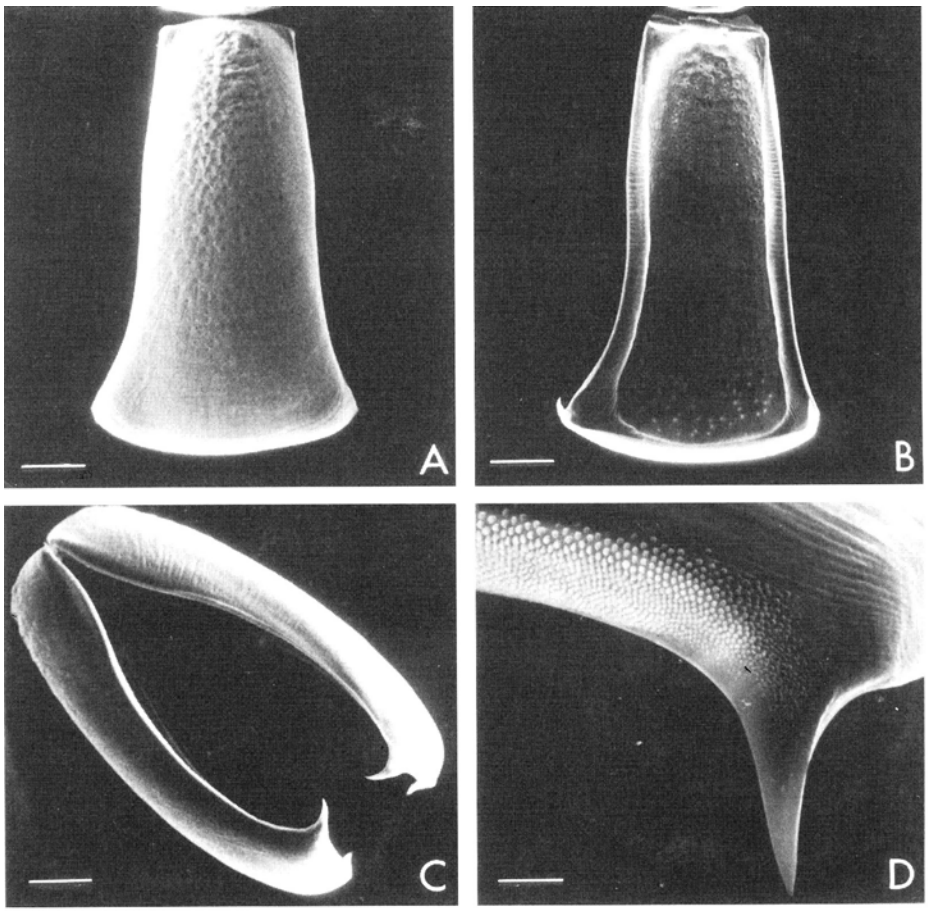
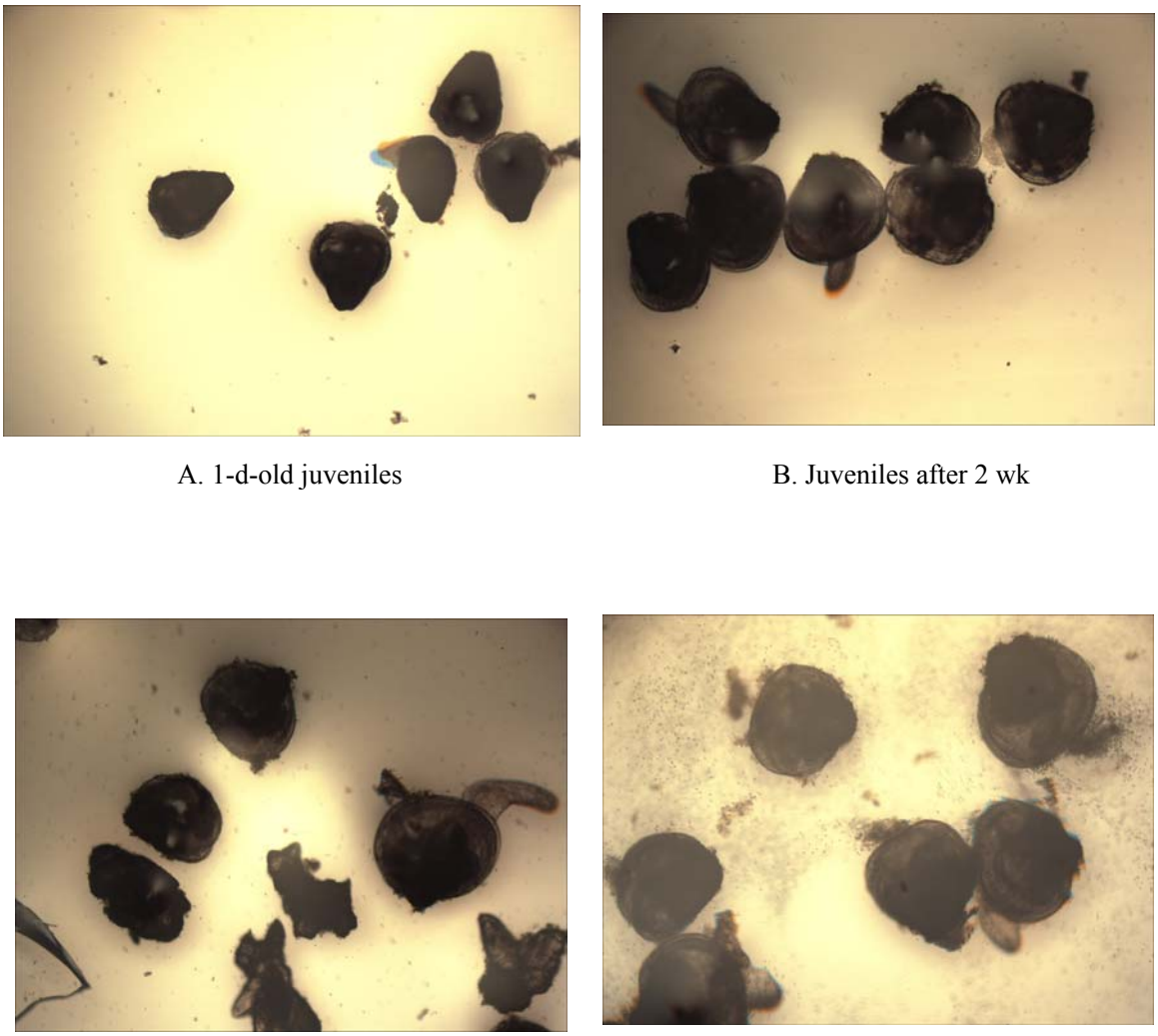


Fig. 3-2. Glochidium of *Potamilus alatus* A. exterior valve, bar length = 50 µm; B. interior valve, bar length = 50 µm; C. lateral view, bar length = 50 µm; D. lanciform hook, bar length = 5 µm (Copyright of Malacologia).



A. 1-d-old juveniles

B. Juveniles after 2 wk

C. Juveniles after 3 wk

D. Juveniles after 4 wk

Fig. 3-3. Morphological changes in shell shape of juvenile *P. alatus* during the first month.

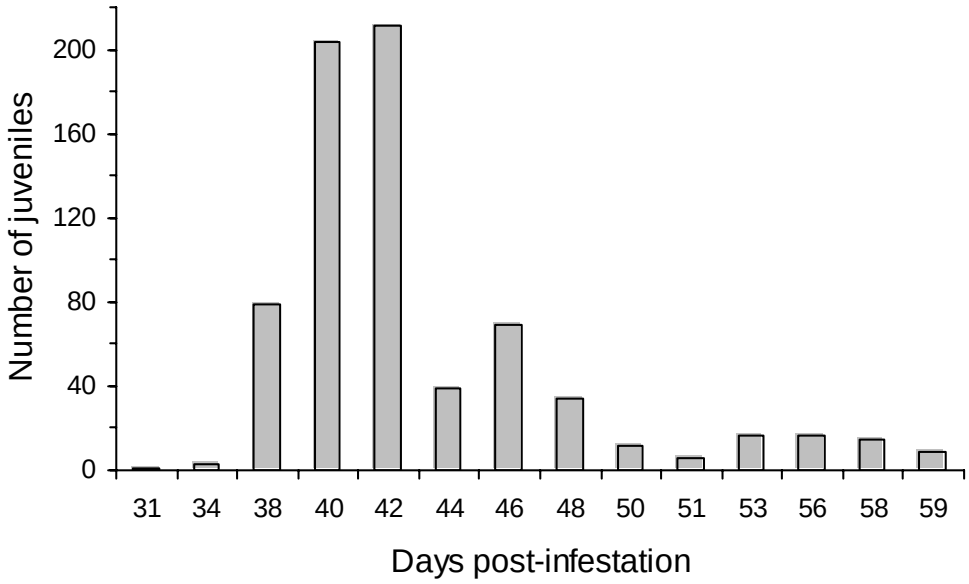


Fig. 3-4. Chronology of metamorphosed juveniles dropping from one host fish (freshwater drum) infested on 3 May 2004, at 17 ℃.

Appendix 3-1. Length and height of a sample of 20 glochidia and juveniles (1-d-old) of *P. alatus*.

Glochidia		Juveniles	
L (μm)	H (μm)	L (μm)	H (μm)
205.1	384.6	359.0	359.0
230.8	410.3	359.0	359.0
230.8	384.6	384.6	410.3
205.1	384.6	384.6	384.6
230.8	384.6	359.0	359.0
205.1	384.6	359.0	384.6
205.1	384.6	384.6	384.6
230.8	384.6	410.3	384.6
230.8	384.6	384.6	384.6
230.8	384.6	384.6	410.3
205.1	410.3	384.6	384.6
205.1	410.3	359.0	384.6
230.8	410.3	384.6	384.6
205.1	384.6	359.0	359.0
230.8	410.3	384.6	384.6
230.8	384.6	410.3	410.3
230.8	384.6	359.0	384.6
230.8	410.3	384.6	384.6
230.8	384.6	384.6	384.6
230.8	384.6	384.6	384.6
* 221.8 ± 12.6	* 392.3 ± 12.1	* 378.2 ± 16.4	* 383.3 ± 15.5

* $\overline{X}$ ± SE (μm)

Appendix 3-2. Juveniles collected from large (L) and small (S) freshwater drum infested on 15 and 21 November 2004. Collection lasted from 6 to 12 December 2004.

Date	Tank1	Tank2	Tank3	Tank4	Tank5	Tank6	Tank7	Tank8	Tank9	Tank10	Tank11	Tank12	Tank13	Tank14
Fish in tanks	3 (L)	2 (L)	2 (L)	8 (L)	3 (S)	4 (S)	5 (S)	4 (S)	4 (S)	4 (S)	5 (S)	5 (S)	5 (S)	4 (Sculpin)
6/12/2004	3					102								
8/12/2004	3				93	170	280		170					
9/12/2004				6				267						
10/12/2004					112	213	265			31	0	21	31	0
11/12/2004	1	10	66	15				11	23					
13/12/2004	0	0	60	46		268				2834				0
16/12/2004	0	57	39	108		43				2294				0
18/12/2004	0			208						241				0
21/12/2004	1	9		58						390				0
23/12/2004		5		170		11				193				0
30/12/2004				76		4				185				0
Subtotal	8	319		687		1561				6691				
Infested date	15/11	11/15	11/21	11/21	15/11	15/11	15/11	11/15	11/15	11/21	11/21	11/21	11/21	11/21

Appendix 3-3. Summary of data for six fish species tested as potential hosts for *P. alatus.*

species	No. of fish used	No. of juveniles	Days to metamorphosis	T(℃)	Host fish ?
A. grunniens [a]	54	9,266	22 - 46	19 - 20	Yes
S. ocellatus [b]	21	43	13	25 - 28	Yes
P. nigromaculatus	3	0	-	19 - 20	No
C. carolinae	4	0	-	19 - 20	No
O. nilotica	26	0	-	20	No
S. canadense	1	0	-	19 - 20	No

[a] data from the fourth and fifth infestation.
[b] data from the eighth infestation. Death of host fish occurred post-infestation.

CHAPTER 4: EFFECTS OF DIET AND SUBSTRATE TYPE ON CULTURE OF JUVENILES OF THE PINK HEELSPLITTER (*POTAMILUS ALATUS*) (SAY, 1817) (BIVALVIA: UNIONIDAE)

INTRODUCTION

Juvenile mussels feed in the sediment with foot ciliation, until their gills become completely developed for filter-feeding (Morton 1976, Lasee 1991, Yeager et al. 1994), and sediment particle size has been considered relevant to growth and survival rate of juveniles. Gatenby et al. (1996) found that rainbow mussel (*Villosa iris*) and giant floaters (*Pyganodon grandis*) reared on sediment (fine sand to clay < 130 µm) had significantly faster growth and higher survival rate than those reared without sediment. Similarly, Hudson and Isom (1984) reported that survival rate of juvenile *Anodonta imbecilis* reached a high of 90 % with an addition of 700 mg silt/L water. However, Brady and Layzer (2000) reported that juveniles of *Lampsilis cardium* preferred larger particle sizes (2 - 4 mm) versus finer sediment (≤ 0.25 mm). Likewise, Hanlon (2000) reared *Lampsilis fasciola* in limestone substrate (1.0 - 2.5 mm) in a river-fed hatchery raceway, resulting in a high survival rate of 80.4 % after 32 d. Rogers (1999) found that juveniles of *V. iris* reared in fine sand (500 - 800 µm) had higher survival than those in fine sediment (< 120 µm). Likewise, Liberty (2004) concluded that juveniles of *V. iris* had higher survival rate in coarse sand, although juveniles exhibited greatest growth in fine sediment. Moreover, Beaty and Neves (2004) reported that growth and survival of juveniles of *V. iris* cultured in fine sediment (< 120 µm) and coarse sediment (120 - 600 µm) were not significantly different. With there results, preference of juveniles likely varies not only by particle size, but by species.

Algae are the common food resources for juvenile and adult mussels. To date, algae have been widely used as a major dietary component for rearing juvenile mussels (Hudson and Isom 1984, Gatenby et al. 1996). The importance of using various species of algae to rear juvenile mussels is due to their physical characteristics and nutritional properties. *Nannochloropsis oculata* has been reported to produce high levels of polyunsaturated fatty acids, which increase survival and growth among a suite of cultured aquatic organisms (Hoff and Snell 1999, Zou et al. 2000). Likewise, *Neochloris oleoabundans* reportly contains high protein (Gatenby 2003) and lipid level as well (Tornabene et al. 1983), such that this species has been extensively used in aquaculture (Gatenby 1997, Sakamoto et al. 1998). Gatenby (1997) studied 9 species of algae in diet combinations, and found that the tri-algal diet of *N. oleoabundans, Bracteacoccus grandis* and *Phaeodactylum tricornutum*, associated with fine sediment, supported the best juvenile growth over 40 d. A second study showed that *Nannochloropsis oculata* was preferentially ingested by the rainbow mussel (*V. iris*), compared to other diets with the larger algal species, *Scenedesmus quadricauda* and *Selenastrum capricornutum* (Beck and Neves 2003). Jones et al. (2005) reported that both *N. oculata* and *N. oleoabundans* are beneficial to growth and survival of juvenile *Epioblasma capsaeformis* and *Villosa iris*. In addition, Beck and Neves (2003) stated that juveniles selectively fed by algal size rather than by species; however, when algae of similar size are compared, juveniles then seemingly select algal species according to nutritional value.

The objective of this study was to compare survival of juvenile *P. alatus* among diets and substrate treatments. Because the survivorship and growth of juveniles of *P. alatus* has not been studied, I sought to test the suitability of two algal species, *Neochloris*

oleoabundans and *N. oculata*; and two types of substrate, for rearing juveniles of *P. alatus*.

METHODS AND MATERIALS

Study site

All experiments were conducted in recirculating culture systems at the FMCC laboratory. These indoor recirculating systems were designed to rear, culture, and hold juvenile and adult freshwater mussels (O' Beirn et al. 1998). Major components of these systems included water-recirculating pumps, air distribution lines, water conditioning and distribution tanks, and algal culture unit. Water recirculating units consisted of troughs, drums, return tubes and airlifts. Air was delivered to the recirculation units from an outside 1.86-kw Sweetwater air blower via 3.8 cm PVC pipes. Water was supplied to the interconnected water conditioning tanks (2270 L) and consisted of a mix of dechlorinated municipal water and well water.

Substrate preparation and algal culture

Fine sediments were collected from the New River, and sand substrates were prepared from crushed limestone. Fine sediments were sieved by 200 μm mesh, and sands (particle-size ≥ 2 mm) were used as substrates. Substrates were autoclaved to kill predators and pathogens. They were then aerated with airstones for 2 - 3 d to facilitate oxidation and to remove excess organic acids (Jones and Neves 2002).

Transparent Kalwall tubes (Kalwalls Aquacenter, Leland, MS) were used to culture algae. *Neochloris oleoabundans* and *N. oculata* were cultured using Kent Pro-Culture F2

Algae Culture Formula (parts A and B). Wide spectrum 40 W fluorescent lights and suitable aeration supplied suitable conditions for algal photosynthesis and growth.

Experimental design

Juvenile pink heelsplitters were obtained from the experiment on host fish testing (Chapter 2). Two treatments with three replicates each for substrates (fine or sand) and algal species (*N. oculata* or *N. oleoabundans*) were tested. Juvenile mussels were reared in the tanks (75 L) in culture dishes (ø = 10 cm, height = 7.5 cm) constructed of PVC pipe, with a 105 μm mesh screen bottom. Each culture dish contained 1 mm-depth substrate of either fine sediment (≤ 200 μm) or sand (≥ 2 mm), and randomly selected juveniles.

Juvenile mussels were fed daily with the green algae *N. oculata* or *N. oleoabundans* at a density of about 30,000 cells/mL. Previous research indicated that a ration of approximately 30, 000 cells m/L was sufficient for juvenile mussels (Rogers 1999, Heneley et al. 2001, Jones 2002). Therefore, this algal density was adopted in this experiment. Cell density and feeding quantity were determined using a hemacytometer and microscope. Twelve culture dishes were placed in two aquaria in separate recirculating systems, according to diet treatments (Figure 4-1). Two wk-old juveniles were reared for 28 d (1 - 28 January 2005) in two tanks, with 300 to 340 individuals per dish (Appendix 3-1).

Sampling and measurements

Juvenile mussels were sampled once every 2 wk to estimate survival and growth rate. Twenty individuals per treatment were measured for length and height at 17 and 28 d

from the start date. Fine sediments in the culture dishes were transferred into 200 μm sieves and washed to separate juveniles from the sediment. Juveniles in the sand treatment were separated using a 2 mm mesh sieve. Surviving juveniles were counted and measured using a Bolgorov plankton counting cell and a dissecting microscope. Juveniles were replaced in culture dishes with fresh substrate after sampling. Empty shells distinguished dead juveniles from live individuals and were not counted. Growth of juveniles was calculated by comparing lengths of sampled juveniles minus the initial length of juveniles on day 1.

Temperature, DO, pH, alkalinity, and total ammonia nitrogen were measured during each sample event. Temperature and DO were measured using a DO meter (YSI Model 55/12 FT, Yellow Springs, OH). Alkalinity was measured using a Hach TM test kit (HACH Company, Loveland, CO), and pH was measured with a pH meter (Model 9024, Apopka, FL). Total ammonia nitrogen was determined by the Nessler method using a HACH DR/2400 spectrophotometer (HACH Company, Loveland, CO).

Data analyses

Survival rate was compared among treatments of substrate and diet using JMP (SAS Institute Inc. Cary, NC, 2001). The normality of each data set was tested using Kolmogorov - Smirnov test (Sall et al. 2001) due to the small sample size. Thereafter, Analysis of Variance and Multiple Comparisons (HSD: Tukey's Honest Significant Difference) were used to test for significant differences at the $\alpha = 0.05$ level.

Data on juvenile growth was compared using Two-way ANOVA to test for significance of differences in growth of juveniles among treatments of algae and substrate. Repeated measurements can cause an incorrect test with type II error. One solution is to

reduce the experimental unit by grouping the data by means of repeated measures instead of by individual values (Sall et al. 2001). Therefore, mean values of growth increases were computed and analyzed for comparison among treatments.

RESULTS

Juvenile survival

The survival rate was determined at 2 wk sampling intervals (17 and 28 January 2005). Survival rate of juveniles at 17 d after stocking ranged from 23.8 to 66.8 %, with an average value of 48.5 %. However, juvenile survival rate dramatically declined after 2 wk, ranging from 1.8 to 7.8 % (mean = 5.8 %) after 28 d.

Survival rates of juveniles among diet and substrate treatments were significantly different ($p = 0.027$, $n = 3$) at the first 2 wk sampling interval (17 January), with a mean of 56.3 % in fine sediment and 40.8 % in sand substrate. The interaction effects also were statistically significant ($p = 0.033$, $n = 3$), in which survival between algae (*N. oculata* and *N. oleoabundans*) did not differ ($p = 0.52$, $n = 3$), but substrates (fine sediment and sands) did differ significantly ($p = 0.018$, $n = 3$). Juveniles reared in sand substrate and fed *N. oculata* had a significantly lower survival rate of 32.2 % (Table 4-1, Figure 4-2).

There were no significant differences among diets and substrates in final survival rates of juveniles sampled on 28 January ($p = 0.128$). Interaction effects were not statistically significant ($p = 0.156$), either between algae ($p = 0.830$) or substrates ($p = 0.053$). However, the treatment with sand associated with the alga *N. oculata* exhibited a similar pattern as the first sampling interval, with the lowest mean survival rate of 3.6 %

(Table 4-1, Figure 4-2).

Juvenile growth

Mean length of juveniles on day 1 was 500.4 ± 48.3 μm (range: 384.6 to 589.7 μm), and mean height was 474.7 ± 28.1 μm (range: 410.3 to 512.8 μm). Juvenile growth was determined by percent increase in shell length. Gains in length were significantly different among treatments of algae and substrates ($p = 0.004$) at 17 d; differences between substrate types were statistically significant ($p = 0.002$), while those between algae (*N. oculata* and *N. oleoabundans*) were not statistically different ($p = 0.113$) nor the interaction of substrates and algae ($p = 0.056$). Juveniles grew faster in fine sediment, with a mean length increase of 23.0 % compared to growth in sand substrates, with a mean length increase of only 10.5 % (Table 4-2). Multiple comparison analysis was not applied, since there was not a significant difference in the interaction of algae and substrate.

When final sizes of juveniles were measured and compared, there was no statistically significant difference of growth rates among 28 d samples ($p = 0.051$), although growth rate increased by 18.6 % in fine sediment versus 15.3 % in sand substrate (Table 4-3).

Juveniles increased by 83.8 μm in mean length after 17 d (16.8 %); therefore mean growth rate was 4.9μm/d, but decreased to 0.2 μm/d during the second 2 wk interval. Overall, juveniles grew faster in the first 2 wk than in the second 2 wk period.

Water quality

Mean water temperature was 20.8℃ (± 0.3℃) during the culture trial. Dissolved oxygen ranged from 8.0 to 8.4 mg/L (mean = 8.2 mg/L), and pH values (mean = 8.2 ±

0.02) remained relatively stable throughout the study. Mean alkalinity was 181 mg/L $CaCO_3$ (± 6.34) and fluctuated from 160 to 198 (mg/L $CaCO_3$). Total ammonia was very low, with a mean value of 0.08 mg/L (± 0.009), and range of 0.05 to 0.11. Water chemistry values were normal and within ranges found suitable for the culture of juvenile mussels (Table 4-3).

DISCUSSION

Juveniles in fine sediment exhibited significantly higher survival rates (61.3 % in *N. oculata,* 51.3 % in *N. oleoabundans*) than those in sands (32.3 % in *N. oculata,* 49.2 % in *N. oleoabundans*) at 2 wk (Table 4-1). This result corroborates those of previous studies; i. e., that juvenile mussels survive better in fine sediment (Hudson and Isom 1984, Gatenby et al. 1996, Henley et al. 2000, Mummert 2001, Zimmerman 2003). Perhaps associated bacteria or organic matter in sediment serves as a supplemental food source for juveniles (Lopez and Holopainen 1987, Gatenby et al. 1997), or it may assist mechanical digestion of algae by the enzymatic activity of adhered bacteria (Gatenby et al. 1996, O'Beirn et al.1998, Beck 2001).

Lasee (1991) found that 2 d-old *Lampsilis ventricosa* have a mouth size of 16 μm and an esophagus of 6 μm in length, suggesting that juveniles need to ingest food within a small size range. My observations agree with this; after 1 d of feeding, the guts of juveniles in fine sediment were colored green and brown, indicating that both algae and fine particles were ingested by juveniles. However, guts of juveniles were mostly colored green in sand dishes, indicating that available food for juveniles was mostly algae. In

addition, sand substrate in this experiment may not allow juveniles to burrow due to coarse particles size (> 2mm), whereas juveniles positioned in fine sediment may move and feed more readily (Barnhart 2005).

Survival rates of juveniles were not significantly different between algal diets, although the model is significantly different between treatments of diet and substrate. This result supports those of a previous study, that survival and growth of juveniles fed *N. oculata* and *N. oleoabundans* were not different (Jones et al. 2005), since both algae contain high levels of polyunsaturated fatty acids and are considered ideal foods for newly metamorphosed juveniles (Gatenby et al. 1997). In addition, size of algal cells is an important factor in juvenile feeding activity. Beck (2001) stated that juveniles prefer small algal cells, smaller than 10 μm. Both *N. oculata* and *N. oleoabundans* are within the proper size range from 3-10 μm, and therefore are readily ingested by juveniles. Consequently, these algae were ingested by *P. alatus* and provided similar survival rates to those of previous studies.

According to analysis by multiple comparisons, juveniles cultured in sand substrate fed *N. oculata* had the lowest survival rate (32.3 %) among the treatments (Table 4-1) (Figure 4-2). There were no significant differences between diets of *N. oculata* and *N. oleoabundans*; however, the interaction effects of diets and substrate were significantly different, as well as between substrates, indicating the lowest survival of juveniles in sand may relate to interaction effects of diets and substrate. This result supports Beck's (2001) hypothesis that juveniles do not assimilate *N. oculata* due to rigid a cell wall, although they easily ingested *N. oculata*. Moreover, Numaguchi (2000) and Payne and Rippingale (2000) reported that the alga *N. oculata* is poorly digested by

oyster spat and copepods. A possible reason indigestibility of algal cell walls may be absence of particular digestive enzymes in mussels. Therefore, fine silt or enzyme in bacteria in fine sediment may aid breakdown of the cell wall, resulting in a higher survival rate (Beck 2001). Previous studies also concluded that juveniles fed *N. oculata* and reared in fine sediment (< 350 μm) had higher growth and survival (Hudson and Isom 1984, Gatenby et al. 1996, O'Beirn et al.1998).

Survival rate declined rapidly after 17 d in this experiment, and mean survival rates dropped from 48.5 % to 5.8 % during the last 2 wk (Figure 4-2). High mortality occurred in all treatments at the end of 28 d (Table 4-1), with the highest survival of only 8.2 %. Moreover, differences in survival rate were not significant among the treatments of diets and substrates. Beck (2001) stated that it was difficult to distinguish statistical differences in survival when mortality was exceedingly high among treatments after 40 d. Two main factors are thought to result in high mortality; when juveniles are not fed a proper diet or when the rearing environment is unsuitable (Buddensiek 1995, Gatenby et al. 1996, Gatenby et al. 1997, O'Beirn 1998, Henley et al. 2001). Although juveniles fed *N. oculata* and *N. oleoabundans* did not differ in survival and growth, it does not guarantee that these are adequate in themselves for juvenile culture at all stages. An inadequate diet may be a factor causing low survival, since water quality in the recirculating systems was suitable and within normal ranges (Table 4-4), and comparable to those in previous studies (Table 4-5). Additionally, flatworms were observed at the final sampling, but not in the first sampling. Flatworms are known predators of juvenile mussels (Sickel 1998, Zimmerman et al. 2003). Thus, juveniles were lost to predation, resulting in low survival at the end of the experiment. Moreover, disturbance caused by sampling can influence

juvenile survival. Liberty (2004) reported that unsampled juveniles had significantly greater survival than sampled juveniles. Therefore, the combination of these effects may have caused high mortality after 28 d.

Growth rate of juveniles among diet and substrate treatments exhibited a trend similar to survival, with 23.0 % greater mean growth in fine sediment than in sand substrate (10.5 %) during the first 2 wk culture period (Table 4-2). Results supported the conclusion that fine sediment can provide additional benefits to juveniles, and possibly aids in digestion. Juveniles generally grew faster, with a mean increase of 16.8 %, during the first 2 wk than the second 2 wk (0.4 %) (Table 4-3). Poor growth was exhibited in fine sediment associated with *N. oculata* after 2 wk, while others exhibited positive growth (Figure 4-3). Barnhart (2005) noted that high mortality could bias the comparison of growth rates. Therefore, data of growth of juveniles for the first 2 wk instead of the second 2 wk was used for analyses.

With a daily growth rate of 4.9 µm/d in the first 2 wk, and 0.2 µm/d in the second 2 wk, the growth rate of juvenile *P. alatus* in this study was low compared to other studies using similar culture systems. Mean growth rate of rainbow mussel juveniles reached 20.7µm/d during a 60 d culture period (Jones et al. 2005). However, growth rate may be mostly determined by difference among species. Barnhart (2005) reported that the growth rates of 5 species of juveniles ranged from 4.7 to 12.2 µm/d, when they were reared in the same recirculating system and fed algae.

Juvenile growth showed a trend similar to survival, as growth rate declined rapidly after 2 wk. Lasee (1991) stated that slow growth of juveniles might be related to loss of stored lipids in the digestive gland. In my study, juveniles may have utilized their lipid

stores after 2 wk, and were unable to obtain adequate nutrition in the second 2 wk period to support further growth and survival. In addition, pedal-feeding occurs early in the developmental stages of juveniles (Reid et al. 1992), until their gills are fully developed (Morton 1976, Lasee 1991, Yeager et al. 1994). Gatenby et al. (1997) reported that juveniles of *V. iris* exhibited pedal-feeding for 140 d. The transition from pedal-feeding to suspension-feeding is undocumented, but is thought to be related to food suitability (Beck 2001) and development of suspension-feeding anatomy. Therefore, juvenile *P. alatus* may have exhibited slow growth after 2 wk because of an unsuitable diet or environment during the transition from pedal-feeding to suspension-feeding.

Previous studies suggested multiple algal diets to promote survival and growth of juveniles (Gatenby et al. 1997, Beck 2003). Howard (1922) reported that outdoor-cultured juveniles were larger than those cultured indoors in a laboratory. Juvenile *Lampsilis siliquoidea* in a river were several-fold larger than those reared in laboratory aquaria. Barnhart (2005) concluded that lampsiline juveniles grew faster by using natural water. In China, intensive juvenile culture using pond or river water has successfully produced high numbers of juveniles of the triangle sail mussel (*Hyriopsis cumingii*), cockscomb mussel (*Cristaria plicata*), and round mussel (*Anodonta woodiana*) in outdoor systems with high densities of around 10,000 - 30,000 individuals/m^2. For example, growth rate of juveniles of *H. cumingii* can reach 1 - 2 cm/mo, with survival of over 90 % (Hua 2003b). Outdoor culture systems using pond water likely provide adequate food with a balanced nutrition to meet the nutritional demands of juveniles rather than single or mixed diets under laboratory conditions.

My experiment was conducted in a recirculating aquaculture system previously used

for rearing endangered and common species of juveniles and adults (O'Beirn 1998, Henley et al. 2001, Jones and Neves 2005). Water quality parameters of these experiments were comparable to those in my study (Table 4-5). Temperature was maintained at 20.8℃, and dissolved oxygen (8.2 mg/L) was close to O'Beirn's (1998) values. Freshwater mussels have a pH tolerance limit of about 6.0 (Peterson 1987), and my pH values (8.2) were greater than this threshold value. Total ammonia nitrogen (TAN) in this study (range: 0.05 - 0.11 mg/L) was less than the U. S. EPA continuous criterion concentration at 1.24 mg/L TAN (pH = 8, T = 25℃) (EPA 1999), and less than the suggested minimum of chronic ammonia exposure for unionids at 0.3 mg/L (pH = 8) (Augspurger et al. 2003). When compared to data of previous studies, water quality parameters appeared acceptable and suitable for rearing juveniles in my experiment.

CONCLUSIONS ND COMMENTS

Overall, juveniles reared in fine sediment exhibited significantly higher survival and growth than those in sand; however, there were no significant differences in growth and survival between diets of *N. oculata and N. oleoabundans* after 17 d culture period. The lowest survival rate (32.3 %) was found in sand associated with a diet of *N. oculata*. Likewise, juveniles grew faster, with mean increase of 115.01 μm (23.0 %) in fine sediment, than those in sand substrate with mean increase of 52.6 μm (10.5 %).

Juveniles exhibited high mortality and slow growth after 2 wk, likely due to flatworm predation, sampling disturbance, and probably inadequate nutrition. The lowest mean survival rate at 3.6 % in sand substrate after 28 d was associated with the diet of *N. oculata*, showing a similar pattern as the first 2 wk. Growth rates of juveniles were not

significantly different between diets and substrate type, although juvenile growth rate was slightly higher (18.6 %) in fine sediment than growth (15.3 %) in sand after 28 d. Juveniles of *P. alatus* grew faster in the first 2 wk than in the second 2 wk, with a growth rate of 4.9µm/d in 17 d, and only 0.2 µm/d from 17 d to 28 d.

Based on these results, I recommend rearing juveniles in fine sediment instead of sand due to the apparent benefits of fine particles. Future research should focus on improving diets and environmental conditions to increase survival and growth rate of juveniles. Nutrition demands of transition from pedal-feeding to suspension-feeding are not clear and should be studied further. Frequent sampling disturbance can jeopardize juvenile survival (Liberty 2004); therefore, a new or minimal sampling method with minimal disturbance should be sought.

A previous study using a bi-algal diet of *N. oculata* and *N. oleoabundans* associated with fine sediment showed excellent survival and growth rate for juvenile mussels (Beck and Neves 2003). Therefore, the species *N. oculata* and *N. oleoabundans* may be used to feed juveniles in the laboratory if pond water or river water is not available.

I recommend outdoor propagation and juvenile culture using natural water recirculation, where pond water or river water would provide the main supply of food (algae, organic debris, and some essential nutrition component) for juveniles. To enhance juvenile health, other nutritional supplements, such as soybean milk (Hua et al. 2001), may be applied during the advanced juvenile culture period. Information on nutritional requirements of freshwater mussels is limited (Gatenby 1996), and environmental requirements also are poorly known. Therefore, further research should focus on essential

nutrition and environment requirements of juveniles in order to establish a successful outdoor propagation protocol.

Table 4-1. Mean survival rate (± SE) of juveniles after 2 wk and 4 wk reared in two substrates and fed two algal species; Analyses of Variance and Multiple Comparisons (HSD: Tukey's Honest Significant Difference) are applied to analysis, with $\alpha = 0.05$ significant level. Means followed by the same letter are not significantly different at the 0.05 level (experimentwise), Using Tukey's HSD.

Sample date	Substrates	Algae	$\overline{X}$ ± SE (%)
17/1/2005	Fine sediment	*N. oculata*	61.3 ± 4.7 [a]
			(51.9 - 66.8)
17/1/2005	Fine sediment	*N. oleoabundans*	51.3 ± 6.5 [a]
			(38.7 - 60.3)
17/1/2005	Sand	*N. oculata*	32.3 ± 5.2 [bc]
			(23.8 - 41.8)
17/1/2005	Sand	*N. oleoabundans*	49.2 ± 4.2 [b]
			(40.8 - 54.1)
28/1/2005	Fine sediment	*N. oculata*	8.2 ± 0.4
			(7.8 - 8.9)
28/1/2005	Fine sediment	*N. oleoabundans*	6.0 ± 1.4
			(4.2 - 8.7)
28/1/2005	Sand	*N. oculata*	3.6 ± 1.3
			(1.8 - 6.0)
28/1/2005	Sand	*N. oleoabundans*	5.2 ± 1.5
			(2.3 -7.3)

Table 4-2. Comparison of growth (increase in mean length ± SE) of juveniles reared in two substrates after 2 wk and 4 wk. Percent increase was the ratio of mean length increase compared to the initial length of juveniles. Significant difference is represented by * symbol.

Date	Substrate	*N*	$\overline{X}$ ± SE (μm)	% increase	
17/1/2005	Find sediment	120	115.0± 6.2	23.0 ± 1.2	*
17/1/2005	Sand	120	52.6 ± 4.2	10.5 ± 0.8	
17/1/2005	Subtotal	240	83.8 ± 4.2	16.8 ± 0.9	
28/1/2005	Find sediment	109	93.1 ± 5.6	18.6 ± 1.1	
28/1/2005	Sand	82	76.6 ± 6.2	15.3 ± 1.2	
28/1/2005	Subtotal	191	86.0 ± 4.2	17.2 ± 0.8	

Table 4-3. Increase in mean length (± SE) of juveniles after 2 wk and 4 wk reared in two substrates and fed two algal species. Percentage increase was the ratio of mean length increase compared to the initial length of juveniles. Analyses of Variance is applied to analysis data, with $\alpha = 0.05$ significant level.

Date	Substrate	Algae	*N*	$\overline{X} \pm$ SE (μm)	% increase
17/1/2005	Fine sediment	*N. oculata*	60	141.5 ± 7.5	28.3 ± 1.5
17/1/2005	Fine sediment	*N. oleoabundans*	60	88.5 ± 8.8	17.7 ± 1.7
17/1/2005	Sand	*N. oculata*	60	49.6 ± 5.9	9.9 ± 1.2
17/1/2005	Sand	*N. oleoabundans*	60	55.6 ± 5.9	11.1 ± 1.2
28/1/2005	Fine sediment	*N. oculata*	60	72.7 ± 5.5	14.5 ± 1.1
28/1/2005	Fine sediment	*N. oleoabundans*	49	118.2 ± 9.1	26.6 ± 1.9
28/1/2005	Sand	*N. oculata*	36	65.9 ± 7.6	13.2 ± 1.5
28/1/2005	Sand	*N. oleoabundans*	46	84.9 ± 9.1	17.0 ± 1.8

Table 4-4. Values of water quality parameters in juvenile culture system during experimental period, January 2005.

Date	Algae	DO (mg/L)	T (℃)	pH	Alkalinity (mg/ L)	TAN * (mg/L)
1/1	*N. oleoabundans*	8.2	20.0	8.3	192.0	0.05
1/1	*N. oculata*	8.3	20.0	8.3	160.0	0.09
1/17	*N. oleoabundans*	8.2	20.5	8.3	192.0	0.08
1/17	*N. oculata*	8.0	21.0	8.2	178.0	0.10
1/28	*N. oleoabundans*	8.4	22.0	8.2	198.0	0.07
1/28	*N. oculata*	8.1	21.00	8.1	166.0	0.11

* TAN = Total ammonia nitrogen.

Table 4-5. Comparison of mean values (with ranges) of water quality parameters in this recirculating aquaculture systems with those of previous studies.

Parameters	O'Beirn (1998)	Henley et al. (2001)	Jones et al. (2005)	This study
DO (mg/L)	8.3 (5.8 - 10.9)	7.8 (SD = 0.6)	—	8.2 (8.0 - 8.4)
pH	8.3 (7.3 - 8.9)	8.7 (SD = 0.01)	8.5 (SE = 0.04)	8.2 (8.1 - 8.3)
Alkalinity (mg/L as $CaCO_3$)	213 (138 - 277)	262.6 (SD = 43.3)	147.42 (SE = 5.5)	181 (160 - 198)
TAN*	—	0.1 (SD = 0.07)	0.27 (SE = 0.02)	0.08 (0.05 - 0.11)
Temperature (℃)	20.4 (15.4 - 26.4)	18.4 (SD = 1.8)	21.9 (SE = 0.59)	20.8 (20 - 22)

* TAN = Total Ammonia nitrogen

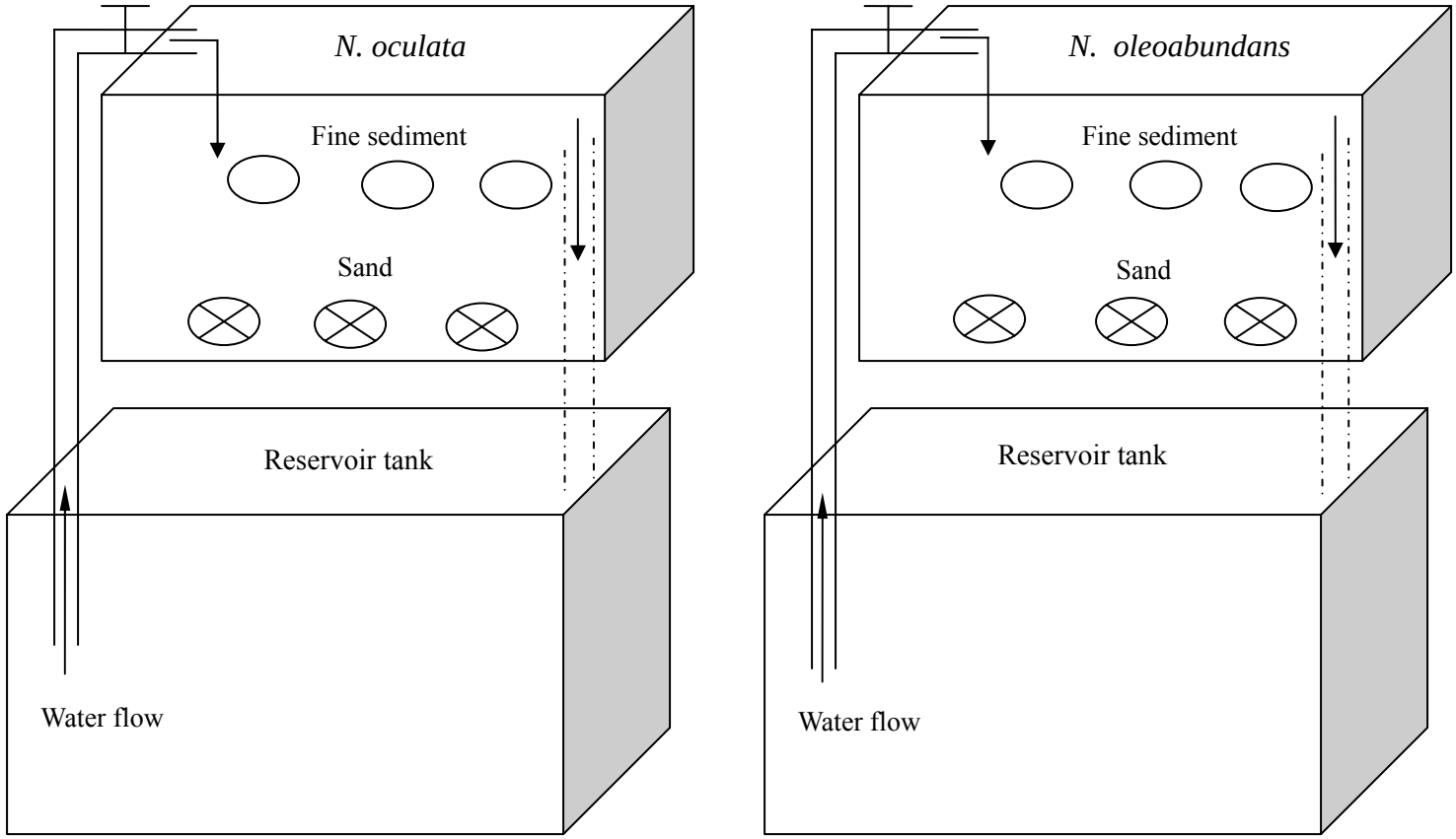


Fig. 4-1. The recirculating system for rearing juveniles in dishes with sand or fine sediment in two tanks (75 L), and fed *N. oleoabundans* and *N. oculata*.

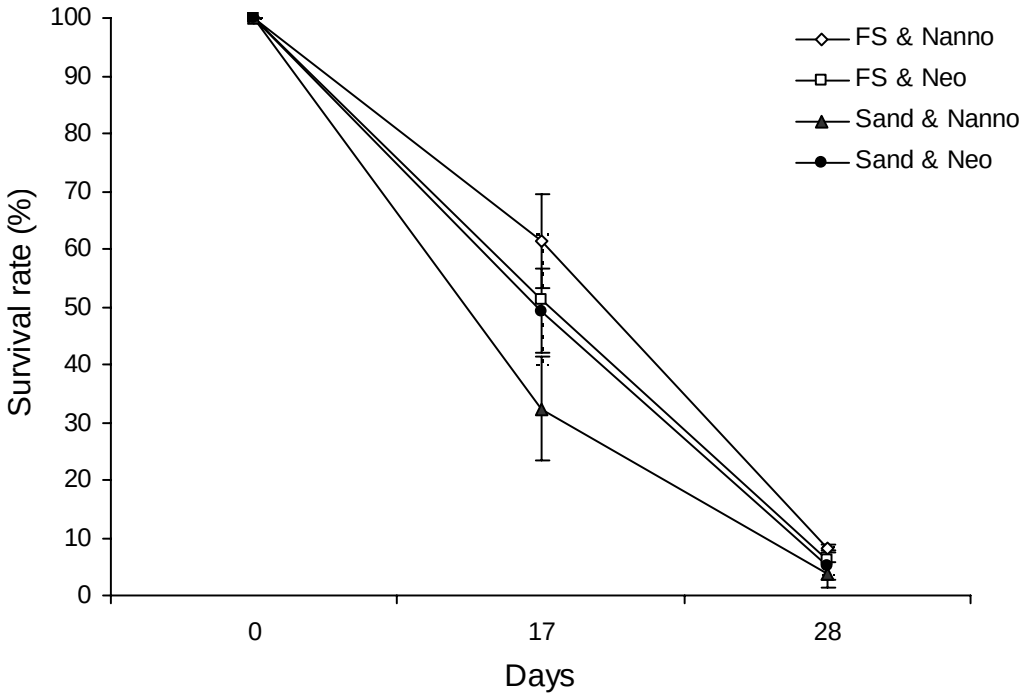


Fig. 4-2. Mean survival rate (± SE) of juveniles reared in fine sediment (FS) and sand substrate and fed *N. oculata* (*Nanno*), and *N. oleoabundans* (*Neo*), respectively, over a 28-d culture period.

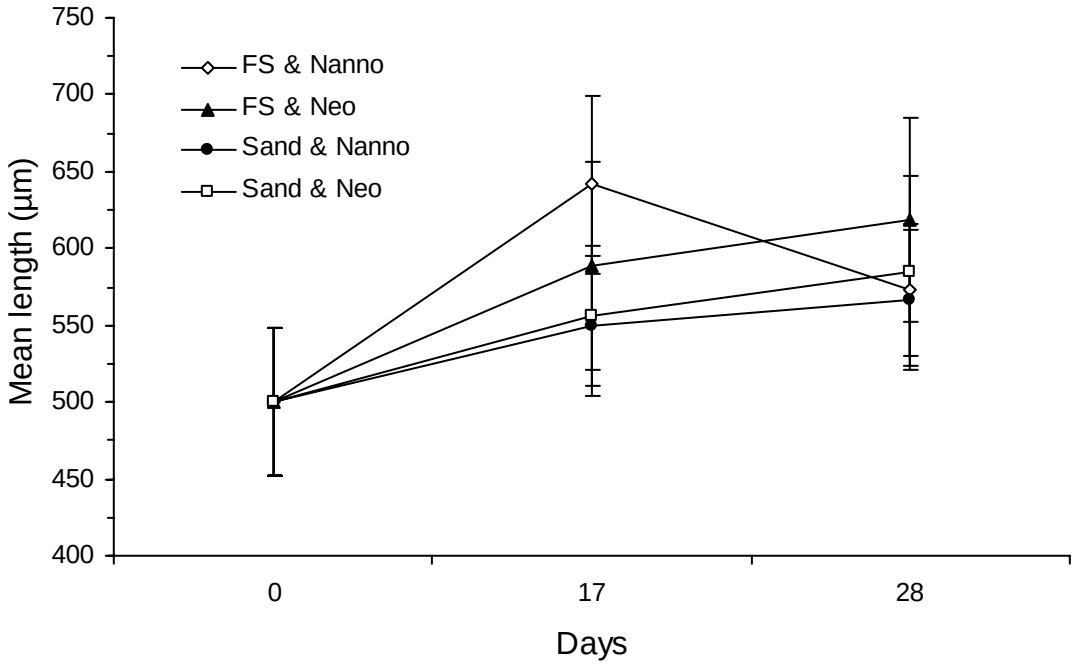


Fig. 4-3. Mean length (±SE) (µm) of juveniles reared in fine sediment (FS) and sand substrate and fed *N. oculata* (*Nanno*) and *N. oleoabundans* (*Neo*), respectively, over a 28-d culture period.

Appendix 4-1. Numbers of juveniles stocked in diet and substrate treatments, with three replicates each on 1 January 2005, and sampled on 17 and 28 January 2005.

Sampled	1/1/2005							
Algae	*N. oleoabundans*				*N. oculata*			
Substrate	Fine sediment		Sand		Fine sediment		Sand	
	Dish	No. of Juv.	Dish	No. of Juv.	Dish	No. of Juv.	Dish	No. of Juv.
	1_1	300	2_1	316	2_1	314	1_1	335
	1_2	305	2_2	303	2_2	322	1_2	340
	1_3	309	2_3	307	2_3	304	1_3	328

Sampled	17/1/2005							
Algae	*N. oleoabundans*				*N. oculata*			
Substrate	Fine sediment		Sand		Fine sediment		Sand	
	Dish	No. of Juv.	Dish	No. of Juv.	Dish	No. of Juv.	Dish	No. of Juv.
	1_1	116	2_1	129	2_1	163	1_1	105
	1_2	184	2_2	160	2_2	215	1_2	142
	1_3	170	2_3	166	2_3	198	1_3	78

Sampled	28/1/2005							
Algae	*N. oleoabundans*				*N. oculata*			
Substrate	Fine sediment		Sand		Fine sediment		Sand	
	Dish	No. of Juv.	Dish	No. of Juv.	Dish	No. of Juv.	Dish	No. of Juv.
	1_1	26	2_1	19	2_1	28	1_1	20
	1_2	16	2_2	22	2_2	25	1_2	10
	1_3	13	2_3	7	2_3	24	1_3	6

Appendix 4-2. Measurements of length and height of juveniles reared in fine sediment and fed *N. oculata*.

Date	01/17/2005					
Algae	*N. oculata*					
Substrate	Fine sediment < 200µm					
Replicates	1		2		3	
Growth	Length (µm)	Height (µm)	Length (µm)	Height (µm)	Length (µm)	Height (µm)
	615.4	538.5	666.7	564.1	512.8	512.8
	615.4	538.5	615.4	564.1	538.5	538.5
	717.9	641.0	666.7	589.7	512.8	512.8
	692.3	666.7	717.9	615.4	589.7	512.8
	641.0	564.1	641.0	564.1	512.8	512.8
	641.0	564.1	589.7	512.8	692.3	589.7
	641.0	538.5	512.8	512.8	641.0	615.4
	666.7	564.1	641.0	589.7	743.6	641.0
	641.0	564.1	666.7	589.7	666.7	564.1
	666.7	589.7	692.3	615.4	692.3	589.7
	692.3	589.7	666.7	589.7	615.4	589.7
	666.7	589.7	641.0	589.7	769.2	615.4
	615.4	512.8	615.4	564.1	538.5	512.8
	589.7	487.2	692.3	589.7	589.7	538.5
	641.0	564.1	666.7	564.1	666.7	564.1
	589.7	512.8	666.7	589.7	692.3	615.4
	615.4	512.8	641.0	564.1	589.7	512.8
	589.7	487.2	692.3	589.7	641.0	589.7
	666.7	641.0	717.9	615.4	692.3	615.4
	641.0	615.4	615.4	564.1	743.6	615.4
$\overline{X}$ ± SE (µm)	546.2± 7.2	515.4± 3.7	564.1± 12.9	516.7± 7.0	539.7± 9.4	509.0± 7.3
N	20	20	20	20	20	20

Appendix 4-2 (continued). Measurements of length and height of juveniles reared in sand substrate and fed *N. oculata*.

Date	01/17/2005					
Algae	*N. oculata*					
Substrate	Sand > 2mm					
Replicates	1		2		3	
Growth	Length (µm)	Height (µm)	Length (µm)	Height (µm)	Length (µm)	Height (µm)
	615.4	512.8	717.9	589.7	641.0	589.7
	512.8	564.1	512.8	512.8	615.4	589.7
	538.5	512.8	512.8	487.2	487.2	461.5
	589.7	512.8	512.8	512.8	512.8	487.2
	512.8	487.2	487.2	487.2	512.8	461.5
	538.5	512.8	641.0	589.7	538.5	512.8
	564.1	512.8	564.1	538.5	564.1	512.8
	538.5	487.2	589.7	512.8	512.8	512.8
	564.1	512.8	512.8	487.2	512.8	512.8
	538.5	512.8	564.1	512.8	564.1	512.8
	564.1	538.5	589.7	512.8	564.1	487.2
	512.8	512.8	615.4	538.5	512.8	512.8
	538.5	512.8	641.0	538.5	564.1	512.8
	512.8	512.8	564.1	512.8	564.1	512.8
	512.8	512.8	589.7	512.8	564.1	512.8
	589.7	538.5	512.8	461.5	512.8	487.2
	564.1	512.8	512.8	487.2	487.2	487.2
	589.7	512.8	538.5	512.8	512.8	487.2
	512.8	512.8	564.1	512.8	564.1	512.8
	512.8	512.8	538.5	512.8	487.2	512.8
$\overline{X}$ ± SE (µm)	546.2±7.2	515.4±3.7	564.1±12.9	516.7±7.0	539.7±9.4	509.0±7.3
N	20	20	20	20	20	20

Appendix 4-2 (continued). Measurements of length and height of juveniles reared in fine sediment and fed *N. oleoabundans*.

Date	01/17/2005					
Algae	*N. oleoabundans*					
Substrate	Fine sediment < 200 µm					
Replicates	1		2		3	
Growth	Length (µm)	Height (µm)	Length (µm)	Height (µm)	Length (µm)	Height (µm)
	589.7	512.8	512.8	512.8	589.7	512.8
	692.3	615.4	487.2	512.8	512.8	512.8
	615.4	589.7	717.9	564.1	538.5	512.8
	717.9	589.7	692.3	538.5	564.1	512.8
	666.7	564.1	641.0	538.5	512.8	512.8
	692.3	589.7	641.0	564.1	564.1	512.8
	564.1	512.8	512.8	512.8	589.7	512.8
	589.7	512.8	641.0	564.1	615.4	538.5
	564.1	512.8	564.1	538.5	564.1	512.8
	564.1	512.8	538.5	512.8	538.5	512.8
	717.9	615.4	589.7	512.8	564.1	512.8
	641.0	564.1	564.1	512.8	538.5	512.8
	666.7	589.7	564.1	512.8	512.8	512.8
	589.7	512.8	769.2	615.4	512.8	487.2
	564.1	512.8	512.8	487.2	641.0	589.7
	615.4	512.8	538.5	512.8	615.4	564.1
	666.7	589.7	564.1	512.8	461.5	487.2
	717.9	589.7	589.7	512.8	512.8	487.2
	564.1	512.8	512.8	512.8	564.1	512.8
	589.7	512.8	538.5	512.8	538.5	512.8
$\overline{X}$ ± SE (µm)	629.5±13.0	551.3±9.2	584.6±17.1	528.2±6.6	552.6±9.8	516.7±5.4
N	20	20	20	20	20	20

Appendix 4-2 (continued). Measurements of length and height of juveniles reared in sand substrate and fed *N. oleoabundans.*

Date 01/17/2005

Algae *N. oleoabundans*

Substrate Sand > 2mm

Replicates	1		2		3	
Growth	Length (µm)	Height (µm)	Length (µm)	Height (µm)	Length (µm)	Height (µm)
	512.8	538.5	589.7	538.5	589.7	538.5
	512.8	487.2	538.5	512.8	538.5	512.8
	564.1	512.8	538.5	512.8	512.8	512.8
	538.5	512.8	435.9	410.3	641.0	564.1
	538.5	512.8	512.8	512.8	564.1	512.8
	641.0	564.1	641.0	564.1	538.5	512.8
	641.0	564.1	512.8	512.8	564.1	512.8
	564.1	512.8	538.5	512.8	641.0	589.7
	589.7	538.5	564.1	538.5	615.4	564.1
	564.1	512.8	512.8	512.8	615.4	564.1
	589.7	512.8	512.8	487.2	589.7	538.5
	564.1	512.8	512.8	512.8	512.8	512.8
	615.4	538.5	461.5	461.5	538.5	512.8
	641.0	538.5	538.5	512.8	589.7	512.8
	512.8	512.8	564.1	512.8	564.1	512.8
	538.5	512.8	512.8	512.8	564.1	538.5
	512.8	512.8	538.5	512.8	564.1	512.8
	589.7	564.1	512.8	512.8	512.8	487.2
	564.1	512.8	538.5	512.8	564.1	538.5
	538.5	512.8	615.4	564.1	512.8	512.8
$\overline{X}$ ± SE (µm)	566.7±9.6	524.4±4.7	534.6±10.4	511.5±7.3	566.7±9.1	528.2±5.7
N	20	20	20	20	20	20

Appendix 4-2 (continued). Measurements of length and height of juveniles reared in fine sediment and fed *N. oculata*.

Date 01/28/205

Algae *N. oculata*

Substrate Fine sediment < 200µm

Replicates	1		2		3	
Growth	Length (µm)	Height (µm)	Length (µm)	Height (µm)	Length (µm)	Height (µm)
	615.4	564.1	615.4	564.1	564.1	512.8
	589.7	538.5	589.7	512.8	641.0	564.1
	564.1	564.1	564.1	538.5	615.4	538.5
	641.0	589.7	615.4	512.8	615.4	564.1
	564.1	512.8	615.4	538.5	564.1	538.5
	512.8	512.8	564.1	512.8	512.8	512.8
	615.4	538.5	641.0	564.1	538.5	512.8
	512.8	538.5	589.7	512.8	512.8	538.5
	564.1	512.8	564.1	538.5	589.7	538.5
	641.0	564.1	538.5	512.8	512.8	512.8
	564.1	538.5	589.7	538.5	512.8	512.8
	615.4	512.8	564.1	564.1	564.1	538.5
	641.0	564.1	538.5	512.8	641.0	564.1
	589.7	512.8	512.8	538.5	589.7	564.1
	589.7	538.5	615.4	538.5	512.8	512.8
	512.8	512.8	512.8	538.5	538.5	512.8
	615.4	538.5	564.1	512.8	589.7	538.5
	512.8	538.5	512.8	512.8	564.1	564.1
	615.4	538.5	538.5	512.8	615.4	538.5
	564.1	512.8	512.8	538.5	589.7	538.5
$\overline{X}$ ± SE (µm)	582.0±9.9	537.2±5.1	567.9±9.0	530.8±4.2	569.2±9.8	535.9±4.5
N	20	20	20	20	20	20

Appendix 4-2 (continued). Measurements of length and height of juveniles reared in sand substrate and fed *N. oculata*.

Date	01/28/205					
Algae	*N. oculata*					
Substrate	Sand > 2mm					
Replicates	1		2		3	
Growth	Length (µm)	Height (µm)	Length (µm)	Height (µm)	Length (µm)	Height (µm)
	564.1	512.8	538.5	512.8	589.7	512.8
	564.1	512.8	564.1	487.2	487.2	512.8
	512.8	487.2	641.0	564.1	512.8	538.5
	641.0	615.4	589.7	512.8	589.7	538.5
	564.1	512.8	512.8	538.5	512.8	512.8
	487.2	512.8	641.0	615.4	538.5	512.8
	589.7	512.8			589.7	512.8
	512.8	512.8			641.0	589.7
	589.7	512.8			564.1	487.2
	589.7	538.5			589.7	512.8
					512.8	512.8
					564.1	512.8
					589.7	538.5
					615.4	512.8
					512.8	487.2
					564.1	512.8
					641.0	564.1
					564.1	512.8
					589.7	538.5
					512.8	487.2
$\overline{X}$ ± SE (µm)	561.5±14.5	523.1±10.9	581.2±21.6	538.5±18.7	564.1±10.0	520.5±5.6
N	10	10	6	6	20	20

Appendix 4-2 (continued). Measurements of length and height of juveniles reared in fine sediment and fed with *N. oleoabundans*.

Date	01/28/205					
Algae	*N. oleoabundans*					
Substrate	Fine sediment < 200 µm					
Replicates	1		2		3	
Growth	Length (µm)	Height (µm)	Length (µm)	Height (µm)	Length (µm)	Height (µm)
	717.9	589.7	641.0	564.1	641.0	564.1
	666.7	564.1	512.8	538.5	564.1	538.5
	615.4	538.5	512.8	512.8	641.0	564.1
	538.5	512.8	717.9	615.4	769.2	615.4
	641.0	564.1	666.7	589.7	641.0	564.1
	641.0	589.7	641.0	512.8	641.0	589.7
	589.7	538.5	615.4	512.8	589.7	538.5
	641.0	564.1	564.1	564.1	641.0	564.1
	512.8	538.5	692.3	589.7	666.7	564.1
	512.8	512.8	666.7	564.1	564.1	538.5
	538.5	538.5	641.0	538.5	641.0	564.1
	589.7	538.5	589.7	512.8	717.9	615.4
	512.8	538.5	743.6	615.4	538.5	538.5
			641.0	564.1	589.7	538.5
			615.4	512.8	512.8	538.5
			512.8	564.1	641.0	564.1
					692.3	589.7
					589.7	589.7
					641.0	589.7
					692.3	564.1
$\overline{X}$ ± SE (µm)	593.7±18.6	548.3±6.8	623.4±17.6	554.5±9.0	630.8±13.8	566.7±5.5
N	13	13	16	16	20	20

Appendix 4-2 (continued). Measurements of length and height of juveniles reared in sand substrate and fed *N. oleoabundans*.

Date 01/28/205

Algae *N. oleoabundans*

Substrate Sand > 2mm

Replicates	1		2		3	
Growth	Length (µm)	Height (µm)	Length (µm)	Height (µm)	Length (µm)	Height (µm)
	743.6	564.1	512.8	512.8	641.0	512.8
	641.0	538.5	641.0	564.1	564.1	538.5
	641.0	512.8	512.8	512.8	538.5	512.8
	641.0	512.8	589.7	538.5	589.7	564.1
	512.8	538.5	615.4	538.5	564.1	538.5
	589.7	512.8	564.1	512.8	538.5	487.2
	615.4	538.5	641.0	589.7	512.8	461.5
			615.4	564.1	589.7	512.8
			641.0	564.1	512.8	461.5
			794.9	641.0	538.5	512.8
			512.8	487.2	589.7	538.5
			538.5	564.1	512.8	461.5
			641.0	564.1	564.1	538.5
			564.1	487.2	512.8	487.2
			512.8	512.8	641.0	589.7
			564.1	512.8	615.4	564.1
			512.8	487.2	641.0	564.1
			564.1	512.8	564.1	487.2
			538.5	512.8	615.4	538.5
			615.4	564.1		
$\overline{X}$ ± SE (µm)	626.4±26.2	531.1±7.3	584.6±15.5	537.2±8.8	570.8±10.3	519.6±8.7
N	7	7	20	20	19	19

CHAPTER 5: THE FEASIBILITY OF PEARL PRODUCTION USING THE PINK HEELSPLITTER (*POTAMILUS ALATUS*) (SAY, 1817) (BIVALVIA: UNIONIDAE)

INTRODUCTION

The frequency of natural pearl formation in mussels is low. Approximately one in 10,000 mussels may produce a valuable pearl (McGregor and Gordon 1992, Anthony and Downing 2001). The pink heelsplitter *Potamilus alatus* is one of four mussels in which natural pearls were collected in the Clinch River (Davis 2000). This finding leads to interest in the feasibility of using this species for artificial pearl formation. The pink heelsplitter is a large species with moderately thick valves, such that it has potential to produce pearls fairly quickly. Freshwater pearls vary in color, including white, cream, pink, salmon, lavender, and purple; of these, purple, pink, and lavender are most desirable (USGS 2002) and fetch high prices in the market. Pearl color is inherent and mainly associated with the nacre of mussel shells, but affected somewhat by habitat location and water quality. Nacre color of *P. alatus* is rich purple and iridescent, which may result in production of lustrous purple pearls. The purple pearls available in the market place are mostly produced from *Hyriopsis cumingii*, which has a wide variation in nacre color (white, cream, pink, purple, and gold); consequentially, purple pearls are infrequent, and hence, it is more difficult to put together matched strands of pearls. However, *P. alatus* has a uniform purple nacre, and has the capability to produce purple pearls consistently for matched jewelries.

Pearl formation is a complicated process, involving inorganic chemistry, organic chemistry, biochemistry and crystallography. The mantle tissues secrete mineral aragonites and conchiolins, which are the components of nacre. Translucent aragonite

gives pearls and shells their lustrous appearance (Hua et al. 2001). Natural pearls are formed via pathological regeneration or hyperplasia, and can be triggered by the introduction of a foreign stimulus to the mantle of a mollusk. Epidermal cells in mantle tissue secrete crystalline fluid nacre around the foreign stimulus, and subsequently layers of nacre form a pearl (Figure 5-1) (Hua et al. 2001). To culture pearls, pieces of mantle tissue, or a bead nucleus accompanied by mantle pieces, are inserted into the mantle of a live mussel, triggering the nacre-secreting processes. Pearls then are produced in a suitable habitat over a long-term culture period. Therefore, a suitable environment for grafted mussels is essential to recovery, as well as for pearl growth.

Glycogen is the primary energy reserve in adult bivalves and commonly is used to evaluate the physiological condition of freshwater mussels (Gabbott 1983, Leavitt et al. 1990, Makela and Oikari 1995, Newton et al. 2001). Haag et al. (1993) studied survival and fitness in two native bivalves, *Lampsilis radiata* and *Amblema plicata*, fouled by the zebra mussel and concluded that low glycogen is the immediate threat to survival and reproduction of these bivalves. Later, Hallac and Marsden (2001) supported this conclusion by studying the glycogen stores in zebra mussel-fouled *Elliptio complanata*; glycogen content was 50 % lower in fouled mussels than in unfouled ones.

The objectives of this study were as follows: 1) to test the feasibility of pearl formation in *P. alatus* by using three surgical treatments and comparing the survival and pearl formation in two holding ponds, and 2) compare glycogen content of implanted mussels to that of wild specimens to assess health and pearl growth.

METHODS AND MATERIALS

Collection and sites

Mussels were collected at the same location described in Chapter 1. Experimental mussels were shipped under cool temperatures, quarantined in a 1135 L round tank for 1 mo, and checked by biologist Brian Watson (Virginia Department of Game and Inland Fisheries) on 17 May and 14 July 2004. Mussels were tagged and measured after the quarantine. The experiment was conducted in the pond at FMCC (described in Chapter 1) and in the Duck Pond which, is located at the west end of the Virginia Tech campus. The Duck pond is fed by two branches of Stroubles Creek that drain approximately 2.89 km^2, including most of Blacksburg and the Virginia Tech campus.

Surgical operation

The surgeries for non-nucleated and nucleated insertion were conducted in two steps: the first step was making pieces of mantle tissue, and the second was the transplantation of these pieces into live mussels. Mantle tissues at the edge of a sacrificed mussel were split from the mussel body and separated into two layers. The epidermal strips of the mantle tissues were cut into square pieces of approximately 2 mm × 2 mm for transplantation. For the treatment of non-nucleated operation, ten pieces of mantle tissue were transplanted into a living mussel at the posterior end. The transplanted mantle tissues were connected tightly with the recipient live mussel's mantle tissue, so that cell division and multiplication would create a pocket-like pearl sac for depositing nacre. The nacre was initially soft and glue-like, and later hardened to became a pearl. Pearls then grew gradually as more nacre was deposited. For the insertion of nuclei, 4-5 shaped shell

nuclei (i. e., shell beads) were transplanted into a living mussel along with mantle tissue pieces. The transplanted mantle tissue pieces then experienced cell division and multiplication to form a pearl sac surrounding each nucleus. At the same time, the pearl sac secreted nacre to coat each nucleus with a pearl layer (Figure 5-2). Image pearls (an image covered by pearly nacre) were obtained by inserting a sculpted nucleus into the cavity between the shell and mantle of the operated mussel, then to be covered by the secreted nacre. The inserted images were made from wax and oval-shaped (25 mm × 35 mm), with a distinct convex surface design (Hua and Gu 2002). Surgical operations on *P. alatus* by the three methods were performed in the laboratory at 20℃ from 11 to 16 July 2004. Surgeries were performed using special tools, including needles, hooks, knives, forceps, mussel opener, scissors, sponger, glass board and mussel holder (Figure 5-3). Twenty implanted mussels for each operation then were held in pocket nets and suspended in the two ponds for pearl culture. Mussels were suspended at a depth of 80 cm below the water surface with floats and fixed lines.

Sampling and measurements

Survival rate and pearl formation were monitored monthly, except January and March 2005, because of ice cover on the ponds. Pearl formation was assessed by sampling 2 pearls from each living mussel and measuring pearl weights. Glycogen was measured using the same method described in Chapter 1, to compare the difference between wild mussels and mussels stocked in the ponds.

To quantify environmental conditions, pH, DO, temperature, alkalinity, and total ammonia nitrogen (TAN) were measured. Dominant did algae were identified during the culture period, and amount of suspended organic matter was determined by ash-free dry

weight. A Hobo temperature logger was placed in the pond to continuously record temperature. Sampling was conducted each month, except January and March 2005, due to ice cover. DO, temperature, alkalinity, pH, and TAN were measured following methods described in Chapter 4.

Experimental design

One hundred and twenty mussels were randomly assigned to the 3 treatments (20 mussels/pocket nets) in two ponds. The surgically implanted mussels were held in two ponds (t = 2 X 3, n = 20 per treatment). Data for two surgical treatments and one control (no surgery) in two holding ponds were compared. This study lasted for 10 mo, from July 2004 to May 2005.

Data analyses

Data were analyzed by JMP (SAS Institute Inc. Cary, NC, 2001) to compare survival rates of implanted mussels for the two types of transplantation (non-nucleated and image insertion) and the two holding ponds, and to assess significance of differences in pearl formation among treatments. Monthly survival rate of mussels was determined as the percentage of live mussels compared to the precious month. Glycogen content was measured at the end of the experiment and compared among treatments. Additionally, pH, DO, temperature, alkalinity, and TAN were analyzed. Due to small sample size, the normality of these data was tested using Kolmogorov - Smirnov test (Sall et al. 2001). I then used the rank-based, nonparametric Wilcoxon test to test the probability of significant differences among non-normal distributions of survival rates; and ANOVA for normal distribution of glycogen content of mussels in two pond and treatments, compared to wild mussels. Ultimately, data were analyzed by LSD to compare differences among

variables. A Paired *t*-test was used to test significance of differences of pearl growth over time in two ponds; and ANOVA was used for analysis of water quality except for pH (Wilcoxon test).

RESULTS

Monthly survival rates of mussels in the Duck pond (94.7 %) and the FMCC pond (97.6 %) were not significantly different ($p = 0.0512$, $n = 9$) at $\alpha = 0.05$ level. Furthermore, monthly survival rate of mussels between the two surgical operations and the no surgery control (NSC) were not significantly different in two ponds ($p = 0.881$, $n = 18$), as well as in Duck pond ($p = 0.851$, $n = 9$) and FMCC pond ($p = 0.915$, $n = 9$) (Table 5-1). Final survival rates through 10 mo (Figure 5-4) were higher in the pond at FMCC (80 %) than in the Duck pond (60 %) (Table 5-2), but statistical analysis was not possible due to inadequate sample size ($n = 1$).

Non-nucleated implanted (NNI) pearls were obtained after 44 d from captive mussels in the two ponds. At monthly intervals, sampled pearls were weighed and compared, with the average weight (mg) per pearl, as some tiny pearls could not be weighed individually. Statistical analysis showed no significant difference in pearl weight between the two ponds ($p = 0.562$, $n = 14$). Overall, pearls in the mantle tissue of mussels were growing incrementally through the 10 mo period (Figure 5-5), although variation in pearl weights was high (Appendix 5-1). Image pearls (IP) also began to form after 40 d, and the surface pearl layer grew over time, with purple nacre or purplish coating on the nucleus surface (Figure 5-6). The nucleated operation failed because the quarantined mussels were unable to sustain shell nuclei within their weak mantle tissues.

Comparison of glycogen content in mussels that experienced the two surgical operations, the NSC in both ponds, and wild specimens resulted in highly significant differences ($p < 0.0001$, $n = 3$) between ponds and the wild specimens (Figure 5-7). The lower glycogen content occurred in FMCC pond mussels (262.6 ± 43.4 mg/g DWT), but there was no significant difference between the wild mussels (868.2 ± 39.0 mg/g DWT) and mussels held in the Duck pond (688.7 ± 60.6 mg/g DWT) (Table 5-3). Comparison of mussels subjected to surgery and the NSC mussels showed no significant difference in both ponds (Duck pond: $p = 0.418$, $n = 3$; FMCC pond: $p = 0.791$, $n = 3$) (Table 5-4).

Water quality parameters of dissolved oxygen, temperature, pH, alkalinity, total ammonia nitrogen and suspended organic matter showed some differences between the two ponds. Mean values differed significantly in alkalinity, TAN and organic matter ($p < 0.05$, $n = 10$), but not significantly in dissolved oxygen, water temperature and pH ($p > 0.05$, $n = 10$) (Table 5-5) (Appendix 5-3).

Daily temperature (Figure 5-8A), the average of measurements from hourly records in the Duck pond, were comparable to temperatures in the FMCC pond during the experiment (Figure 5-8B). Highest temperature was 24.1 ℃ in the Duck pond on 4 August 2004, while it was 28.1 ℃ on 3 August in the FMCC pond. Lowest temperature was 2.0℃ in the Duck pond on 20 December 2004, while it was 2.3 ℃ in the FMCC pond on 15 December 2004. Because water temperature data in Duck pond from 26 August to 24 October 2004 were lost due to malfunction of the Hobo temperature logger, I applied a trendline (dashed in Figure 5-8A, 7B) to connect the discontinuous data.

Algae were collected and idendified monthly from July 2004 to May 2005 to assess

species richness and identify dominant algae in the ponds. The dominant algal genera were *Bracteacoccus, Chlorella, Cryptomonas, Euglena, Melosira, Navicula, Scenedesmus* and *Cryptomonas* in the Duck pond; while *Cylindrotheca, Navicula,* and *Dinobryon* dominated in the FMCC pond (Appendix 5-2).

DISCUSSION

Mean survival rate of implanted mussels was comparable to for the no surgery control mussels in both ponds during the 10 mo period (Table 5-1), indicating that neither the NNI nor the image insertion influenced survival. Final survival also did not differ among them, with survival of 80 % in FMCC pond and 65 %, 50 %, and 65 % among the respective treatments in the Duck pond. Although there was no significant difference in survival among the three treatments, somewhat lower survival occurred in mussels implanted for the IP surgery (Figure 5-4 Table 5-1). Mussels used for producing IP were filled with a large wax insert of 25 mm × 35 mm in size. This large insert may have stressed the mussel and contributed to higher mortality. By comparison, only soft, thin, and small (2 mm × 2 mm) pieces of mantle tissue were transplanted into the grafted mussel for the NNI. In this study, survival of mussels from the NNI was as high as for mussels of NSC (Tables 5-1 and 5-2). Pieces of mantle tissue from the same species usually are accepted by grafted mussels, since antigens from the mantle piece are likely to be compatible to antibodies of the grafted mussels. For example, mantle tissues of the pearl mussels, *H. cumingii, H. schlegeli, Anodonta woodiana,* and *Anodonta calipygo* can create pearl sacs and produce pearls in grafted mussels when they are transplanted within the species (Zhang 1975, Xiong et al. 1980, Hua et al. 2001).

The survival rate of mussels in FMCC pond was slightly higher than that of mussels in the Duck pond, although not statistically different. Likewise, survival rates through 10 mo showed higher survival in the FMCC pond. The difference in survival may relate to the content of TAN, with a concentration of 0.25 (± 0.04) mg/L in the Duck pond and only 0.04 (± 0.01) mg/L in FMCC pond (Table 5-5). The TAN includes the toxic un-ionized species of ammonia (NH_3) and the ionized species (NH_4^+). The un-ionized ammonia concentration can differ widely with pH and temperature. Previous research suggests that unionid mussels are more sensitive to un-ionized ammonia (NH_3) relative to fish (Arthur et al, 1987, Hickey and Vickers 1994, Downing and Merkens 1995). Scheller (1997) found the average 96-hr LC50s of juvenile *Villosa iris* and adult *Pyganodon grandis were* 0.49 and 0.52 mg/L NH_3, respectively. Augspurger et al. (2003), using TAN concentration to reflect the ammonia toxicity on ten species in eight unionid genera, found 24 to 96 h LC50s for mean acute values ranged from 1.75 to 2.50 mg/L TAN (at pH = 8). Furthermore, the chronic ammonia exposure value was estimated at ranges of 0.3 - 1.0 mg/L TAN (at pH = 8). Although the LC50 for TAN to adult *P. alatus* is unknown, the mean of 0.25 mg/L TAN in the Duck pond was less than the USEPA continuous criteria concentration (CCC) of 1.24 mg/L total ammonia as N (at pH = 8, T = 25℃) (EPA 1999). The suggested minimum of chronic ammonia exposure for unionids is 0.3 mg/L TAN (pH = 8) (Augspurger et al. 2003). In this study, TAN concentrations exceeded 0.3 mg/L several times, 0.31 mg/L (pH = 7.85, T = 23.8 ℃), 0.51 mg/L (pH = 6.6, T = 18.3), and 0.41 mg/L (pH = 7.64, T = 6.5), respectively, in the Duck pond in July and September 2004, and February 2005 (Appendix 5-3). Thus, the periodic high TAN concentrations may have induced greater mortality of mussels in the Duck pond. Because

the Duck pond receives storm runoff, contaminants can enter the pond to affect mussel survival as well. Further, toxic blue-green algae of the genera *Microcystis* and *Nodularia* were common in the Duck pond (Appendix 5-2), and may have affected feeding and condition of mussels during blooming periods.

The NNI pearls were first obtained in mussels with transplanted pieces of mantle tissue after 44 d (16 July to 29 August 2004) when water temperature was around 21℃. However, some pearls were not detected from their pearl sacs, and the pearl sacs were still soft in the same mussels in which some pearls had been formed, due to the complicated processes of pearl formation. Therefore, I estimated that pearl formation in *P. alatus* requires approximately 40 d after transplantation, and pearl sac formation should occur in less than 40 d, since pearls were generated from the nacre deposited by the pearl sac. Pearl sac formation varies among mussel species and environmental conditions such as temperature and season; it requires 14 d in *A. woodiana* and 20 d in *H. cumingii* as water temperature is 20℃ in spring (Hu and Shi 1995). The rate of pearl sac formation increases when temperature rises (Hua et al. 2001). Likewise, it takes some time for nacre (pearl) to be deposited after pearl sac formation; for example, it requires about 10 - 12 d for *H. cumininqii* at 18 - 20℃. Compared to *H. cumingii*, the pink heelsplitter may form a pearl sac in 25 d at 21℃, and 15 - 20 d for pearl deposition after the pearl sac is formed. Therefore, *P. alatus* likely has suitable traits for pearl sac formation and pearl deposition, comparable to the pearl - producing mussel *H. cumingii,* although rate of pearl formation is somewhat slower.

Variations in pearl formation or production by mussels were very high (Appendix 5-1), as some pearls were still tiny and unmeasurable in November 2004. The main reason

is the complex process of pearl formation, involving inorganic chemistry, organic chemistry, biochemistry and crystallography (Hua et al. 2001, Hu and Shi 1994). Pearl growth rate is based on the rate of nacre secretion and deposition by the pearl sac, which varies by mussel species, health condition, and environmental quality. Hu and Shi (1995) found that even within a pearl sac in grafted mussels of *Anodonta woodiana*, epithelial cells showed inconsistent development; a few irregular cells occurred after 35 - 42 d, while others exhibited orderly columnar shapes. The irregular cells can negatively affect pearl quality. In this experiment, pearls sampled from the same mussels had high variation in size and weight, which supports previously reported results. My limitation in pearl sampling was the small sample size of implanted mussels, which may have masked minor differences between ponds, varying in environmental conditions among culture seasons. However, results of monthly pearl samples in both ponds show an increasing trend with culture time (Figure 5-5). The largest pearl reached 1mm × 3 mm from a mussel (A406) in the FMCC pond sampled on 2 April 2005. Pearls were colored purple and pink, the same as the nacre color of sacrificed mussels used for mantle tissue preparation (Figure 5-6), as pearls were deposited by the pearl sac epithelium cells from the transplanted mantle tissues.

Mantle tissue of mussels is critical for pearl production (Hua 2001), shell growth (McMahon 1991), and physiological function and energy storage (Gabbott & Bayne 1973, Fraser 1989); therefore, it was used for glycogen determination in this study. The surgically implanted mussels would presumably consume more energy to support pearl growth, as reported for gravid female mussels or for mussels fouled by zebra mussels (Haag et al. 1993, Hallac and Marsden 2001). However, the glycogen data for mussels

from the Duck pond showed no significant difference in glycogen content among surgically implanted, control and wild mussels, but the values were significantly higher than those in mussels at the FMCC pond, indicating that environmental conditions in the Duck pond generally met the needs of *P. alatus*. Water quality in both ponds was mostly suitable for mussel growth, with sufficient DO (8.0 mg/L), stable pH (< 8), alkalinity (140 mg/L CaCO3), and mean water temperature (15℃). However, algal richness and abundance and organic matter differed. Algae and detritus (organic and inorganic) are the main diet components for mussels (Way 1989, Gatenby et al. 2000), and particle concentration and size of food affects filtration rate (Navarro and Winter 1982, Roper and Hickey 1995, Vanderploeg et al. 1995). Therefore, mussel nutrition was affected by differences in the abundance of the food resources in the ponds. Algae data, based on abundance and diversity of species, indicates that algae in the Duck pond provide more food when compared to the FMCC pond (Appendix 5-2). Furthermore, dominant genera varied between the two ponds throughout the experiment, with 8 genera in the Duck pond and only 3 in the FMCC pond. *Cylindrotheca* bloomed in the FMCC pond in August 2004, but biomass dropped rapidly in September 2004. Algae in the FMCC pond were scarce in October 2004 and May 2005.

Additionally, the significant difference in organic matter between ponds, with a mean value 4.2 mg/L in the Duck pond and 1.3 mg/L in the FMCC pond, would tend to support better growth in the Duck pond, resulting in good physiological condition and high glycogen content in mussels. Conversely, water in the FMCC pond was clear and low in suspended organic matter, resulting in oligotrophic conditions and low glycogen content in mussel tissues. Previous studies on the selection of organic matter by bivalves reported

that organic matter was an important food resource, capable of being ingested and then digested or expelled (Hylleberg and Gallucci1975, Newell et al. 1989, Kasai 2004). Therefore, the suspended organic matter, as a food supplement as well as an important food resource, may contribute to improved condition of mussels for pearl culture.

The implanted shell nuclei were not retained in the surgically implanted mussels during the nucleated operation since the mantle tissue seemingly became very weak and thin after one month quarantine, incapable of sustaining implanted shell nuclei. Therefore, successful nucleated implants require further research.

CONCLUSION AND COMMENTS

NNI pearls and image pearls were successfully produced in *P. alatus,* and pearl formation was not significantly different between the Duck pond and the FMCC pond. Purple NNI pearls and image pearls were produced, although growth rate of pearl in *P. alatus* was lower than in *H. cumingii*. Therefore, additional research should focus on increasing pearl growth rate by: 1) propagating juveniles or collecting young mussels for surgical operation since young mussels have faster growth rates; i. e., the suitable size of *H. cumingii* for surgery of pearl production is about 6 -10 cm (Hua 2003b); 2) optimizing food availability in the pond to meet the requirements of mussel and pearl growth; 3) modifying the surgical procedure to form a roll of mantle tissue for transplantation; 4) and experimenting with wax nuclei instead of heavy shell nuclei, and enhancing surgical mussel health condition for the nucleated implants to produce commercial pearls within a shortened period. Based on the results of this study, valuable image pearls could be produced by implanting fine-designed nuclei within two years.

Mussels in the Duck pond contained high glycogen levels comparable to those in wild mussels, suggesting a suitable pond environment for holding implanted mussels in captivity. However, the TAN level of 0.25 mg/L (pH < 8) could be problematic for long-term holding of *P. alatu*s. Mussels in the FMCC pond exhibited low glycogen, and limited richness and abundance of algae indicated oligotrophic conditions, unable to meet the optimal food demand of mussels. Therefore, intensive pond management should be implemented in the future, to include such options as addition of soy bean milk or other additives which have been shown to facilitate mussel growth (Hua et al. 2001). A fertilization regimen is the most important management recommendation to increase algae growth and abundance. Therefore, a suitable fertilizer should to be selected based on water quality, substrate conditions, and water temperatures. Stocking more fish and additional species, and providing pelleted food to those fish will also fertilize pond water. Higher fertilization should increase phytoplankton and impair photosynthesis of submersed vascular plants by reducing water transparency. Other management schemes to maximize suspended algae and decrease rooted macrophytes should be evaluated.

Table 5-1. Mean monthly survival rates (± 1 SE) of mussels surgically implanted with image, non-nucleated implants, and control mussels during the 10 mo period in two ponds. Survival rate was determined monthly as the percent of live mussels. *P*-values was determined by Kruskal-Wallis tests, with $\alpha = 0.05$ significant level ($n = 9$).

Pond	Control (%)	Image (%)	Non-nucleated (%)	P value
Duck pond	95.5 ± 2.0	93.1 ± 3.2	95.5 ± 1.7	0.851
FMCC pond	97.6 ± 1.3	97.6 ± 1.3	97.6 ± 1.0	0.915
Between two ponds				0.051

Table 5-2. Comparison of survival rates of mussels surgically implanted with image and non-nucleated implant and control mussels through 10 mo in two ponds.

Pond	Control (%)	Image (%)	Non-nucleated (%)
Duck pond	65	50	65
FMCC pond	80	80	80

Table 5-3. Comparison of glycogen content (± 1SE) in dry weight (DWT) of mussels cultured in two ponds for pearl formation and in wild mussels collected at the end of the experiment. *P*-values were determined by ANOVA tests, with $\alpha = 0.05$ significant level. Means followed by the same letter are not significantly different at $\alpha = 0.05$ level using LSD.

Sample site	*N*	Glycogen ± SE (mg/g)
Duck pond	9	688.7 ± 60.6 [a]
FMCC pond	9	262.6 ± 43.4 [b]
Wild *	3	868.2 ± 38.9 [a]

* Wild = Kentucky Lake

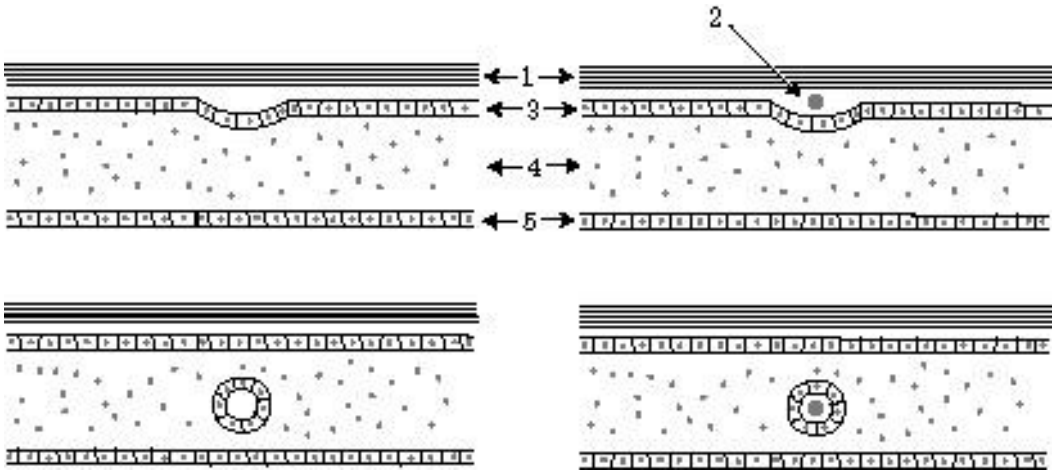
Table 5-4. Comparison of glycogen content (± 1SE) in dry weight (DWT) of mussels among treatments of non-nucleated implanted (NNI), image pearl implanted (IPI) and no surgery control (NSC) in both ponds at the end of the experiment. *P*-values were determined by ANOVA tests, with $\alpha = 0.05$ significant level ($n = 3$).

Pond	NNI (mg/g)	IPI (mg/g)	NSC (mg/g)	P value
Duk pond	734.7 ± 60.5	568.1 ± 161.8	763.2 ± 55.7	0.418
FMCC pond	273.1 ± 51.2	297.6 ± 129.9	217.2 ± 37.0	0.791
Between two ponds				< 0.0001

Table 5-5. Mean values (± 1SE) of water quality in both ponds during the experimental period (July 2004 to May 2005). P-values were determined by ANOVA, except for pH (Wilcoxon test), with $\alpha = 0.05$ significant level. Significant differences are represented by * in *P* value column ($n = 10$).

Parameters	Duck pond	FMCC pond	P value
DO (mg/L)	8.0 ± 0.9	9.3 ± 0.9	0.2855
T (℃)	14.3 ± 1.9	15.4 ± 2.4	0.7417
pH	7.7 ± 0.1	7.9 ± 0.1	0.0886
Alkalinity (mg/L)	182.8 ± 8.1	139.7 ± 6.0	0.0005 *
TAN (mg/L)	0.25 ± 0.04	0.04 ± 0.01	< 0.0001 *
OM (mg/L)	4.2 ± 0.6	1.3 ± 0.2	0.0002 *

TAN = total ammonia nitrogen
OM = organic matter.



Natural pearl formation without nucleus Natural pearl formation with irritant

Fig. 5-1. Diagram of the process of natural pearl formation in the mantle tissue of bivalves. 1. Shell. 2. Irritant (nucleus). 3. Outer epidermis cells of mantle. 4. Tissue of mantle 5. Inner epidermis cells of mantle.

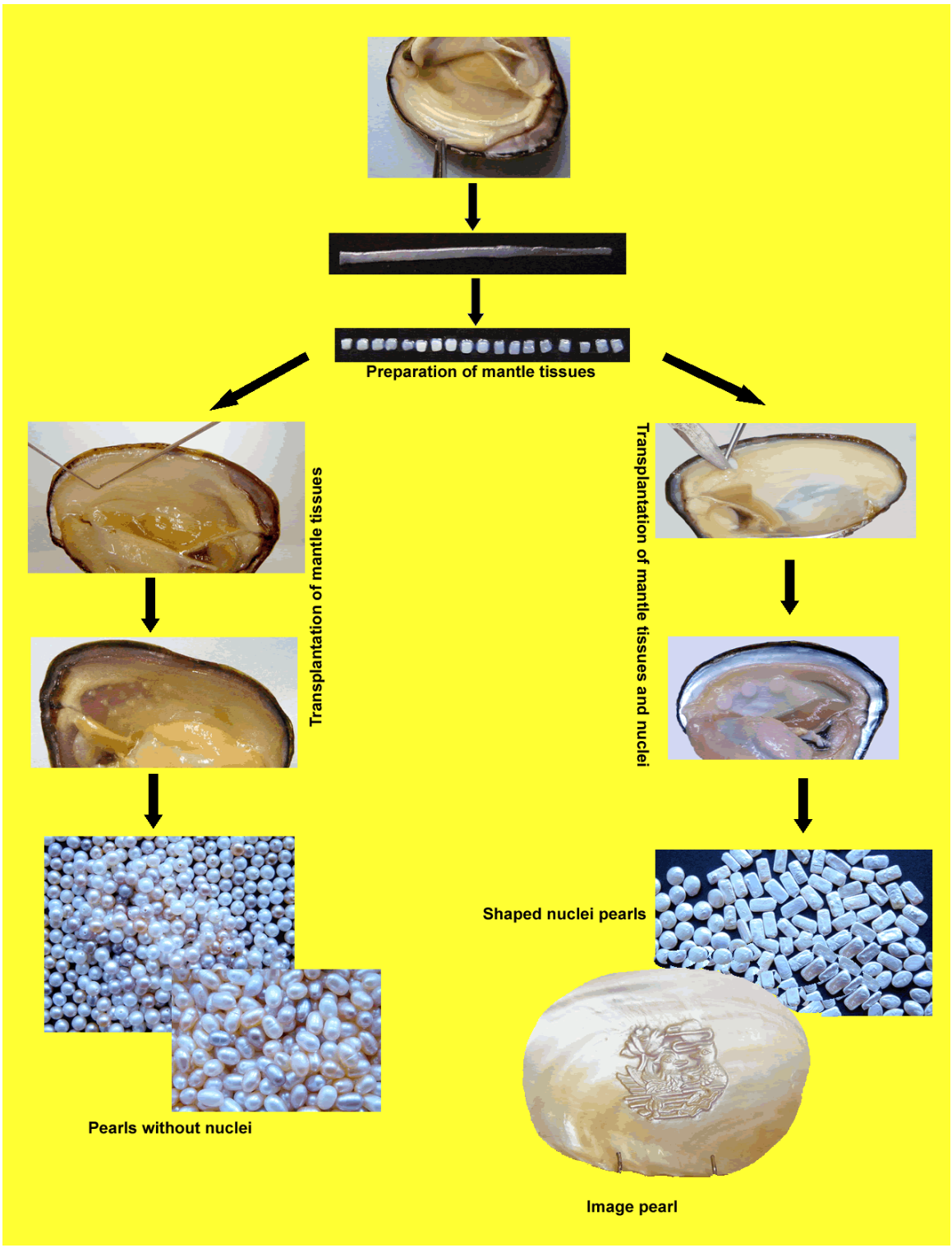


Fig. 5-2. Process of surgical operation for non-nucleated and nucleated pearl formation.

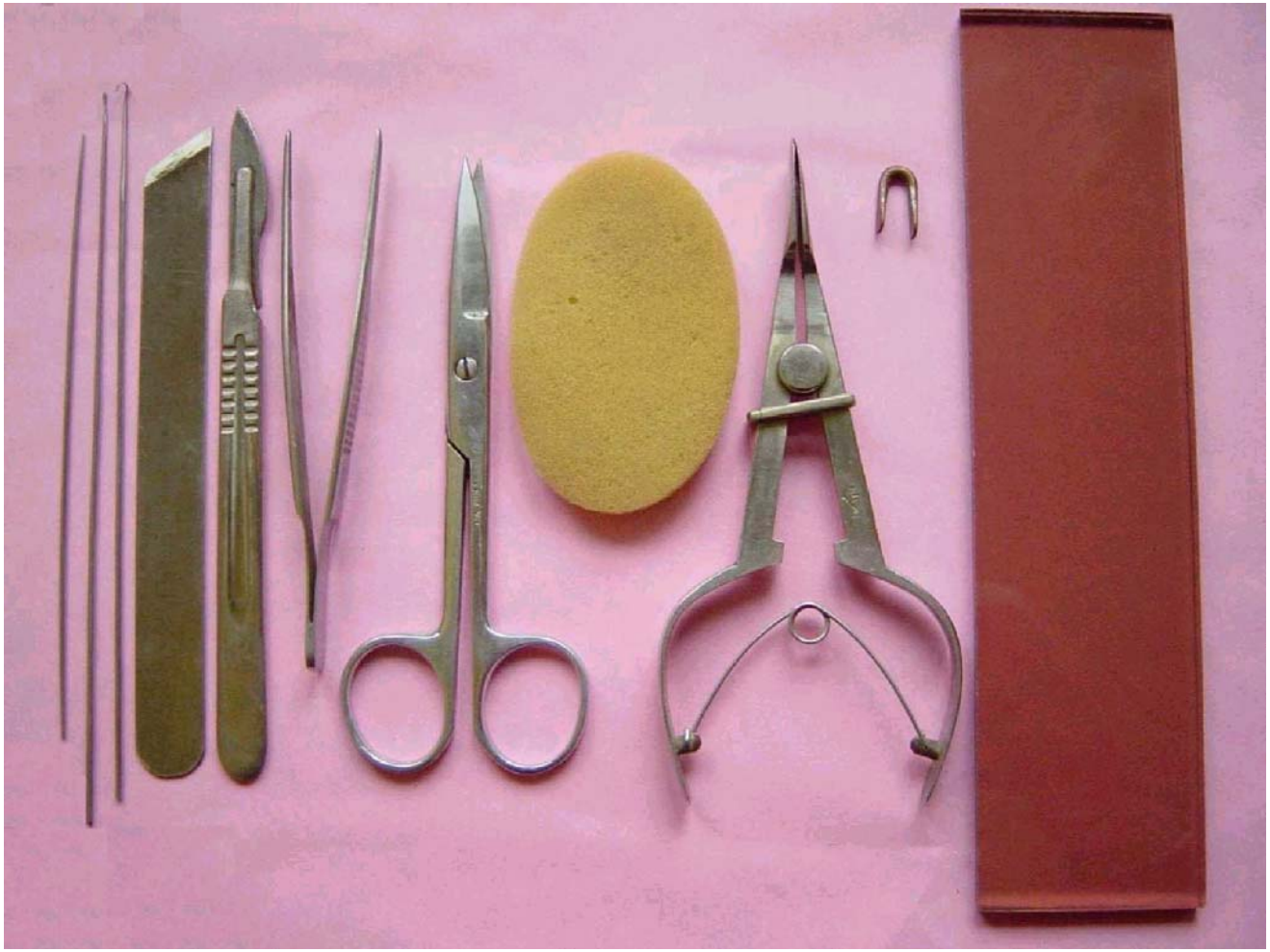


Fig. 5-3. Surgical tools use for mantle tissue preparation and transplantation.

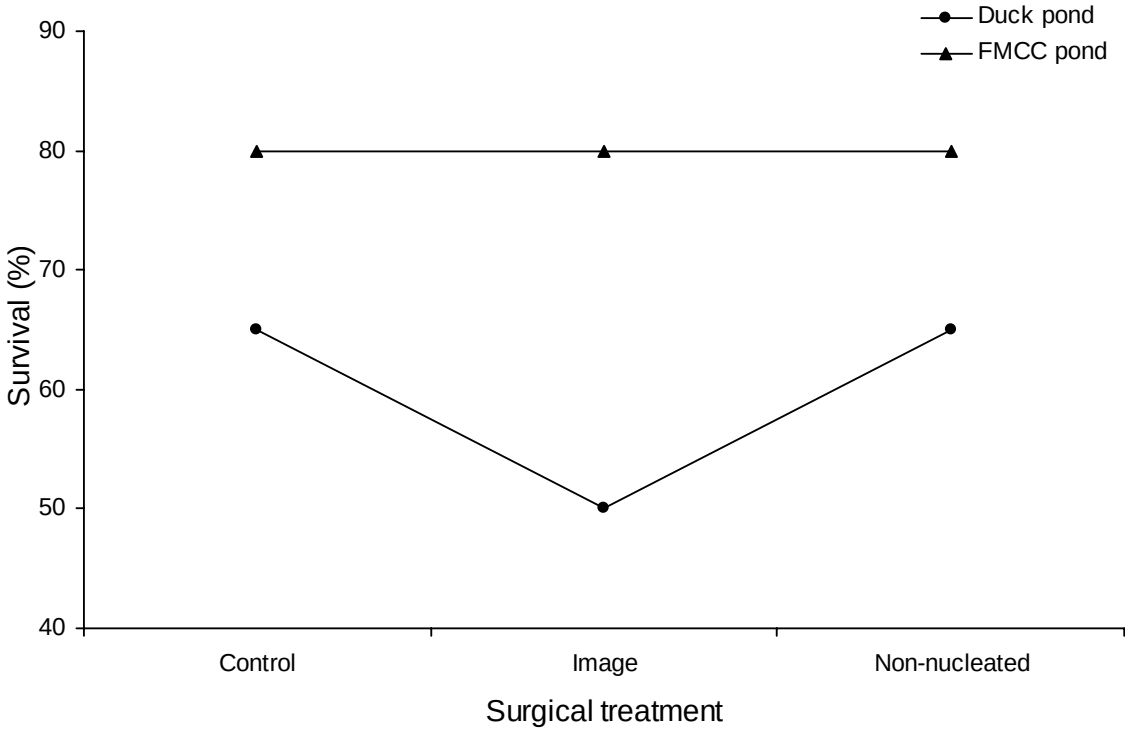


Fig. 5-4. Survival of mussels surgically implanted with image and non-nucleated implants, compared to control mussels after 10 mo in the Duck pond and FMCC pond.

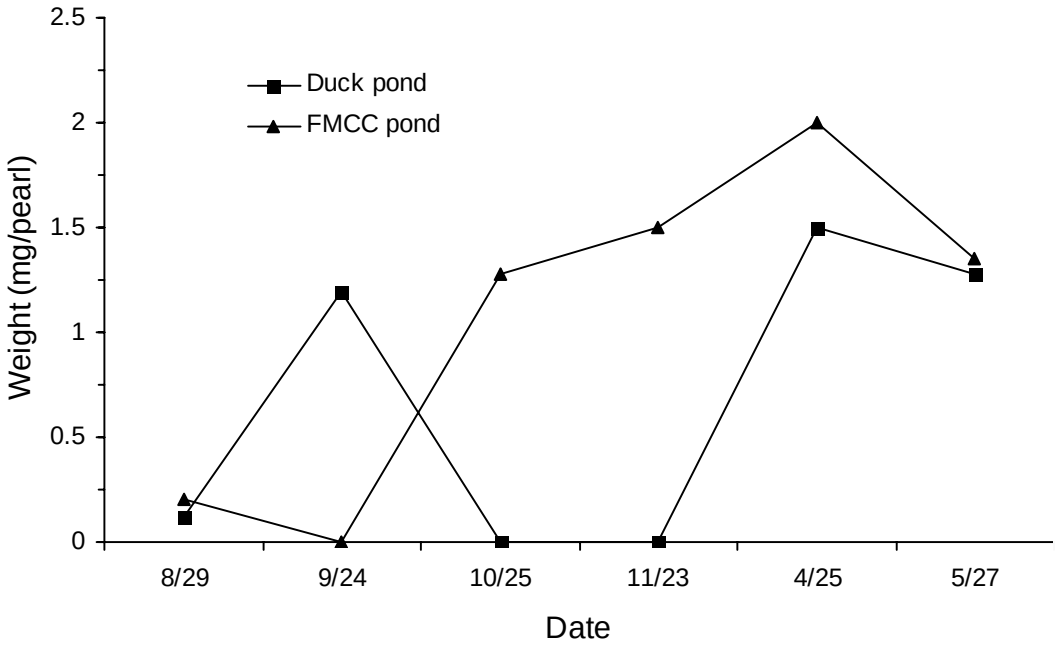
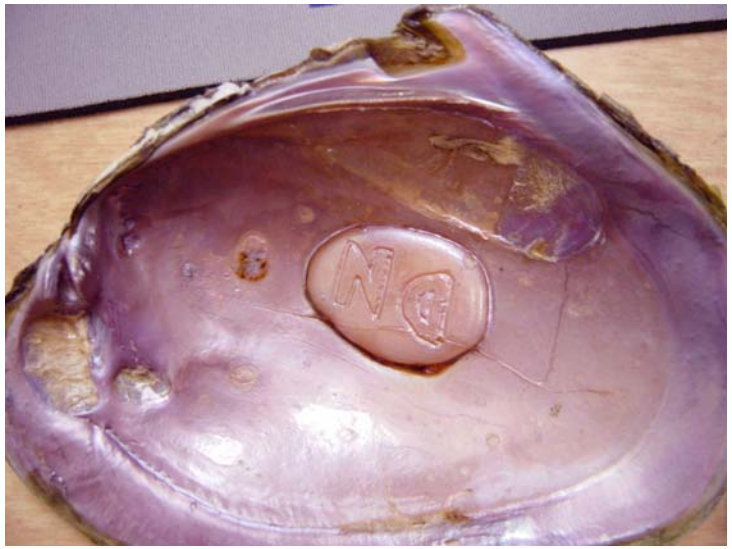


Fig. 5-5. Mean pearl weight (mg/pearl) in mussels surgically implanted with mantle tissue (non-nucleated) in the two ponds over 10 mo, from August 2004 to May 2005.



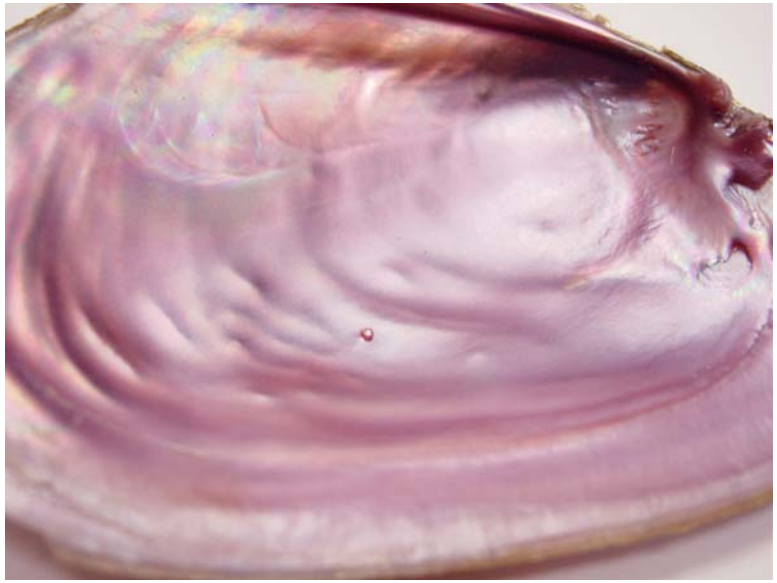


Fig. 5-6. Purple image pearl with “DN” symbol (top left), purplish image pearl with “VT” symbol (top right), and one non-nucleated pearl (bottom left) with purple color sampled on 27 May 2005.

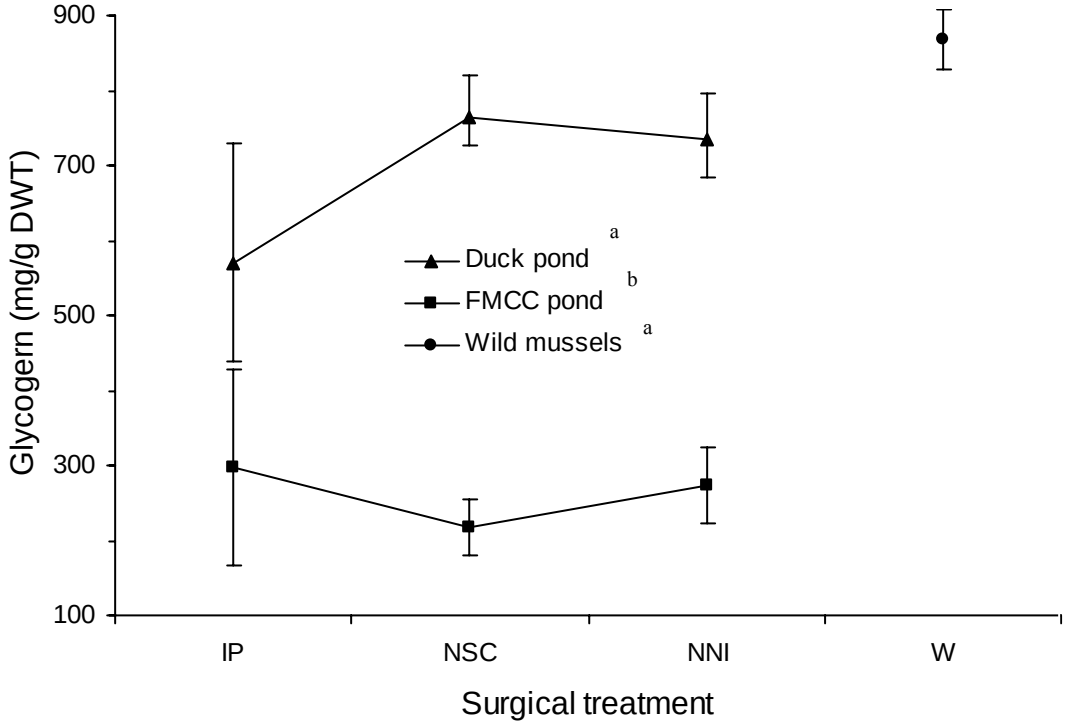


Fig. 5-7. Mean glycogen content (± 1 SE) in dry weight (DWT) of mussel tissue for pearl production in two ponds and wild mussels sampled at the end of the experiment on 27 May 2005. (IP = Image pearl implant, NSC = No surgery control, NNI = Non-nucleated implant, W = wild mussels from Kentucky Lake). The same letter is represented that mean glycogen contents of mussels do not differ at $\alpha = 0.05$ level using LSD.

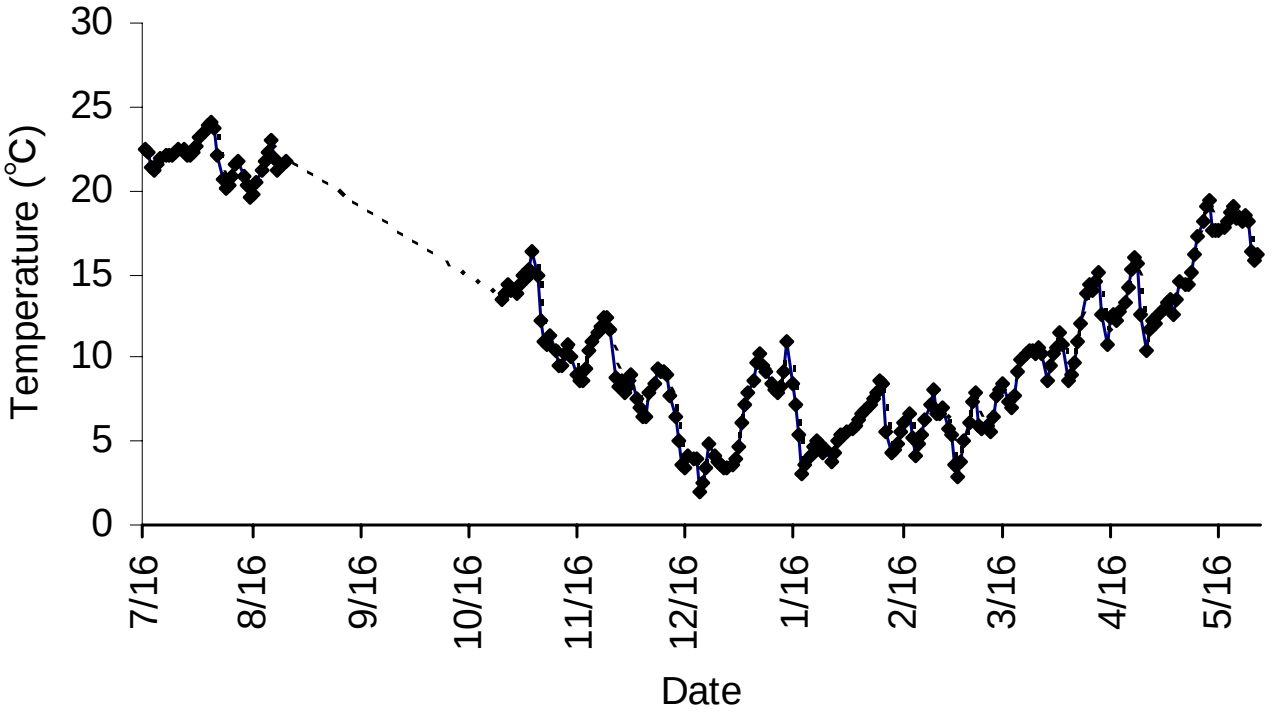


Fig. 5-8A. Water temperatures in the Duck pond for 10 mo from July 2004 to May 2005. A trendline was used to connect the missing date for 26 August to 24 October 2004, due to malfunction of temperature logger.

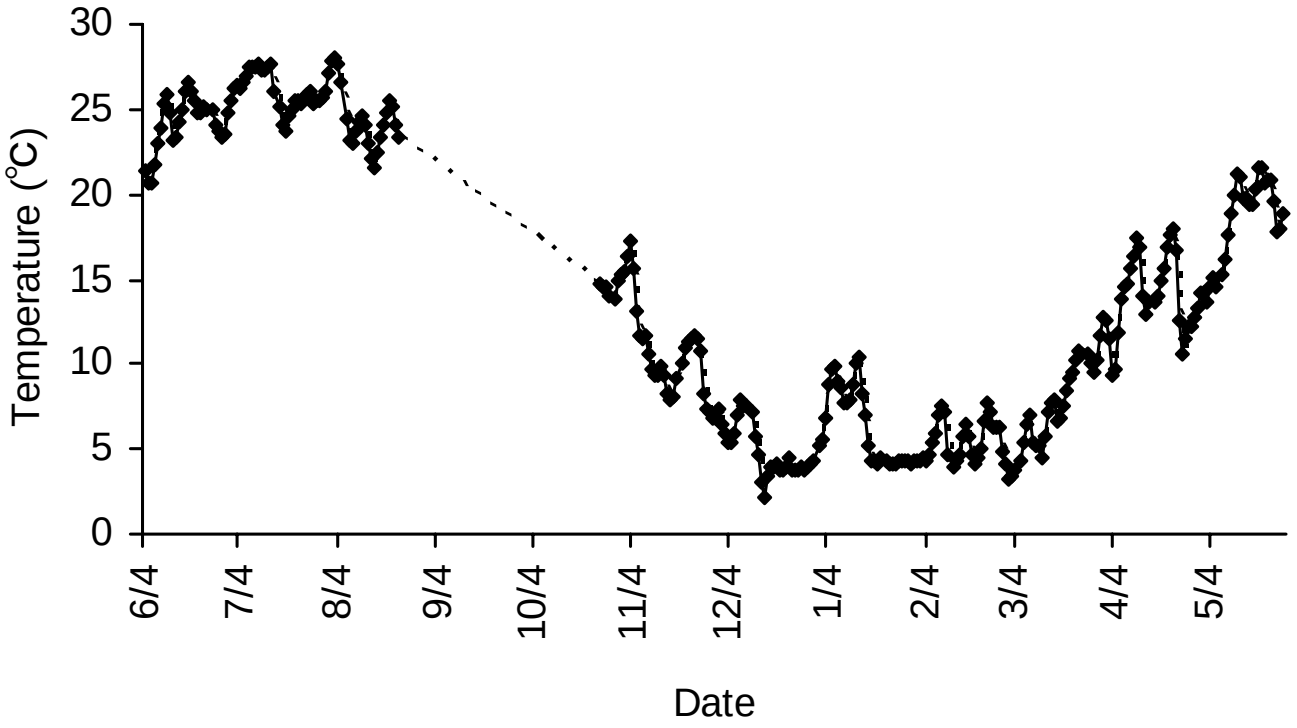


Fig. 5-8B. Water temperature in FMCC pond throughout 1 yr from June 2004 to May 2005. A trendline was used to connect the missing date for 24 August to 24 October 2004, due to the malfunction of temperature logger.

Appendix 5-1. Weights of pearls from sampled mussels with non-nucleated implants during the experimental period from August 2004 to May 2005 ($p = 0.562$, $n = 14$).

	Duck pond				FMCC pond			
Date	Mussel No.	No. of pearls	W (mg)	W (mg/pearl)	Mussel No.	No. of pearls	W (mg)	W (mg/pearl)
29/08/2004	A011	2	0.20	0.10	A038	2	1.00	0.50
29/08/2004	A099	2	0.50	0.25	A110	2	0.00	0.00
29/08/2004	N3	1	0.00	0.00	A108	1	0.10	0.10
24/09/2004	A119	3	5.30	1.77	A036	1	0.00	0.00
24/09/2004	A092	2	3.60	1.80	A039	2	0.00	0.00
24/09/2004	A037	2	0.00	0.00	A118	2	4.60	2.30
25/10/2004	A043	2	0.00	0.00	A102	2	0.00	0.00
25/10/2004	A101	1	0.00	0.00	A104	2	3.10	1.55
23/11/2004	N6	1	0.00	0.00	A021	4	6.00	1.50
25/04/2005	A044	2	5.80	2.90	A406	1	4.00	4.00
25/04/2005	53	1	0.10	0.10	A012	1	2.00	2.00
27/05/2005	A044	4	8.10	2.03	A046	1	0.00	0.00
27/05/2005	53	2	3.60	1.80	N5	4	18.90	4.73
27/05/2005	102	1	0.00	0.00	105	4	6.70	1.68
$\overline{X} \pm$ SE (mg/pearl)				0.77 ± 0.28				1.00 ± 0.26

Appendix 5-2. Algae observed in the Duck pond (D) and FMCC pond (F) from July 2004 to May 2005. Dominant algae are represented by O and other algae are represented by X.

Algae	Date of samples																			
(Phylum)	7/22		8/25		9/20		10/20		11/19		12/23		2/22		3/21		4/21		5/27	
Genus	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F
Blue-green Algae (Cyanoprokaryota)																				
Dactylococcopsis	X																			
Merismopedia			X																	
Microcystis											O									
Nodularia															O					
Oscillatoria								X												
Green Algae (Chlorophyta)																				
Ankistrodesmus																			O	
Asterococcus			X		X															
Bracteacoccus	O		O		X				X	X										
Chlamydomonas	X																			
Chlorella		O	O				X	X	O				X				O		O	
Closteriopsis			O																	

Appendix 5-2 (continued). Algae observed in the Duck pond (D) and FMCC pond (F) from July 2004 to May 2005. Dominant algae are represented by O and other algae are represented by X.

Algae	Date of samples																			
(Phylum)	7/22		8/25		9/20		10/20		11/19		12/23		2/22		3/21		4/21		5/27	
Genus	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F
Coelastrum	X	X	O																	
Crucigenia			X																	
Gloeocystis	X	O																		
Oocyctis			X														X	O		
Scenedesmus	O	X			X						X							X	X	
Schroederia	X	X																		
Selenastrum		X																		
Sphaerellopsis		O																		
Sphaerocystis														X						
Spirogyra	X																			
Stichococcus									X											
Tetradesmus																		X		
Tetraedron														X						X
Volvox			O														X			

Appendix 5-2 (continued). Algae observed in the Duck pond (D) and FMCC pond (F) from July 2004 to May 2005. Dominant algae are represented by O and other algae are represented by X.

Algae	Date of samples																			
(Phylum)	7/22		8/25		9/20		10/20		11/19		12/23		2/22		3/21		4/21		5/27	
Genus	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F
Diatoms																				
(Bacillariophyceae)																				
Cyclotella															X					
Cylindrotheca	X	O		O*	O	O*		O		O		O	X			O		X	O	
Cymbella															X					
Diatomella	O	X																		
Diploneis												X								
Fragilaria																	X			
Frustulia		X																		
Melosira	X	X							X		X								X	
Navicula									X	X		X	X	X	O	O	O	O	X	
Nitzschia													X							
Pinnularia															O					
Surirella											X						X			
Synedra														O	O	X				
Tabellaria															X		O			

Appendix 5-2 (continued). Algae observed in the Duck pond (D) and FMCC pond (F) from July 2004 to May 2005. Dominant algae are represented by O and other algae are represented by X.

Algae	Date of samples																			
(Phylum)	7/22		8/25		9/20		10/20		11/19		12/23		2/22		3/21		4/21		5/27	
Genus	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F
Euglenoids (Euglenophyta)																				
Euglena		O	O				X						X		X	X	X		X	X
Phacus	O	X																		
Trachelomonas	X		O						X				X		X					
Golden Algae (Chrysophyta)																				
Dinobryon	X									O		O		O				O	X	X
Ophiocytium									X											
Cryptomonads (Cryptophyta)																				
Rhodomonas	X						O		O											
Cryptomonas	O	O							O			X					X	X		

Appendix 5-2 (continued). Algae observed in the Duck pond (D) and FMCC pond (F) from July 2004 to May 2005. Dominant algae are represented by O and other algae are represented by X.

Algae	Date of samples																			
(Phylum)	7/22		8/25		9/20		10/20		11/19		12/23		2/22		3/21		4/21		5/27	
Genus	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F
Dinoflagellates (Dinophyta)																				
Ceratium		X																		
Exuviella	X	X																		
Peridinium		O																		

* Cylindrotheca was blooming and dominated biomass in the FMCC pond on 25 August 2004.

Appendix 5-3. Water quality parameters in two ponds during the experimental period (July 2004 to May 2005).

Date	Pond	DO (mg/L)	T (℃)	pH	Alkalinity (mg/L)	TAN[1] (mg/L)	OM[2] (mg/L)
7/22/2004	Duck	4.3	23.8	7.9	160.5	0.31	5.0
8/25/2004	Duck	4.2	22.5	8.3	165.0	0.27	7.6
9/20/2004	Duck	5.8	18.3	6.6	153.0	0.51	6.0
10/20/2004	Duck	6.7	13.2	7.5	184.0	0.13	4.8
11/19/2004	Duck	7.6	10.6	7.6	194.0	0.15	3.7
12/23/2004	Duck	10.5	5.9	7.9	213.0	0.11	1.2
2/22/2005	Duck	12.4	6.5	7.6	146.0	0.41	2.1
3/21/2005	Duck	11.1	9.6	7.9	199.0	0.18	4.3
4/21/2005	Duck	8.3	15.3	7.8	221.0	0.17	3.7
5/27/2005	Duck	8.7	17.4	7.8	192.0	0.27	3.4
7/22/2004	FMCC	7.0	26.1	8.3	113.0	0.07	2.0
8/25/2004	FMCC	5.5	24.6	8.5	157.0	0.09	3.0
9/20/2004	FMCC	8.7	22.2	7.3	144.0	0.05	1.5
10/20/2004	FMCC	10.0	13.4	8.0	130.0	0.00	0.8
11/19/2004	FMCC	9.9	10.3	7.9	127.0	0.01	1.0
12/23/2004	FMCC	14.8	5.1	7.9	126.0	0.01	1.3
2//22/2005	FMCC	12.2	6.5	7.8	163.0	0.00	0.9
3/21/2005	FMCC	10.7	8.9	7.9	173.0	0.04	0.7
4/21/2005	FMCC	7.4	17.3	7.8	132.0	0.06	1.0
5/27/2005	FMCC	7.2	19.1	8.0	132.0	0.03	1.0

[1] TAN = total ammonia nitrogen.
[2] OM= organic matter.

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VITA

Dan Hua was born in Wuxi, Jiangsu, China on November 21, 1965. She graduated from Jiangsu Xishan Senior High School, Wuxi in July 1981. Thereafter she attended Jiangsu Province Aquaculture Institute, Lianyungang, China and earned her B.S in Freshwater Aquaculture in 1985. She started her research career in freshwater fisheries sciences at the Freshwater Fisheries Research Center (FFRC), Chinese Academy of Fishery Sciences (CAFS), China. She enrolled in an international cooperative research project, entitled biology and pond dynamic of integrated fish farming, from 1985 to 1994. In 1995, she extended her research interests to freshwater mussels and was granted her first research project, 'Study on the Effect of Culture Method on Pearl Quality of *Hyriopsis cumingii*'. Subsequently, she was responsible for 8 research projects as a Principal Investigator and Freshwater Mussel Research Unit Leader (FFRC). Meanwhile, she taught an undergraduate class on 'Freshwater and Marine Edible Mollusks: Culture and Management', at Wuxi Fishery College, Nanjing Agricultural University since 2000; and an international training course on Freshwater Pearl Culture Principles and Techniques for Asia-Pacific Regional Research & Training Center, China, since 1999. She was promoted to Associate Professor at FFRC in 2003.

In 2001 she worked for Bangladesh Fisheries Research Institute as a pearl research consultant of World Bank's Agricultural Research Program, and completed the project successfully by developing a protocol on pearl culture using native freshwater mussels.

In fall 1999, she visited Virginia Tech, supported by Dr. R. J. Neves, Professor of Fisheries and Wildlife Sciences, Virginia Tech, and was impressed by the research on endangered mussel propagation. Therefore, she decided to return to pursue her MS degree in this field and came back in August 2003. She will work as Research Specialist for Dr. R. J. Neves at the College of Natural Resources, Virginia Tech, starting in August 2005.