An Evaluation of the Survival and Growth of Juvenile and Adult Freshwater Mussels at the Aquatic Wildlife Conservation Center (AWCC), Marion, Virginia

by

Aaron J. Liberty

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Approved:

Dr. Richard J. Neves, Chairperson	Dr. Ewen McLean
Dr. Steven R. Craig	

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Richard J. Neves, Chairman

Department of Fisheries and Wildlife Sciences

(ABSTRACT)

The decline of many freshwater mussel populations in the United States has brought about the need for facilities in which mussels can be held for purposes of relocation, research, and propagation. The Aquatic Wildlife Conservation Center (AWCC) of the Virginia Department of Game and Inland Fisheries (VDGIF) serves as a freshwater mussel conservation facility in southwest Virginia. The goals of this study were: (1) to determine whether adult freshwater mussels could maintain energy reserves at AWCC (2) to determine whether adults could produce mature gametes at AWCC and (3) to establish suitable rearing conditions for juvenile mussels at the AWCC.

In fall 2002, four species of mussels, *Villosa iris*, *V. vanuxemensis*, *Amblema plicata*, and *Pleurobema oviforme*, served as surrogates for endangered species and were relocated to the AWCC. Three energy reserves (glycogen, protein, and lipid) were measured seasonally (fall 2002 to summer 2004) from mantle tissue and compared between AWCC specimens and those from their wild source populations. The gametogenic stage of each species was also compared to determine whether gametogenesis was occurring in captivity. In summer 2003, the first of two juvenile experiments tested the effects of three rates of water flow (1 L/min, 3 L/min, and 7 L/min) on the survival and growth of *V. iris* and *Epioblasma capsaeformis* reared in flow-

through troughs. In summer 2004, round flow-through tanks were used to assess the effects of three sizes of substrate (fine sediment, fine sand, and coarse sand) and sampling frequency on the survival and growth of *V. iris.* Gut content analyses also were conducted at the end of each experiment to determine which algal species were being consumed.

Overall survival rates were as follows: *A. plicata,* 100 %; *V. vanuxemensis*, 86 %; *V. iris*, 79 %; *P. oviforme* (2002 collection), 53 %; and *P. oviforme* (2003 collection), 50 %. All energy reserves varied among seasons, but every species except *P. oviforme* (2003 collection) had levels higher than those in source populations at the end of this experiment. Glycogen appeared to be the best indicator of condition in these species, with protein also being important in the 2003 collection of *P. oviforme*. Mature gametes were found in all four captive species in 2003 and 2004, with lipids appearing to fuel gametogenesis. Additionally, gametogenesis was occurring earlier in captive long-term brooders than in the wild, possibly due to warmer water temperatures at AWCC.

The first juvenile experiment resulted in 15 % overall survival, with 1 L/min having the greatest survival (18 %), and the 3 L/min having the greatest growth (656 μm). In the second experiment, dishes left unsampled had significantly greater survival (40 %) ($P<0.05$) of juveniles than those which were sampled (27 %). The unsampled fine sand treatment had significantly greater survival than the other two unsampled treatments (52 %) ($P<0.001$). Sampled juveniles in fine sediment had the greatest growth (887 μm). Also, juveniles from Experiment 1 were consuming primarily *Navicula*, with *Coelastrum* and *Chlorella* consumed in greatest abundance in Experiment 2.

Results indicate that most adult mussels maintained energy reserves and produced mature gametes, and that juveniles of *V. iris* had good survival and growth. Only *P. oviforme* had

survival rates lower than expected and did not appear to maintain condition at AWCC. Based on results of the species tested, environmental conditions at AWCC appear suitable for the survival of most adult and juvenile freshwater mussels.

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TABLE OF CONTENTS

ABSTRACT……...….ii
ACKNOWLEDGEMENTS……...……..v
TABLE OF CONTENTS……...….vi
LIST OF TABLES……...viii
LIST OF FIGURES……..…...x

Chapter 1: Chapter 1: Body Condition of Four Species of Freshwater Mussels…….…..1

INTRODUCTION……..…….1
METHODS AND MATERIALS…….6
Collection Sites and Species Selection……...…….6
Sampling…….…….7
Glycogen Determinations……9
Protein Determinations……...……10
Lipid Determinations…….…11
Water Quality…….…12
Data Analysis……...……..12
RESULTS……..12
Water Quality……16
DISCUSSION……17
CONCLUSIONS……...24
RECOMMENDATIONS……..25
LITERATURE CITED……..27
TABLES AND FIGURES…….34

Chapter 2: Gametogenesis in Four Species of Freshwater Mussels in Virginia……...…54

INTRODUCTION……...……..54
METHODS AND MATERIALS……...……56
Data Analysis…….57
RESULTS……..57
DISCUSSION……61
CONCLUSIONS……...……67
RECOMMENDATIONS……..69
LITERATURE CITED……..70
TABLES AND FIGURES…….74

Chapter 3: Survival and Growth of Juvenile Freshwater Mussels Reared in Flow-Through Troughs and Round Tanks…….84

INTRODUCTION…….……84
Species Selection…….……..88
METHODS AND MATERIALS……..88
Experiment 1: Growth and Survival of Juveniles Reared at Three Flow Rates……...…88

Production of Juveniles……..90
Release of Juveniles..…….92
Trough Sampling……...…92
Escapement Sampling……93
Experiment 2: To Determine a Suitable Substrate Size and Sample Frequency for Juvenile Mussels in 4 L Round Flow-Through Tanks…….…..94
Production of Juveniles……..95
Release of Juveniles……..95
Tank Sampling……...……96
Water Chemistry……97
Incoming Sediment Determination……97
Gut Content Analysis……98
Algae Density and Composition……99
Data Analysis……..…100
RESULTS……....101
Experiment 1……...…101
Survival……101
Growth……...……..102
Water Quality……...……102
Incoming Sediment…….……103
Escapement……..103
Gut Content Analysis……...…104
Experiment 2……..……..105
Survival……105
Growth……106
Water Quality…….……..107
Incoming Sediment…….……107
Escapement……..……108
Sampling Effort……108
Gut Content Analysis……..……109
Algae Density and Composition……..109
DISCUSSION…….……110
Experiment 1……...……110
Gut Content Analysis…….……..114
Experiment 2……...……114
Gut Content Analysis…….……..118
Algae Density and Composition……..119
CONCLUSIONS……...……..120
RECOMMENDATIONS……122
LITERATURE CITED……...……124
TABLES AND FIGURES……...……129
APPENDIX 1……...……147
APPENDIX 2……...……149
APPENDIX 3……...……150
VITA……151

LIST OF TABLES

Table 1.1. Percent survival of four species of freshwater mussels held at AWCC from September 2002-July 2004……...……34

Table 1.2. Mean (± 1 SE) glycogen content in mg/g dry weight of two species of short-term brooders held in captivity at AWCC (fall 2002 to summer 2004), and their source populations. P-values determined by Wilcoxon Two-Sample Test, with P<0.05 considered significant. Significant differences are in bold. n=6 except where noted.…….……...…..……35

Table 1.3. Mean (± 1 SE) glycogen content in mg/g dry weight of two species of long-term brooders held in captivity at AWCC (fall 2002 to summer 2004), and their source populations. P-values determined by Wilcoxon Two-Sample Test, with P<0.05 considered significant. Significant differences are in bold. n=6 except where noted……..…….…….....35

Table 1.4. Mean (± 1 SE) protein content in mg/g dry weight of two species of short-term brooders held in captivity at AWCC (fall 2002 to summer 2004), and their source populations. P-values determined by Wilcoxon Two-Sample Test, with P<0.05 considered significant. Significant differences are in bold. n=6 except where noted…….…..…...……..36

Table 1.5. Mean (± 1 SE) protein content in mg/g dry weight of two species of long-term brooders held in captivity at AWCC (fall 2002 to summer 2004), and their source populations. P-values determined by Wilcoxon Two-Sample Test, with P<0.05 considered significant. n=6 except where noted……....36

Table 1.6. Mean (± 1 SE) lipid content in mg/g dry weight of two species of short-term brooders held in captivity at AWCC (fall 2002 to summer 2004), and their source populations. P-values determined by Wilcoxon Two-Sample Test, with P<0.05 considered significant. Significant differences are in bold. n=6 except where noted..……...…….37

Table 1.7. Mean (± 1 SE) lipid content in mg/g dry weight of two species of long-term brooders held in captivity at AWCC (fall 2002 to summer 2004), and their source populations. P-values determined by Wilcoxon Two-Sample Test, with P<0.05 considered significant. Significant differences are in bold. n=6 except where noted..……...…….37

Table 1.8. Water quality values measured with a Hydrolab unit at AWCC, from September 2003 to July 2004……38

Table 2.1. Classification stages of the gamete development index (GDI) for evaluating gametogenic activity in male and female gonads of freshwater mussels. Adapted from Barber (1996) and Henley (2002)…….…….74

Table 2.2. Mean (± 1 SE) glycogen, protein, and lipid content in mg/g dry weight of trematode-infested and uninfested *V. vanuxemensis* sampled from the wild source population (North Fork Holston River) from fall 2002 to summer 2004. P-values determined by Wilcoxon Two-Sample Test, with *P<0.05 considered significant. …….……..……75

Table 2.3. Mean (± 1 SE) glycogen, protein, and lipid content in mg/g dry weight of trematode-infested and uninfested *V. vanuxemensis* sampled from the captive population (AWCC) from winter 2003 to summer 2004. P-values determined by Wilcoxon Two-Sample Test, with *P<0.05 considered significant……...……...……76

Table 2.4. Mean (± 1 SE) glycogen, protein, and lipid content in mg/g dry weight of trematode-infested and uninfested *V. iris* sampled from the wild source population (North Fork Holston River) from winter 2003 to spring 2004. P-values determined by Wilcoxon Two-Sample Test, with *P<0.05 considered significant……..……77

Table 2.5. Mean (± 1 SE) glycogen, protein, and lipid content in mg/g dry weight of trematode-infested and uninfested *V. iris* sampled from the captive population (AWCC) from fall 2002 to winter 2004. P-values determined by Wilcoxon Two-Sample Test, with *P<0.05 considered significant…….……..78

Table 2.6. Results of ANOVA using Tukey's multiple comparison technique between GDI values in captive and source population mussels from September 2002 to July 2004. *P-values <0.05 represent significant differences in gametogenic stages. …….……..….78

Table 3.1. Summary of water chemistry values (n=5) taken from the pond during Experiment 1 in 2003…….……129

Table 3.2. Dissolved oxygen values (n=11) in mg/L measured with a YSI Model 50B DO meter from one replicate in each flow treatment of Experiment 1 in 2003...……..……..129

Table 3.3. Total number and mean lengths of juveniles of *E. capsaeformis* in the catchment containers for each flow treatment during Experiment 1……....130

Table 3.4. Relative abundance (%) of algae in the gut contents of *E. capsaeformis* juveniles in each flow treatment after 56 days, from June 26 to August 21, 2003…….130

Table 3.5. Summary of water chemistry readings (n=4) taken from the reservoir of the recirculating system at FMCC during Experiment 2.……..….131

Table 3.6. Summary of water chemistry readings (n=6) taken from water entering the grow-out system at AWCC for Experiment 2 ….……..…….131

Table 3.7. Total number and mean lengths of juveniles of *V. iris* in the catchment containers for each substrate treatment in Experiment 2 for sampled tanks and after 10 wk for unsampled tanks…….131

Table 3.8. Relative abundance (%) of algae in the gut contents of *E. capsaeformis* juveniles in each flow treatment after 70 days, from June 29 to September 7, 2004……..132

LIST OF FIGURES

Figure 1.1. Seasonal mortality of four species of mussels held in captivity at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer)……..…..39

Figure 1.2. Comparison of mean glycogen content (± 1 SE) of *A. plicata* sampled from the Clinch River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference....40

Figure 1.3. Comparison of mean glycogen content (± 1 SE) of *P. oviforme* sampled from the North Fork Holston River (n=6) and three groups of captive mussels (relocated in 1999, August 2002, and August 2003) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes sig. diff. between wild and 2003 collection, x denotes sig. diff. between 2002 and 2003 collection, and + denotes sig. diff. between 1999 and 2003 collection….……...41

Figure 1.4. Comparision of mean glycogen content (± 1 SE) of *V. iris* sampled from the North Fork Holston River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference……..……..…42

Figure 1.5. Comparison of mean glycogen content (± 1 SE) of *V. vanuxemensis* sampled from the North Fork Holston River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference……..…….….43

Figure 1.6. Comparison of mean protein content (± 1 SE) of *A. plicata* sampled from the Clinch River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference……44

Figure 1.7. Comparison of mean protein content (± 1 SE) of *P. oviforme* sampled from the North Fork Holston River (n=6) and three groups of captive mussels (relocated in 1999, August 2002, and August 2003) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference between 2002 collection and wild population, x denotes sig. diff. between wild and 2003 collection, and + denotes sig. diff. between 2002 and 2003 collection…….……45

Figure 1.8. Comparison of mean protein content (± 1 SE) of *V. vanuxemensis* sampled from the North Fork Holston River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer)……...…..46

Figure 1.9. Comparison of mean protein content (± 1 SE) of *V. iris* sampled from the North Fork Holston River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer)……...…….47

Figure 1.10. Comparison of mean lipid content (± 1 SE) of *A. plicata* sampled from the Clinch River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference……..……...…….48

Figure 1.11. Comparison of mean lipid content (± 1 SE) of *P. oviforme* sampled from the North Fork Holston River (n=6) and three groups of captive mussels (relocated in 1999, August 2002, and August 2003) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference between 1999 and 2003 collection…...….49

Figure 1.12. Comparison of mean lipid content (± 1 SE) of *V. iris* sampled from the North Fork Holston River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference……....……..50

Figure 1.13. Comparison of mean lipid content (± 1 SE) of *V. vanuxemensis* sampled from the North Fork Holston River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference……..……..…51

Figure 1.14. Daily mean temperature (ºC) of the raceway with captive mussels at AWCC during January 2003 to July 2004. Mean temperature (ºC) for duration of experiment indicated by dotted line……...52

Figure 1.15. Daily mean temperature (ºC) of AWCC, Clinch River, and North Fork Holston during May to October 2004……..53

Figure 2.1. Gamete development index (GDI) of gonads of *Villosa vanuxemensis* (*n*=4/sample date) held in captivity at AWCC and from wild source population (North Fork Holston River) during September 2002 to July 2004, showing percent of observations identified in each developmental stage (0: inactive, 1: early active, 2: late active, 3: mature, 4: spawned, and 5: resorbing)……...…79

Figure 2.2. Gamete development index (GDI) of gonads of *Villosa iris* (*n*=4/sample date) held in captivity at AWCC and from wild source population (North Fork Holston River) during September 2002 to July 2004, showing percent of observations identified in each developmental stage (0: inactive, 1: early active, 2: late active, 3: mature, 4: spawned, and 5: resorbing)……..80

Figure 2.3. Gamete development index (GDI) of gonads of *Amblema plicata* (*n*=4/sample date) held in captivity at AWCC and from wild source population (Clinch River) during September 2002 to July 2004, showing percent of observations identified in each developmental stage (0: inactive, 1: early active, 2: late active, 3: mature, 4: spawned, and 5: resorbing)…….81

Figure 2.4. Gamete development index (GDI) of gonads of *Pleurobema oviforme* (*n*=4/sample date) held in captivity at AWCC and from wild source population (North Fork Holston River) during September 2002 to July 2004, showing percent of observations identified in each

developmental stage (0: inactive, 1: early active, 2: late active, 3: mature, 4: spawned, and 5: resorbing)……..…..82

Figure 2.5. Gamete development index (GDI) of gonads infested with digenean trematodes for *V. vanuxemensis* (n=22), *V. iris* (n=17), *P. oviforme* (n=3), and *A. plicata* (n=2) from wild source populations and those held in captivity at AWCC from fall 2002 to summer 2004, with percent of observations identified in each developmental stage (0: inactive, 1: early active, 2: late active, 3: mature, 4: spawned, and 5: resorbing)……..…….83

Figure 3.1. Schematic design of 15 flow-through troughs used in Experiment 1. All species were randomly assigned to troughs. Each trough contained 200 *E. capsaeformis* and either 200 or 100 *V. iris* as indicated by color scheme……...……..133

Figure 3.2. Trough used in Experiment 1 and round tank used in Experiment 2. Arrows indicate direction of water flow in each unit……...…..134

Figure 3.3. Catchment container used to capture escaping juvenile mussels from flow-through troughs in Experiment 1. Arrows indicate direction of water flow out of trough and out of holes cut into catchment container……..……..……134

Figure 3.4. Schematic design of 27 flow-through round tanks used in Experiment 2. All treatments (indicated by letters in illustration) were randomly assigned to tanks. Each unsampled tank had 400 *V. iris*, and each sampled tank had 700 *V. iris*, as indicated by color scheme…...135

Figure 3.5. Mean survival (%) of *E. capsaeformis* reared in flow-through troughs at three rates of flow (1 L/min, 3 L/min, and 7 L/min) in Experiment 1. A Poisson distribution test showed significant differences between 1 L/min and 3 L/min treatments and between 1 L/min and 7 L/min treatments, at 29 and 59 days old……..136

Figure 3.6. Mean survival (%) of *V .iris* reared in flow-through troughs at three rates of flow (1 L/min, 3 L/min, and 7 L/min) in Experiment 1. A Poisson distribution test showed significant differences between 1 L/min and 3 L/min treatments and between 1 L/min and 7 L/min treatments at 29 days old…….…….137

Figure 3.7. Mean shell lengths of *E. capsaeformis* reared in flow-through troughs at three rates of flow (1 L/min, 3 L/min, and 7 L/min). Tukey's student range test showed significant differences between the 3 L/min and 7 L/min treatments at 29 days old, and between 1 L/min and 3 L/min and 1 L/min and 7 L/min treatments at 59 days old……...…138

Figure 3.8. Mean shell lengths of *V. iris* reared in flow-through troughs at three rates of flow (1 L/min, 3 L/min, and 7 L/min). No statistical analysis was done because of excessive mortality……...139

Figure 3.9. Daily mean water temperature in flow-through troughs over the 56 day study period (Experiment 1) from June 26 to August 21, 2003 and in round flow-through tanks over the 70 day study period (Experiment 2) from June 29 to September 7, 2004 for Experiment 2.

Temperatures were significantly different between summer 2003 and 2004 (ANOVA; P<0.0001)……..…140

Figure 3.10. Mean organic and inorganic matter in mg/100 cm^2 of incoming sediment in low (L), medium (m), and high (H) rates of flow (5 replicates per treatment) for 4 sample dates in Experiment 1……...…….141

Figure 3.11. Mean survival (%) of sampled and unsampled juveniles of *V. iris* reared in flow-through tanks and with three different substrate sizes (fine sediment, fine sand, and coarse sand) in Experiment 2. A Poisson distribution test showed significant differences among all three treatments in the tanks that were sampled at every sample event, except between fine and coarse sand at 8 (P=0.4530) and 10 wk (P=0.4368). All three treatments in the unsampled dishes were significantly different from each other at 10 wk (P<0.0001)……..142

Figure 3.12. Mean survival (%) of juvenile mussels held at the FMCC, and sampled and unsampled juveniles of *V. iris* including and excluding fine sediment treatment reared in flow-through tanks in three different substrate sizes (fine sediment, fine sand, and coarse sand) in Experiment 2..……..143

Figure 3.13. Mean shell lengths of sampled juveniles of *V. iris* reared in flow-through tanks in three different substrate sizes (fine sediment, fine sand, and coarse sand) in Experiment 2. Tukey's student range test showed significant differences between the fine sediment and coarse sand at 4 wk, and between the fine sediment and coarse sand, and fine sand and coarse sand at 6 and 8 wk, respectively (P<0.05)…….……144

Figure 3.14. Mean shell lengths of *V. iris* juveniles reared at the FMCC, and in sampled and unsampled flow-through tanks in three different substrate sizes (fine sediment, fine sand, and coarse sand) in Experiment 2 at AWCC……..……145

Figure 3.15. Mean of incoming sediment in mg/100 cm^2 in each of the three substrate treatments for sampled and unsampled round tanks at 4 sample events in Experiment 2……...……146

Chapter 1: Body Condition of Four Species of Freshwater Mussels

INTRODUCTION

Threats to mussels in North America come from a variety of sources. These sources include habitat alteration, lack of host species, competition from non-indigenous species such as the zebra mussel (*Dreissena polymorpha*) or Asian clam (*Corbicula fluminea*), overharvesting, and pollution (Fuller 1974, Neves et al. 1997, National Native Mussel Conservation Committee 1998). In response to these declining populations of freshwater mussels in the southeastern United States, there is a need to have facilities that can hold freshwater mussels that will survive, maintain physiological condition, and reproduce. However, before freshwater mussels are brought into captivity, such a facility must be able to support and maintain the well-being of mussels for the duration of their captive holding. Gabbot and Walker (1971) experimented with the marine oyster *Ostrea edulis*, and reported that maintaining brood stock in the best possible condition during the reproductive process is an important factor in obtaining healthy larvae that will grow and survive. Walne (1964) also reported that, with *Ostrea edulis*, the number of young produced is related to the health and condition of the parents. Success of mussel propagation and mussel relocation in the future will rely upon having suitable facilities to hold these mussels for long periods of time.

Often the stress caused by capture and transport of mussels can impact survival, and in the past, relocation projects have had limited success. Cope and Waller (1995) reported that 37 projects on mussel relocations (90,000 mussels) yielded a mean mortality rate of 50 %. After relocating 20 species into a variety of captive holding facilities, Dunn and Layzer (1997) reported that survival differed greatly among species and the type of holding facility used. Such variability is not unexpected, since there are no guidelines for relocating mussels, and most

monitoring after relocation only occurs for <1 yr (Cope and Waller 1995, Newton et al. 2001). Holding mussels in captivity is confounded by the fact that captive conditions should mimic nature as closely as possible, yet little is known of habitat requirements or the effects of stress from relocation (Cope and Waller 1995).

Layzer and Gordon (1993) reported that relocated mussels had an overall survival rate of nearly 75 % after 2 yr, and that high mortality was not seen until nearly 4 yr after relocation, when survival dropped to 21 %. Considering the extended longevity of many species of freshwater mussels, the effects of relocation and the long-term stress induced by a captive holding facility may not be accurately reflected in the immediate survival rates of relocated mussels. To better assess the suitability of holding facilities, physiological condition has been used in place of mortality to monitor the health of captively-held freshwater mussels (Haag et al. 1993, Newton et al. 2001). Changes in physiological condition should become apparent before mortality is observed (Naimo and Monroe 1999, Monroe and Newton 2001, Newton et al. 2001, Greseth et al. 2003).

Glycogen is the primary storage form of carbohydrates in bivalves and has been reported in nearly all types of animal cells (Stetten and Stetten 1960, De Zwaan and Zandee 1972, Hummel et al. 1989, Naimo and Monroe 1999). Glycogen levels reported in bivalves have been accepted as a good indicator of the energetic status or "condition" of mussels (Naimo and Monroe 1999, Patterson et al. 1999). Condition is defined by Mann (1978) as the "ability of an animal to withstand an adverse environmental stress, be this physical, chemical or biological." Also, glycogen is used as the energy reserve when food is scarce in the winter, and can serve as the main energy substrate when bivalves are exposed to the air (Hummel et al. 1989).

Changes in the glycogen content of mussels can be the result of stress by natural and artificial environmental changes (Hummel et al. 1989). Natural changes in glycogen levels of mussels occur before and during gametogenesis, when glycogen is transferred from the soma to gametes in the gonads. Prior to gametogenesis, energy is stored in the form of glycogen, lipids, and proteins. Typically, the highest levels of glycogen occur prior to and decrease after reproduction, since great amounts of energy are required for this process. After reproduction, the levels of glycogen are usually depleted and at their lowest levels (Bayne 1973, Barber and Blake 1981, Hummel et al. 1989, Monroe and Newton 2001).

Increased stress levels caused by anthropogenic sources include such things as exposure to air, collecting and handling, extreme temperatures or temperature changes, pollutants, or relocation to a non-native habitat (Hummel et al. 1989, Patterson et al. 1999). These changes also result in decreased glycogen levels in mussels. For example, Naimo and Monroe (1999) reported that glycogen levels in the threeridge (*Amblema plicata*) declined 80 % after being relocated to an artificial pond for 24 mo. Gabbot and Walker (1971) reported that high temperatures and low food levels resulted in a decline of body condition, and the use of body reserves such as glycogen in the oyster species *Ostrea edulis* L. Bayne and Thompson (1970) had similar results with the blue mussel *Mytilus edulis*. Haag et al. (1993) reported that glycogen levels in *A. plicata* were reduced significantly after exposure to zebra mussels, but their survival was not significantly different (Naimo and Monroe 1999).

When bivalves are subjected to stress, other forms of energy reserves can be affected. Nearly 85 % of the biochemical content of freshwater mussels is contained within the glycogen, protein, and lipid content (Baker and Hornbach 2001). However, very few studies have examined the total biochemical composition when conducting research. Greseth et al. (2003)

studied the effects of emersion on three species of unionids by monitoring the glycogen, protein, and lipid content of these animals over a range of temperatures and emersion times. Although no consistent trends or drastic changes in biochemical content were noted, they concluded that more research was needed to measure this suite of biochemical indicators. Baker and Hornbach (2000) also monitored glycogen, protein, and lipid levels in *A. plicata* infested with zebra mussels. They concluded that glycogen and protein were the best indicators of stress, and that glycogen was catabolized first, followed by protein. Makela and Oikari (1995) also conducted a study on the glycogen, protein, and lipid content of captively held freshwater mussels and concluded that neither glycogen nor lipid differed significantly between captive and natural populations, but that protein content in captive mussels was significantly lower. Bayne (1973) noted that during prolonged starvation in the blue mussel, there was a significant increase in the use of lipid reserves. Also, Bayne and Scullard (1977) observed that during winter, blue mussels utilized primarily protein reserves, whereas lipid reserves were catabolized during summer.

Many studies have been published on how nutrient reserves are related to reproduction in marine mussels (Berthelin et al. 2000 a, b). However, little research has been conducted on the utilization of these nutrient reserves in freshwater mussels (Boyles 2004). The reproductive process in bivalves is cyclic, and during the rest period, the accumulation of energy reserves is very important for the success of reproduction. In some cases there has been shown to be an inverse relationship between glycogen stores and reserves of proteins and lipids (Bayne 1973, Berthelin et al. 2000 b, Baker and Hornbach 2001). Since glycogen is utilized during reproduction, it typically declines to its lowest point during the reproductive process, as well as during winter when food is often scarce (Gabbot 1983, Hummel et al. 1989). Also during reproduction, Berthelin et al. (2000 b) reported that proteins and lipids were conserved and

stored at their highest levels. However, during summer, after reproduction was complete, proteins and lipids were utilized while glycogen was being stored for the up-coming spawning season. Blackmore (1969) reported similar results, observing increasing lipid reserves before and during gametogenesis in the marine species *Patella vulgata.*

The Aquatic Wildlife Conservation Center (AWCC), operated by the Virginia Department of Game and Inland Fisheries (VDGIF) in Marion, Virginia, contains a series of renovated raceways and tank systems that serve as a freshwater mussel propagation facility in southwest Virginia. Hatchery water is supplied from a reach of the South Fork Holston River at approximately river mile 105. Underground pipes are then used to feed this water from the river into the hatchery system of raceways and holding ponds used. A series of two holding ponds supply all raceways with water at AWCC. The first holding pond is approximately 12,140 m^2 in area and is used to hold muskellunge (*Esox masquinongy*). The second pond is approximately 288 m^2 and is fed with water from the larger muskellunge pond and from a pipe supplying water directly from the river. This pond is lined with a polyethylene liner to prevent leakage. This liner also serves to warm the water to 20.2°C during summer, an average of 2.7° C warmer than the South Fork ambient water temperature. The warming of this water promotes increased algae growth, which is a food source for mussels held at AWCC. The turnover period for this holding pond is 25 hr, and the water flow into the raceways is maintained at 720 l/min (Zimmerman 2003).

Typically, there are two different reproductive groups of freshwater mussels. The first group consists of long-term brooders, or bradytictic breeders. Bradytictic breeders spawn and fertilize eggs in the late spring or summer and release glochidia from early spring to early summer. The second group is short-term brooders, also known as tachytictic breeders, which

spawn and fertilize eggs in late spring and release glochidia in summer (Coker et al. 1921, Jirka and Neves 1992). The objective of this study was to examine the body condition of four species of mussels, two long-term brooders and two short-term brooders, held at AWCC over a 2 yr period. Body condition was assessed by comparing the glycogen, protein, and lipid levels in the captively-held mussels to those from their wild source populations on a seasonal basis. The four species chosen for this study were the rainbow mussel (*Villosa iris*), mountain creekshell (*Villosa vanuxemensis*), threeridge (*A. plicata*), and Tennessee clubshell (*Pleurobema oviforme*).

METHODS AND MATERIALS

Collection Sites and Species Selection

Two long-term brooder and two short-term brooder species were held in captivity as surrogates for endangered species. The species of short-term brooders were the threeridge (*A. plicata*) and Tennessee clubshell (*P. oviforme*), while the rainbow mussel (*V. iris*) and mountain creekshell (*V. vanuxemensis*) were the long-term brooders (Parmalee and Bogan 1998). These species were selected for their abundance in southwestern Virginia, similar habitat preferences to many endangered species, differing reproductive strategies, and representation of two subfamilies of mussels.

Since the objective of this study was to monitor the physiological condition of selected species held at AWCC, the origin of these species was very important. The Clinch River and North Fork Holston River were chosen as collection sites because of their relative closeness to AWCC and Virginia Tech, which minimized transport time and possible stress levels. Three of the four species, V. *vanuxemensis*, *V. iris*, and *P. oviforme*, were collected from the North Fork Holston River. Another abundant short-term brooder could not be easily collected from the North Fork, so the fourth species, *A. plicata*, was collected from the Clinch River. The

threeridge was selected as the fourth species because it is more commonly associated with larger rivers, unlike the other headwater species collected.

Approximately 70 specimens of each of the four species were collected and held at AWCC. This was a suitable population size so that sufficient individuals were available for sacrifice during seasonal physiological testing. However, for unknown reasons, *P. oviforme* suffered high mortality during spring and summer 2003. An additional collection of 50 *P. oviforme* was taken from the original location and brought to AWCC on August 26, 2003. Specimens of *A. plicata* were collected from the Clinch River at the public boat ramp, 1.6 km north on Route 65 in Clinchport, Scott County, VA. Specimens of *P. oviforme, V. vanuxemensis,* and *V. iris* were collected from the North Fork Holston River in Chatham Hill, VA, 1.6 km down Ridgedale Road (Smyth County) off of Route 16.

Sampling

Sampling occurred quarterly after the initial collection period for 2 yr, for a total of eight consecutive seasons. The initial collection, and first fall sample event, occurred in September 2002. Subsequent quarterly sample dates for the winter, spring, summer, and fall then occurred in January, April, July, and October respectively. Each sample event was conducted at the middle of the month, so energy reserves and histological results could be compared between years.

During the initial collection event, individual mussels were located in the wild by using water scopes with clear plastic bottoms. Chen et al. (2001) reported that transporting freshwater mussels in water is less stressful than placing them in wet burlap, so all mussels were transported to AWCC in aerated coolers. Once the mussels arrived at the facility, a tag with a unique number was attached to a smooth part of the periostricum with superglue. All mussels were

placed in plastic tool trays and submerged into three raceways. The tool trays were approximately 15 cm deep with dimensions of 36 x 61 cm. Approximately 12 cm of course substrate; a mixture of sand, gravel, and pebbles, was placed in the bottom of each tray**.** For the smaller species, *V. vanuxemensis* and *V. iris*, approximately 20 individuals were placed into a single tool tray. For the slightly larger species, *P. oviforme*, approximately 12 individuals were placed into a single tray. The largest of the four species, *A. plicata*, required separate Tupperware® containers approximately 15 cm deep and 61 x 91 cm, with eight individuals per container. Trays were used to hold mussels at AWCC to facilitate relocating captive specimens, to prevent dispersal in the raceways, and to minimize the cost and space required by covering the entire raceway bottoms with substrate. These trays have been used in the past at AWCC with adequate survival of some species for over 4 yr (Mike Pinder, VDGIF, pers. comm.). Raceways were divided by 1.9 cm plywood partitions and supplied from with same water source.

During the first sampling trip, six specimens of each species were randomly selected and transported on ice to the Freshwater Mollusk Conservation Center (FMCC) at Virginia Tech. Live specimens were placed in plastic bags and not directly on the chlorinated ice which could potentially alter biochemical analysis results. The shell length of each mussel, its unique number, and whether or not it was gravid was recorded for each specimen before being dissected. During dissection, the entire mantle tissue from each of the six specimens was extracted, placed in a vial, and frozen at -60°C until freeze-dried. This procedure was repeated during every sampling event for both wild and captive mussels.

Vials of mantle tissue were placed in a Labconco freeze drier (model 79480) for approximately 1 wk or until a constant dry weight was obtained. All vials were placed in the freeze-drier without caps to ensure that the tissue was exposed so the air and moisture could exit

the tubes. Common cheesecloth was held in place over the vial tops with elastics to allow moisture to escape without the loss of any tissue. Freeze-drying occurred at a temperature of -40°C and a vacuum pressure of 110 X 10^{-3} torr. After freeze-drying, samples were crushed into powder form using a mortar and pestle, returned to their vials, and stored frozen at -60°C until biochemical analyses were completed.

Glycogen Determinations

Glycogen content of the collected specimens was quantified by using the phenol-sulphuric acid method (Dubois et al. 1956, Naimo et al. 1998). A stock solution of glycogen at a concentration of 5000 mg/L was first made by placing 250 mg of glycogen (*Mytilus edulis,* Type VII, Sigma Chemical Company, St. Louis, MO) in a 50 mL flask, filling it with deionized water, and thoroughly shaking it by hand for 5 min, followed by vortexing for 5 min. Seven standard glycogen concentrations (0, 250, 500, 1000, 2000, 2500, and 5000 mg/L) were made by using appropriate amounts of deionized water to dilute the glycogen stock solution. Each concentration was vortexed, and then a sub-sample of 250 µL was placed in a labeled cryovial. Samples were then prepared by placing 2.5 to 5.0 mg of freeze-dried, pulverized mantle tissue in 2 mL labeled cryovials with 500 µL of 30% potassium hydroxide (KOH). The same amount of KOH also was added to the standards before all cryovials were placed in a 100°C water bath for 20 min. Immediately after removing the samples from the water bath, they were shaken for 20 sec and then placed on ice for 5 min. All cryovials (standards and samples) then received 750 µL of ethyl alcohol (EtOH) and were placed back in the 100°C water bath for 15 min. The contents of each cryovial were poured into labeled test tubes and subsequently rinsed with 5 mL of deionized water, which also was added to the test tube. All test tube volumes were then equalized by adding 350 µL of deionized water to each sample test tube and 100 µL to each

standard test tube. Upon vortexing, two 250 µL aliquots of each sample and standard were placed in labeled test tubes. Each test tube then received 100 µL of 80 % phenol and 5 mL of concentrated sulfuric acid before being allowed to sit at room temperature for 30 min. Finally, a spectrophotometer (Genesis 8; Thermo Spectronic, Rochester, NY) was used to measure the absorbance of the samples at an optical density of 490 nm. The two replicates for each sample were averaged and incorporated into the trend line of the standard curve equation to get the glycogen concentrations in mg/g. The following equation was used to determine glycogen levels:

mg glycogen /g = (mg glycogen /L / 1000 mL) /g dry tissue

Protein Determinations

Approximately 5.0 to 6.5 mg of freeze-dried, pulverized tissue was placed in a labeled test tube with 2 mL of 0.1sodium hydroxide (NaOH). Each sample was then homogenized with a Tekmar Tissuemizer (Tekmar Co., Cincinnati, OH) for 1 min. The tissuemizer was then rinsed with 6 mL of 0.1 NaOH into the test tube (Barber et al. 1988, Kreeger 1993). Protein content of each specimen was quantified using a Pierce Bicinchoninic (BCA) Protein Assay Reagent Kit (# 23225, Pierce, Rockford, IL) based on the methods of Lowry et al. (1951). Seven standard protein concentrations (0, 25, 125, 250, 500, 1000, and 2000 µg/mL) were made using appropriate amounts of 0.1 NaOH as a dilutant and known protein concentrations of bovine serum albumin (BSA). After vortexing for 1 min, two 50 µL subsamples from each sample and standard were placed in two labeled test tubes. Each test tube then received 1.0 mL of a working reagent containing 50 parts reagent A to 1 part reagent B. Reagent A contained sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide, while reagent B contained 4% cupric sulfate. Each test tube was covered with

parafilm and allowed to sit at room temperature for 2 hr. A spectrophotometer (Genesis 8; Thermo Spectronic, Rochester, NY) was used to measure the absorbance of the samples at an optical density of 562 nm. The two replicates for each sample were averaged and incorporated into the trend line of the standard curve equation to get the protein concentration in mg/g. The following equation was used to compute the protein levels:

$$\text{mg protein /g} = [(\mu\text{g/mL} / 1000)*8]/ \text{ g dry tissue}$$

Lipid Determinations

The lipid content of the mussels was quantified using a chloroform-methanol extraction method (Folch 1957, Barber et. al 1988). Each sample had 50 mg of freeze-dried, pulverized tissue placed into two glass filter funnels to serve as replicates. Each filter then had 10 mL of a 2:1 chloroform:methanol solution poured into it and allowed to drain into a polypropylene centrifuge tube. After filtration, any remaining liquid was forced through the filter using nitrogen gas. Next, 2 mL of 0.9 sodium chloride (NaCl) was added to the centrifuge tube before it was vortexed for 1 min. All samples were then centrifuged for 10 min at 3500 rpm and 8°C. Centrifuging caused the separation of methanol from chloroform, so that the top layer (methanol) could be removed with a Pasteur pipette. The chloroform volume in the centrifuge tube was then raised to 10 mL using mostly chloroform and a few drops of methanol to wash down the sides of the centrifuge tube. Aluminum weigh boats (57 mL) were labeled and put into a 120°C oven and allowed to cool before their weights were recorded. Each weigh boat then received 5 mL of the sample and was placed in the 120°C oven for 10 min. Lipid weight was calculated by subtracting the weight of the original weigh boat from the final cooled weigh boat. Lipid in mg/g dry weight was then calculated by using the following formula:

$$\text{mg lipid/g} = [\,(\text{lipid weight/aliquot volume}) \times (\text{total volume})]/\text{dry tissue} * 1000$$

Water Quality

Water temperature in the raceway with mussels was monitored daily using Hobo temperature loggers (Onset Computer Corporation, 536 MacArthur Blvd. P.O. Box 3450 Pocasset, MA). A Hydrolab unit, located 4 m upstream of where captive mussels were held, also was used to take hourly measurements of dissolved oxygen (DO), pH, salinity, and conductivity in the raceways throughout the experiment.

Data Analysis

Glycogen, protein, and lipid comparisons were made among specimens held at AWCC, and samples from the source populations during every sample event for all four species. Since the original collection in fall 2002 was used as a reference point, no statistics were completed for this sample. Data from the remaining seven seasons were analyzed with Wilcoxon rank sums two-sample tests using the SAS software package (SAS Institute Inc. 2002). This test was used because it compares pairs (AWCC vs. wild source population) of measurements, taking into account that these data had a non-normal distribution. Statistical differences were indicated by using $P<0.05$ for all three energy substrate comparisons.

RESULTS

Survival rates of the four species of mussels held in captivity are as follows: *A. plicata,* 100 %; *V. vanuxemensis*, 86 %; *V. iris*, 79 %; *P. oviforme* (2002 collection), 53 %; and *P. oviforme* (2003 collection), 50 % (Table 1.1 and Figure 1.1). The 2002 collection of *P. oviforme* experienced a high rate of mortality during May to July 2003. Similarly, after no mortality for nearly 10 mo in captivity for the 2003 collection, a high mortality event of 50 % occurred during June and July of 2004.

Overall, glycogen was the most variable energy substrate measured for all four species. Threeridge mussels maintained their glycogen levels, and experienced a slight increase in levels over the course of the experiment (Table 1.2, Figure 1.2). This species had a significantly higher glycogen level at AWCC than those in the wild during summer 2004 (P=0.0172). The other short-term brooder, *P. oviforme,* showed declining glycogen levels for year 1 in captivity (Table 1.2, Figure 1.3). At the same time, glycogen levels decreased in wild *P. oviforme*, resulting in no significant differences. Glycogen in *P. oviforme* in the wild rebounded very quickly in fall 2003, while those in captivity only increased slightly in glycogen levels (Table 1.2, Figure 1.3). Due to the high mortality in summer 2003, the final few individuals of *P. oviforme* from the original collection were sacrificed in winter 2004 for analysis, such that the next two seasonal comparisons could not be completed.

After it was evident that the original 2002 collection of *P. oviforme* would not survive the duration of the experiment, more *P. oviforme* were collected in August 2003 and used for comparisons. The *P. oviforme* collected in 2003 at AWCC had significantly higher glycogen levels than those in the wild in summer 2003 (P=0.0172) and winter 2004 (P=0.0404) (Figure 1.3). The 2003 collection also had significantly higher glycogen levels than the 2002 collection of *P. oviforme* in fall 2003 (P=0.0229) (Table 1.2, Figure 1.3). Coincidentally, a collection of *P. oviforme* from the Clinch River in 1999 had been held at AWCC for the past 5 yr with 'good' survival (Mike Pinder, VDGIF, pers. comm.). Six individuals of this 1999 collection were sacrificed in summer of 2004 for additional energy substrate comparisons. Although no significant differences were reported in glycogen levels between this population and the 2003 collection at AWCC, the source population had significantly higher glycogen levels (P=0.305) (Table 1.2, Figure 1.3). Although percent survival is unknown for this 1999 collection, three

dead specimens from this collection were observed in the raceway when collected for biochemical analysis.

Both species of long-term brooders had higher glycogen levels in captivity than in the wild at the last sample event taken in summer 2004 (Table 1.3, Figures 1.4 and 1.5). However, source populations had significantly higher glycogen levels than those at AWCC for *V. iris* in spring 2003 (P=0.0127) and spring 2004 (P=0.0404) (Table 1.3, Figure 1.4), and for *V. vanuxemensis*, in spring 2003 (P=0.0404) (Table 1.3, Figure 1.5). Glycogen levels were consistently lower in *V. iris* at every sample event except for fall 2003 and summer 2004 (Figure 1.4), and for *V. vanuxemensis* at every sample event except for summer and fall 2003, and summer 2004 (Figure 1.5). Of the four species, glycogen values in *V. vanuxemensis* exhibited the least fluctuation on a seasonal and yearly basis.

Protein levels were far less variable than glycogen reserves in both short-term and long-term brooders. *Amblema plicata* experienced highest protein levels during summer and fall, and lowest protein levels in spring (Table 1.4, Figure 1.6). However, *A. plicata* at AWCC seemed to maintain low protein levels from spring into summer 2004, resulting in protein levels significantly lower than those in the wild (P=0.0172) (Table 1.4, Figure 1.6). Since the experiment concluded in summer 2004, it is unknown whether these levels would have rebounded the following season. All *P. oviforme* peeked in protein levels in summer 2003 and spring 2004 (Table 1.4, Figure 1.7). *Pleurobema oviforme* in the 2002 collection had significantly higher protein levels at AWCC in spring 2003 (P=0.0244) (Table 1.4, Figure 1.7). In summer 2003, the source population had significantly higher (P=0.0172) protein levels than their captive counterparts, and the 2002 collection had significantly higher protein levels than those in the 2003 collection (P=0.0305) (Table 1.4, Figure 1.7).

Both species of long-term brooders seemed to follow the same trend in protein levels throughout the duration of this experiment. The species peeked in protein levels during winter 2003 and showed an overall decrease in protein levels, becoming more pronounced after fall 2003 (Table 1.5, Figures 1.8 and 1.9). However, *V. vanuxemensis* increased in protein levels once during this decline in spring 2004 (Table 1.5, Figure 1.8). Since protein levels in captive mussels mimicked those in the source populations so well, no significant differences were reported for either species.

Lipid levels for the short-term and long-term brooders showed least variability and exhibited the most significant differences of the three energy substrates measured. Lipid levels in *A. plicata* fluctuated little in wild and AWCC specimens, yet had lower levels in captivity at every sample event (Table 1.6, Figure 1.10). Lipid levels for *A. plicata* at AWCC were significantly lower than those in the wild during winter 2003 (P=0.0244), summer 2003 (P=0.0229), fall 2003 (P=0.0305), and winter 2004 (P=0.0305) (Table 1.6, Figure 1.10). All *P. oviforme* peaked in lipid levels in fall 2002 and 2003, and experienced their lowest levels in spring 2003 and 2004, except for the source population which exhibited its lowest levels in summer 2004 (Table 1.6, Figure 1.11). The only significant difference in levels for *P. oviforme* was that the 1999 collection had significantly higher lipid levels than those in the wild during summer 2004 (P=0.0172) (Table 1.6, Figure 1.11). Lipid levels were also lower in captive populations (2002 and 2003 collection) at every sample event, except for summer 2004.

Lipid levels in the long-term brooders in the wild exhibited the same trend over the course of the experiment, and both species at AWCC also seemed to follow the same trend (Table 1.7, Figures 1.12 and 1.13). Mussels in the wild peaked in lipid levels in winter, and exhibited their lowest levels in spring and summer. Mussels held at AWCC peaked in lipid

levels in winter 2004 with levels decreasing thereafter, and had their lowest levels in fall 2003. This resulted in *V. iris* having significantly higher (P=0.0244) lipid levels at AWCC in spring 2003 (Table 1.7, Figure 1.12). *Villosa vanuxemensis* at AWCC had significantly higher lipid levels in winter 2004 (P=0.0404), and those in the wild have significantly higher lipid levels in fall (P=0.0172) and winter 2003 (P=0.0456) (Table 1.7, Figure 1.13).

Water Quality

Water temperatures at AWCC throughout the experiment ranged between 0.23ºC and 25.6ºC, with a mean of 11.6ºC (Figure 1.14). Due to a malfunction of the Hobo temperature logger, temperature data prior to January 9, 2003 were lost. Temperature data loggers for the Clinch and North Fork Holston rivers were lost in the spring floods of 2004, and no data was recovered prior to May 18, 2004. From May 18 to July 15, 2004, water temperatures did not differ significantly between AWCC and the Clinch River (ANOVA; P=0.2882), but did differ significantly between AWCC and the North Fork Holston River (ANOVA; P<0.0001) (Figure 1.15). Three months of additional water temperature data were collected from the Clinch and North Fork Holston rivers between July 15 and October 11, 2004 (Figure 1.15). During this time, the Clinch averaged 3.2.ºC warmer than the North Fork Holston, which was significantly different (ANOVA; P<0.0001).

Dissolved oxygen (mg/L) and pH had mean values of 9.07 ± 1.67 mg/L and 7.29 ± 0.79, respectively (Table 1.8). These mean values were within the range typically considered suitable for freshwater mussels (Gatenby 2000). However, pH values less than 6 were observed on multiple occasions in January 2004. Peterson (1987) observed that freshwater mussels were not present in water with a pH less than 6, indicating that pH values less than 6 are not suitable for freshwater mussels. Suitable values of salinity (ppt), dissolved solids (g/L), and specific

conductivity (μS/cm) for freshwater mussels are undetermined, and it is unknown whether my reported values were well-suited (Table 1.8).

DISCUSSION

Seasonal variations in the biochemical composition for all four species of mussels at AWCC and in the source populations consistently agreed with results of previous research (Baker and Hornbach 2001, Monroe and Newton 2001, Gatenby and Neves 2002). Comparisons between years and annual trends were difficult to interpret and possibly were confounded by the effects of drought conditions in 2002, and above-average rainfall in 2003. Comparisons between wild and captive populations also were not consistent. It has been suggested that the glycogen content of wild populations of mussels makes up the largest energy substrate of these animals, but once brought into captivity, glycogen plays a lesser role and other energy reserves become more important (Monroe and Newton 2001). This hypothesis may explain the inconsistent patterns in my study. However, many of the seasonal patterns, relationships between the three energy reserves, and overall values of the energy substrates indicate that AWCC can support adult mussels in captivity.

All species used for this experiment, with the exception of *P. oviforme*, had very good survival over the course of 2 yr in captivity. Survival rates of all species except *P. oviforme* are similar to survival rates reported by Boyles (2004), who held these species at White Sulphur Springs National Fish Hatchery (WSSNFH). After one year, the following survival rates were reported: *A. plicata,* 77 %; *V. vanuxemensis*, 96 %; *V. iris*, 90 %; and *P. oviforme,* 88 %. Overall survival at AWCC after 2 yr was nearly 80 %, slightly higher than the overall 1 yr

survival of 77 % at WSSNFH, which was deemed suitable for the long-term holding of mussels (Boyles 2004).

Captive populations of the long-term brooders seemed to exhibit the same trend in seasonal glycogen values. *Villosa iris* and *V. vanuxemensis* had consistently lower glycogen levels in captivity at every sample event, except for in fall 2003 and summer 2004 (Figure 1.4) for *V. iris*, and in summer and fall 2003, and summer 2004 (Figure 1.5) for *V. vanuxemensis*. A steady decrease in glycogen levels was also seen during the first two seasons in captivity, indicating perhaps stress was evident after the initial relocation to AWCC. However, the three species that had >79 % survival in captivity had higher glycogen levels at the end of this experiment at AWCC than in the wild. Both species had their lowest glycogen values in spring 2003 and summer 2004, and highest levels in the fall. In a study with the long-term brooder *Actinonaias ligamentina*, Gatenby and Neves (2002) suggested that condition increases with spawning, followed by a subsequent drop in condition after fertilization and glochidial development. This conclusion seems to fit these species at AWCC, since they spawned in late summer and fall. However, the wild populations exhibited a very different trend than those in captivity, generally peaking in condition in spring and having their lowest levels in fall. These findings agree with Baker and Hornbach (2001), who reported the same pattern in *A. ligamentina*. Despite seasonal fluctuations, the only significant differences in glycogen for the two long-term brooders were in the spring, indicating a possible difference in captive conditions during spring. This possible difference is unlikely related to food or temperature, since algal densities were higher at AWCC than those in the North Fork during the spring (Appendix 1 and 2), and temperatures at AWCC in spring are higher than those in the North Fork at that time (Figure 1.18).

Trends in glycogen values for *A. plicata* were less apparent, but it appeared this species increased in glycogen levels while at AWCC Glycogen content (617-945 mg/g dry wt) at AWCC was greater than reported in other studies: 243-434 mg/g dry wt (Naimo and Monroe 1999) and 339-705 mg/g dry wt (Boyles 2004). Glycogen levels for *P. oviforme* (2002 collection) showed the opposite trend, and consistently decreased in its first year in captivity. From spring to summer 2003, glycogen levels in captive specimens plummeted sharply, and at the same time suffered high mortality. However, the wild population also exhibited a sharp decrease in glycogen levels during this same time, resulting in no significant differences with the captive population. In fall 2003, glycogen levels in the wild population rebounded, whereas the captive population increased only slightly. Possibly, specimens of *P. oviforme* were overly stressed by drought conditions of 2002 when brought into captivity, and coupled with the harsh winter of 2003, were unable to survive. The increase in condition in *A. plicata* and its excellent survival, versus the decrease in condition in *P. oviforme* and its poor survival, support the hypothesis that glycogen is a sensitive biochemical endpoint to anticipate mortality (Naimo et al. 1998, Monroe and Newton 2001, Greseth et al. 2003).

In contrast to the 2002 collection, the 2003 collection of *P. oviforme* increased in glycogen content in its first season in captivity, decreased during the following fall and winter 2004, and then exhibited little fluctuation thereafter. Even though glycogen values only decreased slightly in spring and summer 2004, mortality for this captive population was again high during this time period. While glycogen levels did not change greatly, protein levels decreased drastically at this point, concurring with other studies that identify protein as the primary energy source and a potential indicator of stress (Riley 1976, Baker and Hornbach 2000). High mortality during summer 2002 coincided with decreasing glycogen values, and high

mortality in summer 2003 coincided with decreasing protein levels. These findings support the hypothesis that either glycogen or protein could be used as the main energy substrate in some instances. However, since a large percentage of *P. oviforme* died immediately prior to sampling in July 2004, the ones that survived and were sampled may have been the hardiest specimens with the highest glycogen content, resulting in a higher average glycogen level at this time.

No seasonal trends were apparent with protein levels in long-term brooders, and there were no significant differences between either species in captivity or in the wild. These results confirm that these two species did very well in captivity at AWCC, since protein was not being catabolized. The threeridge seemingly showed a seasonal trend in that levels of protein in the wild and captive populations peaked in summer and had their lowest values in spring. Gatenby and Neves (2002) reported similar findings with the short-term brooder *E. dilatata*, concluding that protein levels peaked in early summer due to gametogenic and spawning activities.

Lipids are a major component of the gametes in bivalves (Barber and Blake 1981, Gabbot 1983, Makela and Oikari 1995), and levels are related to the reproductive cycle (Nagabhushanam and Dhamne 1977, Pollero et al. 1979). For this study, lipid levels in wild populations of the long-term brooders appeared to be related to gametogenic activity, since peaks were seen during the winter months. Gatenby and Neves (2002) suggested that lipid levels were possibly being stored during fertilization in the fall and then used as an energy reserve by brooding females in winter and spring. My data for these long-term brooders in the wild support this hypothesis. Lipid levels in the captive, long-term brooders appeared to be more variable in their first yr in captivity, yet exhibited trends in their second yr in captivity, peaking in lipid levels in the winter, followed by decreasing levels into summer 2004. In a similar study, Boyles (2004) hypothesized that captive mussels may not catabolize lipids for gametogenic purposes.

Lipid levels of *A. plicata* in the wild and AWCC populations exhibited little fluctuation over time, but levels were consistently lower in the captive population at every sample event (Figure 1.10). Lack of variability could possibly point to a lack of reproduction for this species, but since mature gametes were found in both males and females during 2003 and 2004, this was not the case. It is more likely that captive conditions at AWCC affected the ability of *A. plicata* to achieve natural levels of lipid reserves. *Pleurobema oviforme* also exhibited a clear trend with all collections of wild and captive mussels peaking in lipid content in fall and decreasing in spring, with levels in captivity being consistently lower than those in the wild at every sample event, except for summer 2004 (Figure 1.11). This again indicates that perhaps lipids are being used for the production of gametes. Previous studies also confirm that lipids are not only being used during reproduction for this species, but that they could also be used as an additional energy store through the winter months (Berthelin et al. 2000 b, Gatenby and Neves 2002).

When the 1999 collection of *P. oviforme* was sampled at AWCC, I presumed these mussels were in much better condition than those of the 2002 or 2003 collection because they had survived in captivity for nearly five years. However, glycogen levels in these captive mussels were significantly lower than those in the wild population in the North Fork Holston River and did not differ significantly from the 2003 collection. Since glycogen and protein levels were equivalent or lower than the values in the 2003 population, these specimens may have been in poor condition at this time. Three dead mussels were observed in the raceway when this sample was taken, indicating that condition may have been marginal. However, with an undocumented mortality rate, only one sample and no additional data points after summer 2004, a definite conclusion on these captive *P. oviforme* is not possible.

All water quality results of this study, except for pH, all fell within accepted values for adult freshwater mussels (Table 1.8). In January 2004, pH levels < 6 were observed on multiple occasions. These levels are below the recommended pH >6 reported to ideal for freshwater mussels (Peterson 1987). However, it is unlikely that these levels influenced the survival of *P. oviforme* since higher pH levels were observed when mortality was occurring in spring and summer 2003 and 2004. Water temperatures < 28ºC are typically considered suitable for mussels (Boyles 2004), and the highest temperature recorded during this experiment was 25.6ºC. Water temperatures between AWCC and the Clinch were very similar, yet were significantly warmer at AWCC than those found in the North Fork. It is unlikely that these slightly warmer water temperatures affected the condition of captive *P. oviforme* since they are also found in the Clinch River, which had water temperatures more similar to AWCC. However, when high mortality was reported for *P. oviforme* in June and July temperatures averaged 3.5ºC warmer at AWCC than in the North Fork. It could be possible that this population from the North Fork is suited for slightly cooler temperatures during this energetically demanding time of year. The specimens of *P. oviforme* collected in 1999 were from the Clinch River.

Seasonal fluctuations in glycogen, lipids, and proteins occurred for all four species and could be attributed to multiple external factors, including water temperature, water chemistry, substrate conditions, and food availability. Such fluctuations are presumed, but levels of energy substrates, especially glycogen, after 2 yr in captivity are of greater importance. Even though the 2002 collection of *P. oviforme* survived poorly at AWCC, the other three species maintained body condition and good survival. In addition, numerous other species have been held with success at AWCC in previous years. Various species of mussels, residing in a variety of habitats in the wild and considered to be hardy species, can do poorly when brought into captivity (Jess

Jones, FMCC, pers. comm.). In addition, other studies that have held mussels in captivity have shown that survival among species is often quite variable (Dunn and Layzer 1997, Boyles 2004).

Previous captive studies have had varying results, often with significant mortality and greatly reduced energy reserves (Gabbot and Walker 1971, Dunn and Layzer 1997). My study indicated that AWCC was capable of holding a suite of species, except *P. oviforme*, in good body condition with minimal mortality. Reduced energy substrates (glycogen and protein), and significant mortality indicate that mussels of *P. oviforme* experienced above-average mortality at AWCC in year 1. My study also indicated that even if significant differences occurred in energy substrates, mussels were still not necessarily in poor condition since mass mortality events were infrequent and reproduction could still occur. Based on these results, holding freshwater mussels was successful overall, and did not significantly compromise the condition or survival rates of three out of four mussel species over a 2 yr trial period. Future research should focus particularly on the condition of short-term brooders at AWCC, since many of the imperiled species that need to be propagated are short-term brooders.

CONCLUSIONS

Survival rates of three species of mussels held in captivity for 2 yr were high: *A. plicata,* 100 %; *V. vanuxemensis*, 86 %; *V. iris*, 79 %.

Survival rates of *P. oviforme* were lower than expected: *P. oviforme* (2002 collection), 53 %; and *P. oviforme* (2003 collection), 50 %.

Glycogen appeared to be the best indicator of good condition, with protein also potentially reflecting good condition in some cases (2003 collection of *P. oviforme*).

Glycogen was the most variable energy reserve measured, with higher levels at the end of the experiment in all captive specimens, except for the 2002 collection of *P. oviforme*, than in the wild populations.

Protein levels in all species, except for *P. oviforme*, closely mimicked levels in the wild source populations.

Protein levels in both captive long-term brooders peeked in winter 2003 and showed an overall decrease in protein levels thereafter, becoming more pronounced after fall 2003.

Lipid levels were seemingly related to gametogenic activity in long-term brooders from the wild source population, captive long-term brooders in 2004, and in captive and wild populations of *P. oviforme*.

Lipid was the energy substrate with the greatest significant difference between captive and wild source populations, especially for the threeridge mussel.

Since survival, and glycogen and protein, maintained levels relatively close to those in the wild, AWCC is deemed a suitable facility to hold freshwater mussels for at least 2 yr.

RECOMMENDATIONS

Since some mussels brought into captivity for propagation purposes are often long-lived species, it would be beneficial to conduct a periodic energy substrate study with some of these specimens held for longer than a 2 yr period. Also, since there was high variability in the energy substrates and survival in this study, monitoring additional species for survival and condition, especially short-term brooders, should be researched in the future.

Additional research should be completed with other species in the genus *Pleurobema* to determine whether conspecific species experience poor survival at AWCC. In addition, potential variables such as water flow, temperature, and food availability between AWCC and wild populations should be conducted for species reported to do poorly at AWCC. This may help explain potential differences in body condition and survival between captive and source populations.

Body condition studies that assess the suite of energy reserves (glycogen, protein, and lipid) should be encouraged in the future, to incorporate the three energy substrate variables. There also is a need to further develop non-lethal techniques for sampling mantle tissue and to assess the effects of mantle clipping on captive specimens. Tissue amounts needed for protein (5.0 –6.5 mg) and especially lipid (50 mg) analyses were too great for biopsy samples, given current technology. In some cases, not enough tissue was available when conducting lipid analyses on the smaller species, such as *V. iris* and *V. vanuxemensis* to complete sufficient replicates. Refining techniques using smaller amounts of tissue would help minimize mortality and could also be useful in determining mortality associated with mantle clipping in captive specimens.

Although not done in this experiment, monitoring survival in the species sampled from the wild could also be useful in making comparisons between their energy substrate levels, as done with the captive mussels. This would require tagging a large number of mussels in the wild and keeping them contained in an enclosure that would prevent dispersal. In addition, this information would also be helpful in comparing survival rates between those in the wild and those in captivity. These additional data would aid in determining whether captive conditions were in fact promoting mortality.

Studying the effects of substrate composition and different holding containers on the energy substrate levels in mussels could also be beneficial. The containers used for this experiment may have affected body condition by restricting horizontal and vertical movement. Experiments with a variety of substrate types, substrate depths, and holding containers could help determine the effects of such variables on body condition.

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Table 1.1. Percent survival of four species of freshwater mussels held at AWCC from September 2002-July 2004.

	Species				
Season	*A. plicata*	*P. oviforme* (2002 collection)	*P. oviforme* (2003 collection)	*V. iris*	*V. vanuxemensis*
Fall 2002	100.0	98.7	·	100.0	94.5
Winter 2003	100.0	97.5	·	98.3	93.2
Spring 2003	100.0	78.5	·	94.8	91.8
Summer 2003	100.0	55.7	100.0	94.8	90.4
Fall 2003	100.0	55.7	100.0	94.8	90.4
Winter 2004	100.0	53.2	100.0	82.3	90.4
Spring 2004	100.0	·	100.0	79.3	87.7
Summer 2004	100.0	·	50.0	79.3	86.3

Table 1.2. Mean (± 1 SE) glycogen content in mg/g dry weight of two species of short-term brooders held in captivity at AWCC (fall 2002 to summer 2004), and their source populations. P-values determined by Wilcoxon Two-Sample Test, with P<0.05 considered significant. Significant differences are in bold. n=6 except where noted.

	P. oviforme **(2002 Collection)**			***A. plicata***			***P. oviforme*** **(2003 Collection)**		
Season	**Wild**	**Captive**	**P-values**	**Wild**	**Captive**	**P-values**	**Wild**	**Captive**	**P-values**
Fall 2002	805.1 ± 21.7	805.1 ± 21.7		668.5 ± 51.9	668.5 ± 51.9				
Winter 2003	690.8 ± 78.5	452.4 ± 47.1	0.0706	528.8 ± 104.6	617.4 ± 79.0	0.3203			
Spring 2003	528.1 ± 58.1	539.1 ± 99.7	0.2550	686.1 ± 84.2	636.9 ± 71.4	0.9376			
Summer 2003	418.6 ± 67.3	437.3 ± 154.2	0.3973	571.7 ± 101.4	889.0 ± 33.2	0.0706	418.6 ± 67.3	861.2 ± 53.4	**0.0172**
Fall 2003	397.6 ± 42.7	276.5 ± 48.5	0.2550	493.7 ± 59.0[+]	680.7 ± 61.1	0.0836	397.6 ± 42.7	648.5 ± 93.5	0.0927
Winter 2004	788.8 ± 32.4	365.9 ± 185.3	0.0595	989.0 ± 99.7	917.4 ± 88.3	0.5864	788.8 ± 32.4	586.6 ± 64.0	**0.0404**
Spring 2004	727.0 ± 81.9			711.0 ± 126.0	745.1 ± 147.7	0.9376	727.0 ± 81.9	722.1 ± 136.9	0.6966
Summer 2004	844.6 ± 85.1			571.4 ± 54.6	944.6 ± 41.7	**0.0172**	844.6 ± 85.1	640.9 ± 94.9[+]	0.1135

+ n=5

Table 1.3. Mean (± 1 SE) glycogen content in mg/g dry weight of two species of long-term brooders held in captivity at AWCC (fall 2002 to summer 2004), and their source populations. P-values determined by Wilcoxon Two-Sample Test, with P<0.05 considered significant. Significant differences are in bold. n=6 except where noted.

	V. vanuxemensis			***V. iris***		
Season	**Wild**	**Captive**	**P-values**	**Wild**	**Captive**	**P-values**
Fall 2002	461.0 ± 41.1[+]	461.0 ± 41.1[+]		518.7 ± 94.6	518.7 ± 94.6	
Winter 2003	515.8 ± 35.5	369.7 ± 43.2	0.0535	579.3 ± 35.5	481.0 ± 123.1	0.0927
Spring 2003	492.2 ± 31.9	334.4 ± 32.9	**0.0404**	762.1 ± 52.2	347.8 ± 56.2	**0.0172**
Summer 2003	431.3 ± 27.4	443.4 ± 92.8	0.8146	668.8 ± 158.4	452.7 ± 82.6	0.3203
Fall 2003	344.5 ± 36.6	463.8 ± 67.7	0.2550	409.9 ± 83.6	471.1 ± 134.2	1.0000
Winter 2004	384.5 ± 58.4	308.3 ± 23.8	0.2550	505.3 ± 90.9	337.5 ± 77.5	0.3203
Spring 2004	550.2 ± 31.8	462.9 ± 12.3	0.1208	906.4 ± 108.1	600.4 ± 94.0	**0.0404**
Summer 2004	443.9 ± 97.0	635.4 ± 46.6	0.1564	789.1 ± 92.1[+]	991.5 ± 113.4[+]	0.3235

+ n=5

Table 1.4. Mean (± 1 SE) protein content in mg/g dry weight of two species of short-term brooders held in captivity at AWCC (fall 2002 to summer 2004), and their source populations. P-values determined by Wilcoxon Two-Sample Test, with P<0.05 considered significant. Significant differences are in bold. n=6 except where noted.

	P. oviforme (2002 Collection)			*A. plicata*			*P. oviforme* (2003 Collection)		
Season	**Wild**	**Captive**	**P-values**	**Wild**	**Captive**	**P-values**	**Wild**	**Captive**	**P-values**
Fall 2002	491.9 ± 35.2+	491.9 ± 35.2+		413.0 ± 41.3	413.0 ± 41.3				
Winter 2003	392.5 ± 17.4	396.5 ± 25.9	0.9376	410.0 ± 15.1+	372.0 ± 17.6	0.2628			
Spring 2003	293.6 ± 12.9+	449.7 ± 20.7	**0.0244**	309.5 ± 33.2	303.1 ± 18.9	0.8146			
Summer 2003	541.3 ± 15.3	478.4 ± 30.6	0.2007	451.5 ± 30.1	424.7 ± 17.1	0.5864	541.3 ± 15.3	372.6 ± 13.1	**0.0172**
Fall 2003	428.9 ± 24.0	453.8 ± 28.0	0.5864	372.9 ± 8.6	416.1 ± 34.9	0.3973	428.9 ± 24.0	395.2 ± 12.9	0.6966
Winter 2004	395.2 ± 25.0	380.1 ± 12.1	0.9005	336.1 ± 24.8	324.9 ± 15.6	0.8146	395.2 ± 25.0	363.5 ± 18.5	0.3203
Spring 2004	428.9 ± 49.0			259.1 ± 3.5	245.0 ± 14.8	0.3973	428.9 ± 49.0	457.5 ± 33.2	0.8755
Summer 2004	314.5 ± 10.2			302.6 ± 8.8	215.7 ± 13.9	**0.0172**	314.5 ± 10.2	309.0 ± 15.0+	0.9291

+ n=5

Table 1.5. Mean (± 1 SE) protein content in mg/g dry weight of two species of long-term brooders held in captivity at AWCC (fall 2002 to summer 2004), and their source populations. P-values determined by Wilcoxon Two-Sample Test with P<0.05 considered significant. n=6 except where noted.

	V. vanuxemensis			*V. iris*		
Season	**Wild**	**Captive**	**P-values**	**Wild**	**Captive**	**P-values**
Fall 2002	429.0 ± 22.0	429.0 ± 22.0		484.0 ± 54.7	484.0 ± 54.7	
Winter 2003	455.0 ± 13.5	501.0 ± 50.4+	0.9291	469.0 ± 17.6	490.0 ± 31.6	0.3973
Spring 2003	409.0 ± 10.8	434.0 ± 19.9	0.2550	447.0 ± 18.7	420.0 ± 21.5	0.4862
Summer 2003	427.0 ± 18.2	443.4 ± 9.8	0.2550	472.5 ± 21.9	447.1 ± 23.0	0.3203
Fall 2003	422.7 ± 21.0	386.7 ± 18.6	0.2007	454.4 ± 20.5	474.3 ± 41.2	0.8146
Winter 2004	332.4 ± 32.8	381.0 ± 24.7	0.3203	359.2 ± 15.3	408.2 ± 31.3	0.2550
Spring 2004	392.0 ± 14.5	433.6 ± 17.2	0.1208	385.6 ± 10.9	401.9 ± 15.2	0.8146
Summer 2004	344.6 ± 16.1	322.7 ±19.9	0.3978	358.4 ± 13.7+	353.4 ± 23.1+	1.0000

+ n=5

Table 1.6. Mean (± 1 SE) lipid content in mg/g dry weight of two species of short-term brooders held in captivity at AWCC (fall 2002 to summer 2004), and their source populations. P-values determined by Wilcoxon Two-Sample Test, with P<0.05 considered significant. Significant differences are in bold. n=6 except where noted.

	P. oviforme (2002 Collection)			*A. plicata*			*P. oviforme* (2003 Collection)		
Season	**Wild**	**Captive**	**P-values**	**Wild**	**Captive**	**P-values**	**Wild**	**Captive**	**P-values**
Fall 2002	62.3 ± 4.7[+]	62.3 ± 4.7[+]		52.4 ± 7.7	52.4 ± 7.7				
Winter 2003	49.1 ± 3.6	35.1 ± 6.2	0.1208	63.9 ± 3.5	28.0 ± 2.9[+]	**0.0244**			
Spring 2003	28.7 ± 4.7	28.6 ± 3.1	0.9376	43.5 ± 6.1	32.7 ± 2.3	0.1208			
Summer 2003	42.5 ± 7.8	34.9 ± 5.8	0.4862	63.2 ± 5.2	34.4 ± 3.7	**0.0229**	42.5 ± 7.8	36.4 ± 6.0	0.6966
Fall 2003	49.7 ± 4.1	40.5 ± 4.6	0.3203	48.6 ± 2.6	36.0 ± 2.5	**0.0305**	49.7 ± 4.1	47.3 ± 0.5	0.6966
Winter 2004	48.7 ± 4.2	32.5 ± 6.6	0.1318	58.0 ± 2.3	20.7 ± 3.6	**0.0305**	48.7 ± 4.2	35.2 ± 3.8	0.0927
Spring 2004	41.4 ± 2.8			45.2 ± 4.5	43.8 ± 3.1	0.0938	41.4 ± 2.8	31.7 ± 5.2	1.2080
Summer 2004	29.2 ± 4.5			41.1 ± 2.0	38.1 ± 2.5	0.3973	29.2 ± 4.5	42.4 ± 3.3[+]	0.1517

+ n=5

Table 1.7. Mean (± 1 SE) lipid content in mg/g dry weight of two species of long-term brooders held in captivity at AWCC (fall 2002 to summer 2004), and their source populations. P-values determined by Wilcoxon Two-Sample Test, with P<0.05 considered significant. Significant differences are in bold. n=6 except where noted.

	V. vanuxemensis			*V. iris*		
Season	**Wild**	**Captive**	**P-values**	**Wild**	**Captive**	**P-values**
Fall 2002	53.3 ± 4.5[++]	53.3 ± 4.5[++]		55.6 ± 6.1[++]	55.6 ± 6.1[++]	
Winter 2003	65.3 ± 5.7	41.9 ± 2.0[+]	**0.0456**	72.5 ± 7.4[+]	45.6 ± 5.0[+]	0.0928
Spring 2003	24.5 ± 5.8[+]	53.2 ± 8.7	0.0842	29.8 ± 1.8	53.7 ± 7.5[+]	**0.0244**
Summer 2003	33.4 ± 9.9	58.1 ± 6.5	0.0706	31.5 ± 9.4	39.8 ± 12.8[++]	0.5367
Fall 2003	47.6 ± 4.7	17.2 ± 3.8	**0.0172**	38.1 ± 4.1[+]	22.0 ± 2.5[+]	0.0928
Winter 2004	45.8 ± 4.9	78.0 ± 5.1	**0.0404**	53.5 ± 4.0	53.8 ± 12.4[+++]	1.0000
Spring 2004	28.4 ± 5.2	42.6 ± 3.6	0.0927	36.9 ± 6.3	41.4 ± 9.4[+]	0.5372
Summer 2004	24.0 ± 6.0[+]	40.1 ± 6.2	0.2009	31.1 ± 6.4[+]	28.0 ± 6.5[++]	0.7228

+ n=5

++ n=4

+++ n=3

Table 1.8. Water quality values measured with a Hydrolab unit at AWCC, from September 2003 to July 2004.

Parameters	n	Mean	Min	Max
pH	3535	7.29 ± 0.79	5.34	9.45
Specific Conductivity (µS/cm)	3436	140.71 ± 20.63	61	179.90
Total Dissolved Solids (g/L)	3444	0.0900 ± 0.0140	0.0001	0.2382
Dissolved Oxygen (mg/L)	3441	9.07 ± 1.67	5.95	12.01
Salinity (ppt)	3535	0.06 ± 0.02	0	0.18

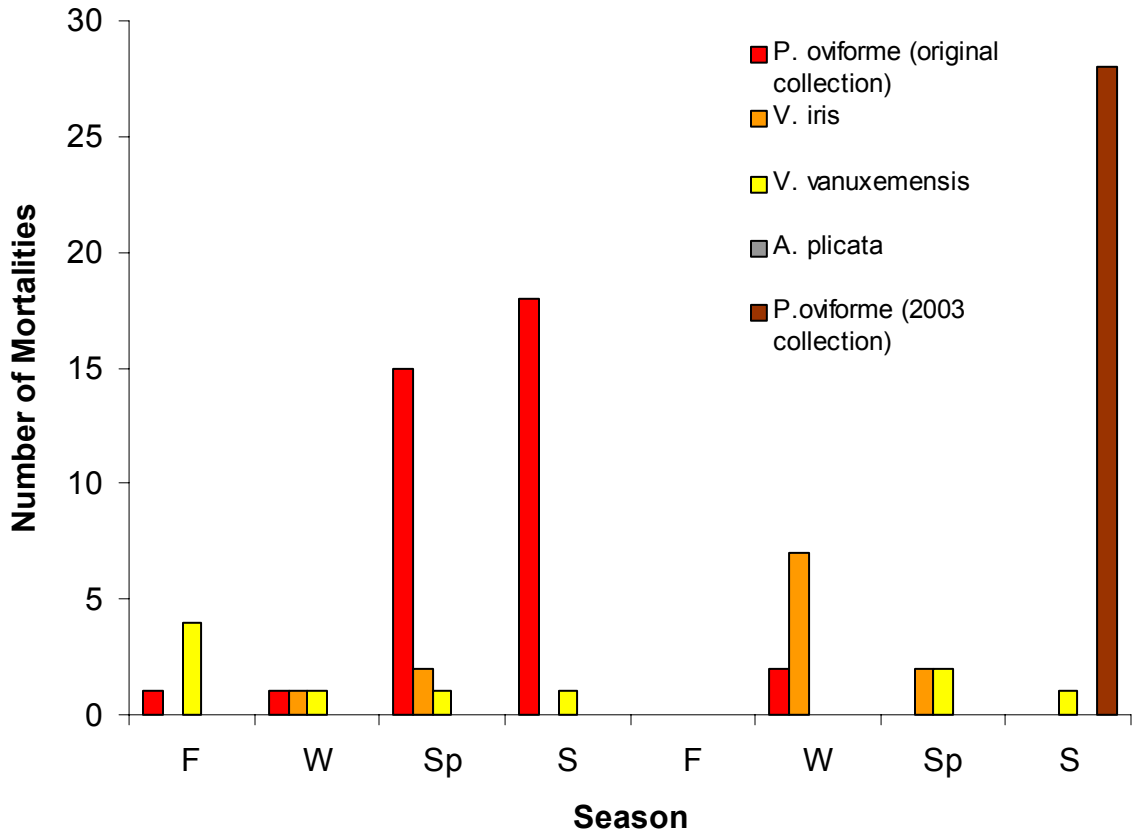

Figure 1.1. Seasonal mortality of four species of mussels held in captivity at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer).

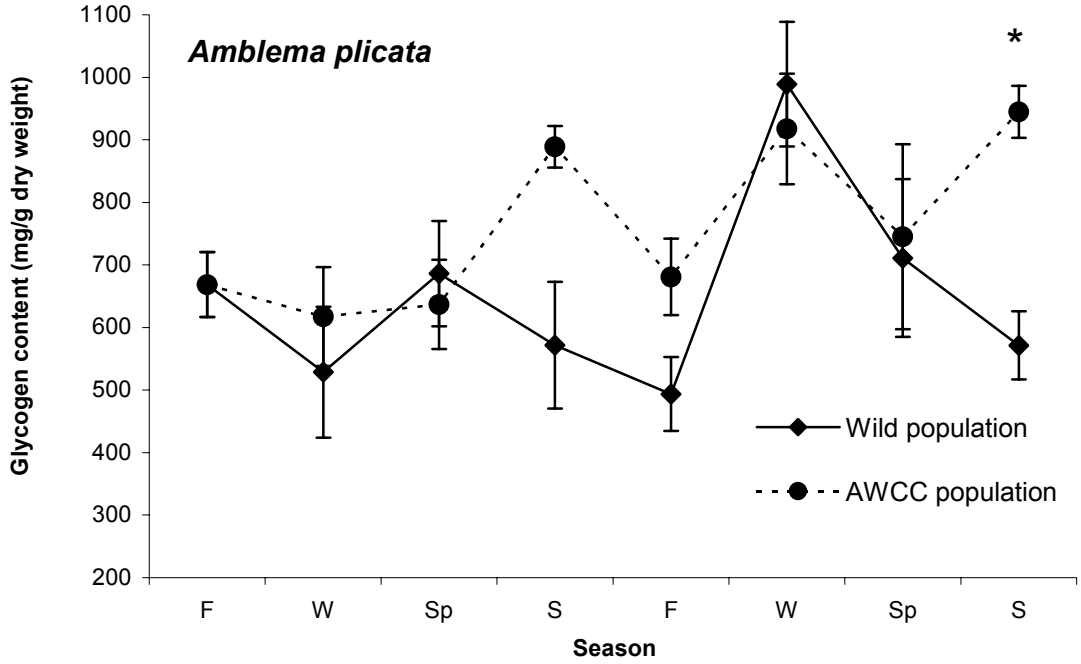

Figure 1.2. Comparison of mean glycogen content (± 1 SE) of *A. plicata* sampled from the Clinch River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference.

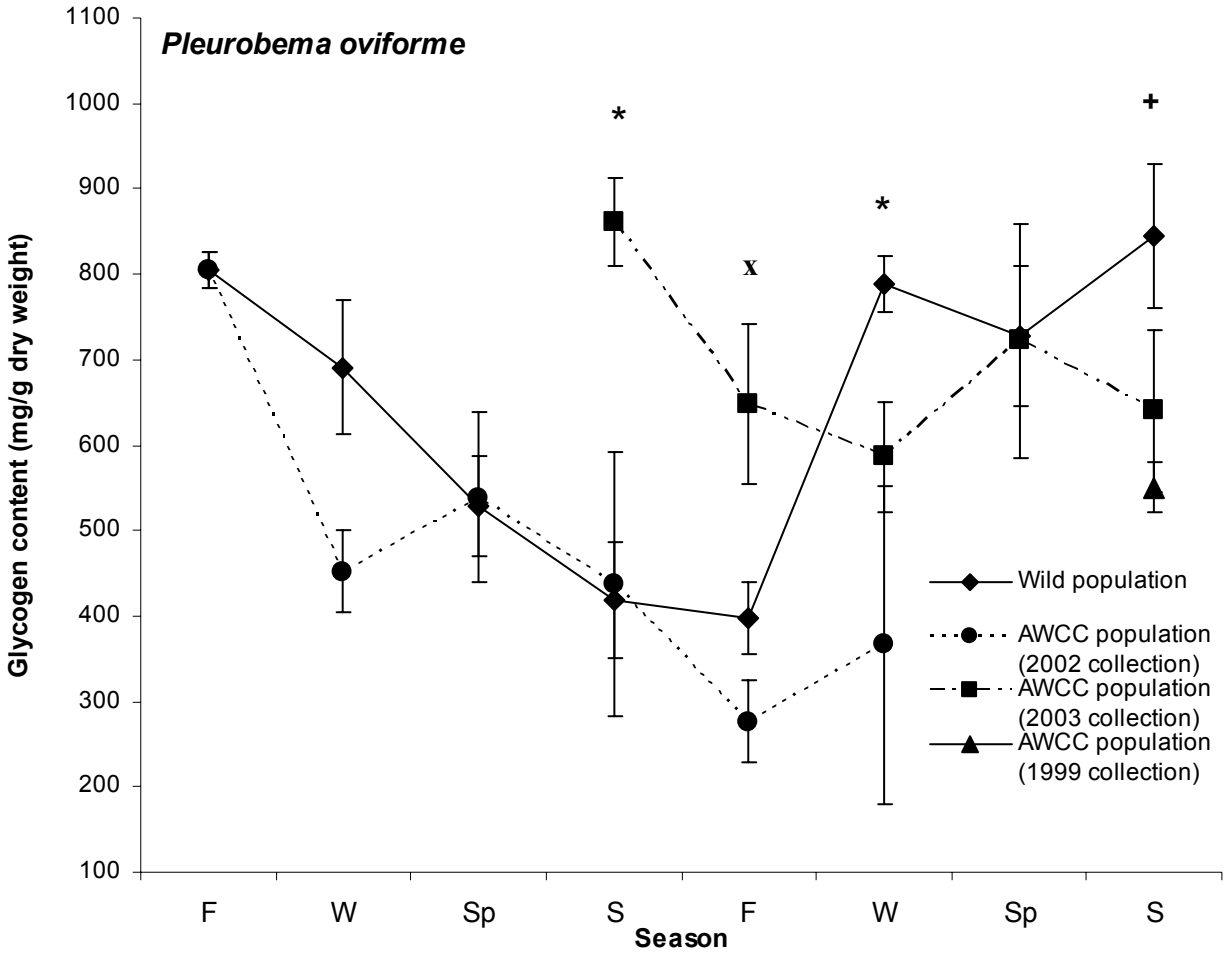

Figure 1.3. Comparison of mean glycogen content (± 1 SE) of *P. oviforme* sampled from the North Fork Holston River (n=6) and three groups of captive mussels (n=6) (relocated in 1999, August 2002, and August 2003) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes sig. diff. between wild and 2003 collection, **x** denotes sig. diff. between 2002 and 2003 collection, and + indicates sig. diff. between 1999 and 2003 collection.

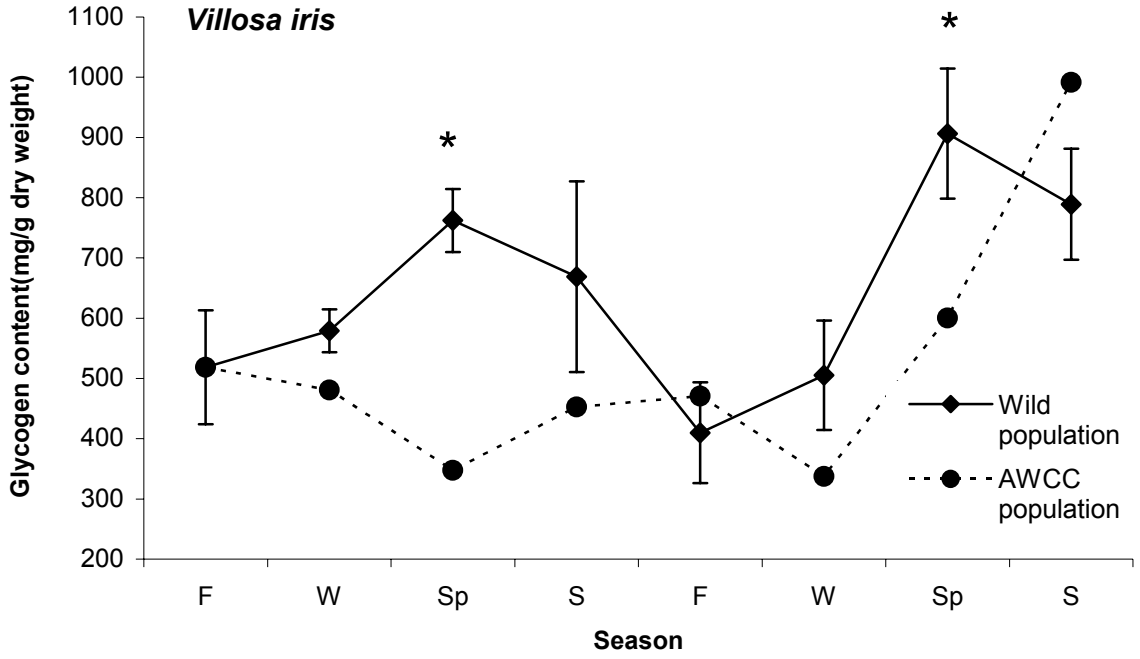

Figure 1.4. Comparision of mean glycogen content (± 1 SE) of *V. iris* sampled from the North Fork Holston River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference.

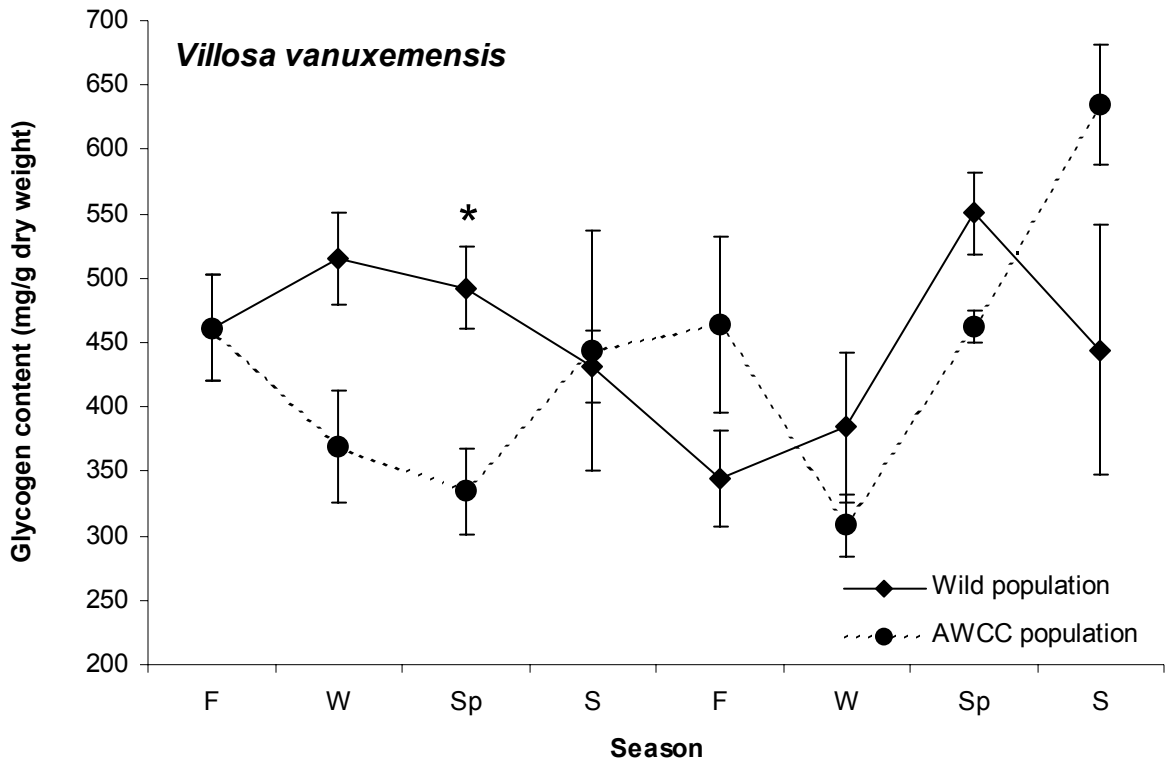

Figure 1.5. Comparison of mean glycogen content (± 1 SE) of *V. vanuxemensis* sampled from the North Fork Holston River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference.

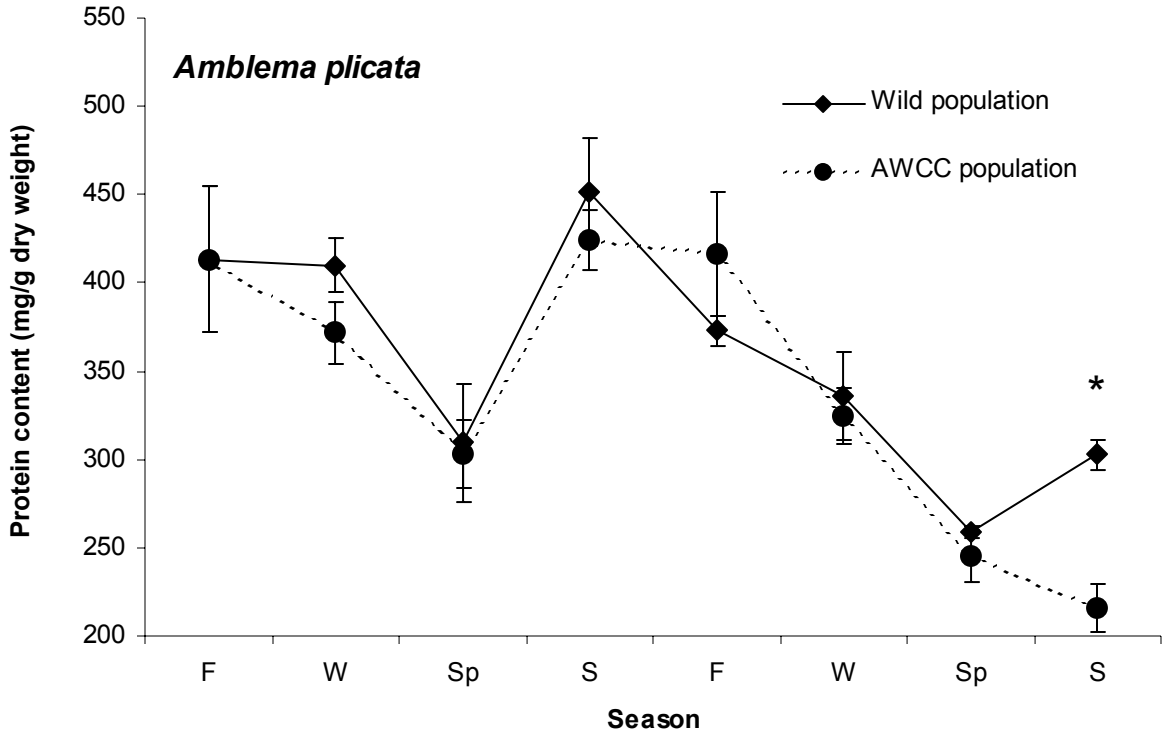

Figure 1.6. Comparison of mean protein content (± 1 SE) of *A. plicata* sampled from the Clinch River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference.

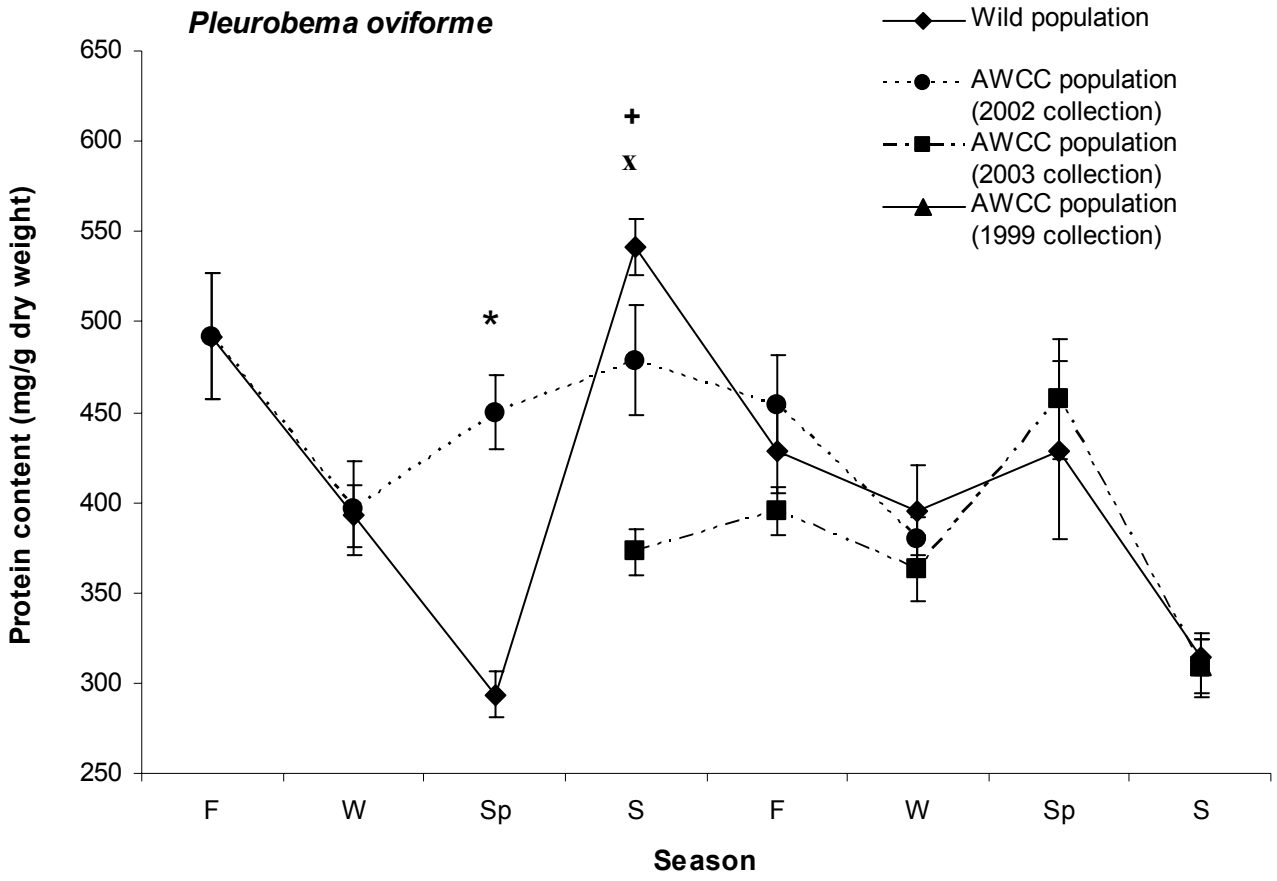

Figure 1.7. Comparison of mean protein content (± 1 SE) of *P. oviforme* sampled from the North Fork Holston River (n=6) and three groups of captive mussels (relocated in 1999, August 2002, and August 2003) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference between 2002 collection and wild population, **x** denotes sig. diff. between wild and 2003 collection, and + denotes sig. diff. between 2002 and 2003 collection.

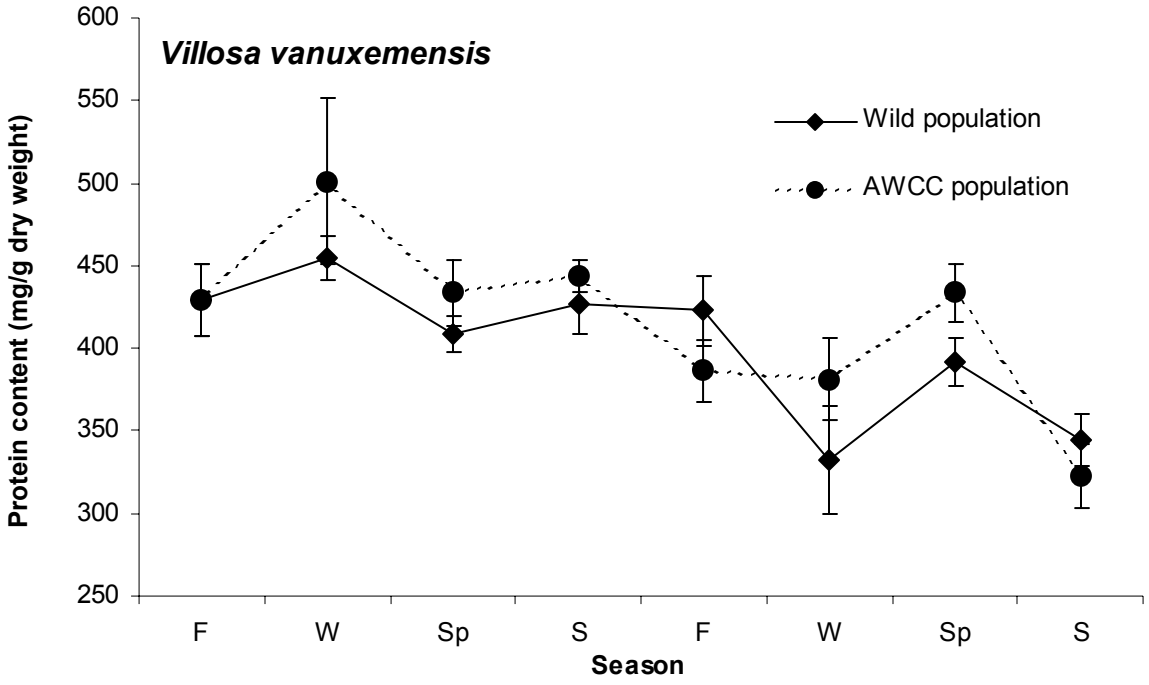

Figure 1.8. Comparison of mean protein content (± 1 SE) of *V. vanuxemensis* sampled from the North Fork Holston River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer).

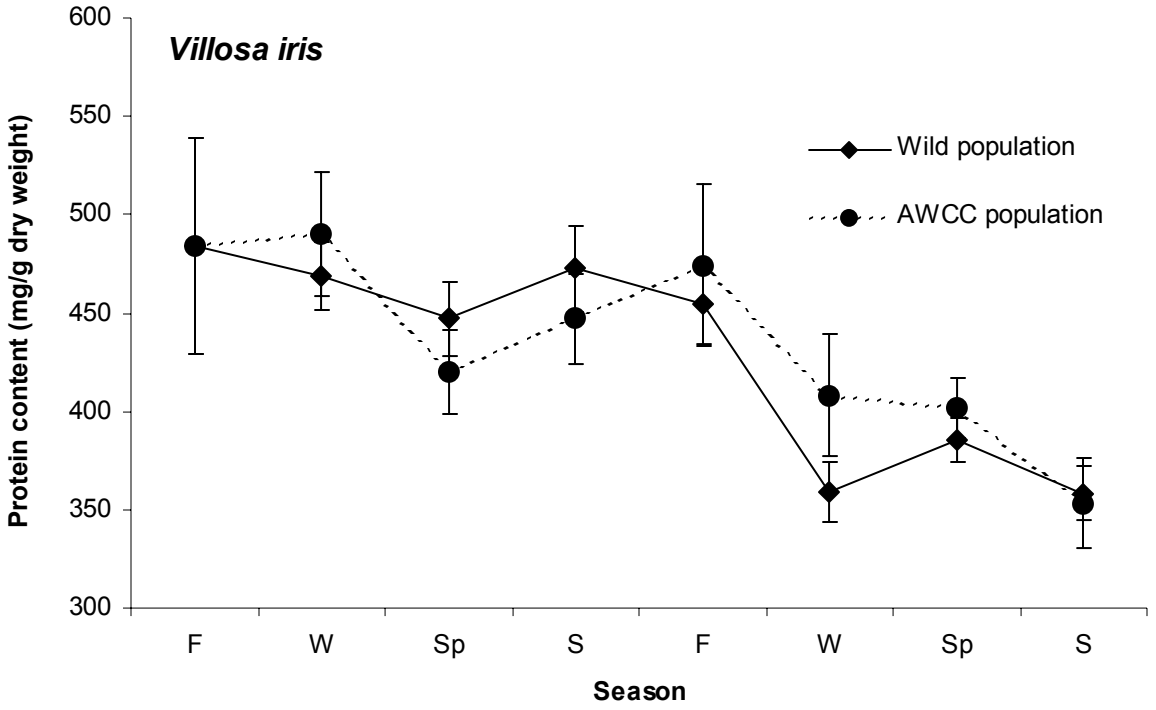

Figure 1.9. Comparison of mean protein content (± 1 SE) of *V. iris* sampled from the North Fork Holston River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer).

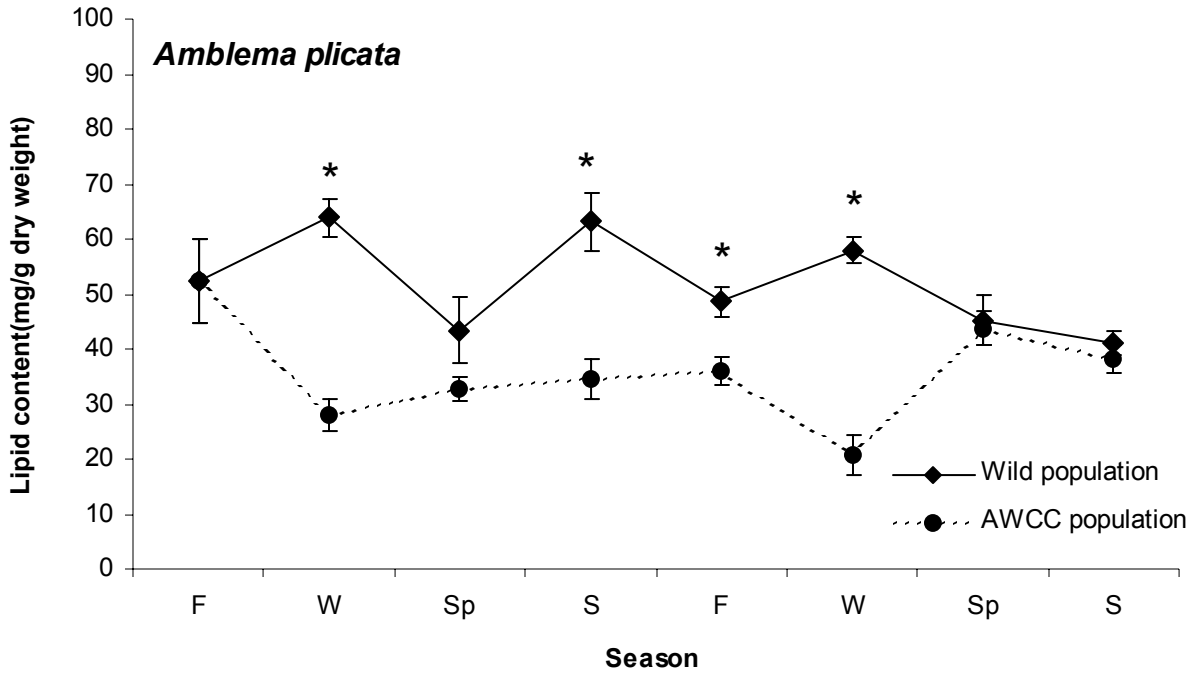

Figure 1.10. Comparison of mean lipid content (± 1 SE) of *A. plicata* sampled from the Clinch River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference.

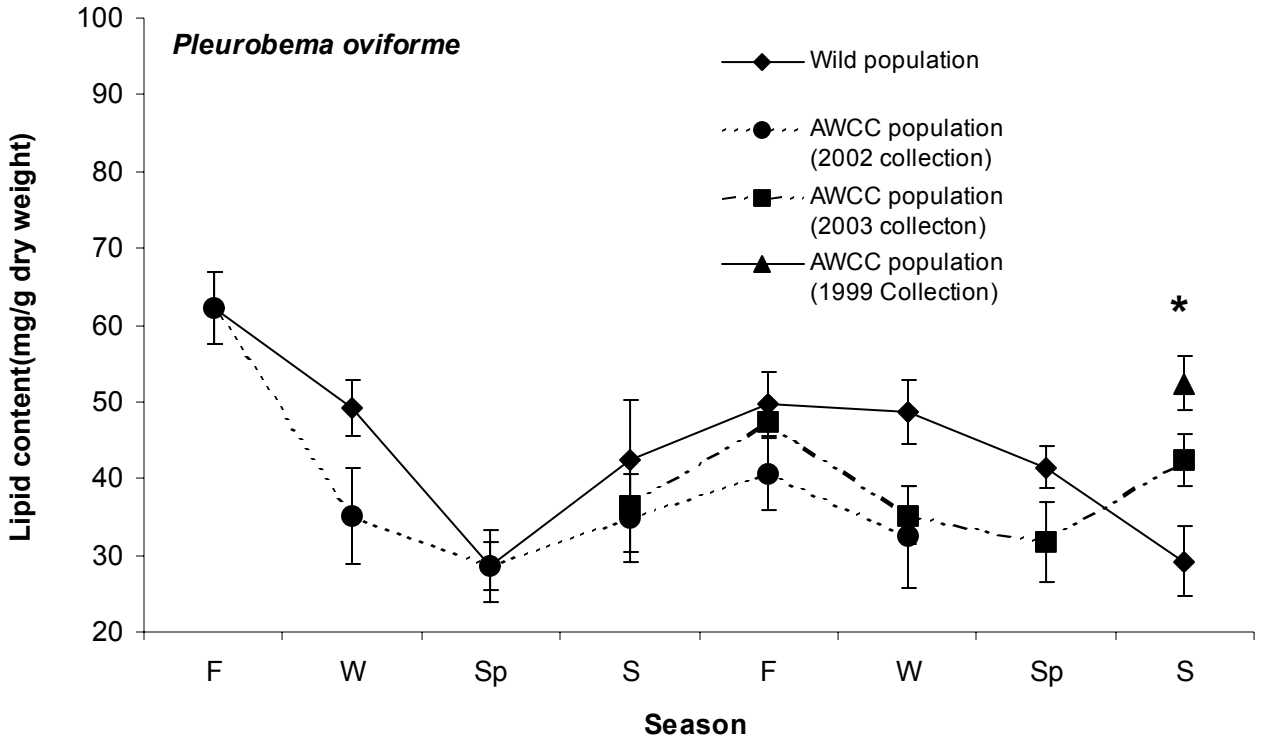

Figure 1.11. Comparison of mean lipid content (± 1 SE) of *P. oviforme* sampled from the North Fork Holston River (n=6) and three groups of captive mussels (relocated in 1999, August 2002, and August 2003) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference between 1999 and 2003 collection.

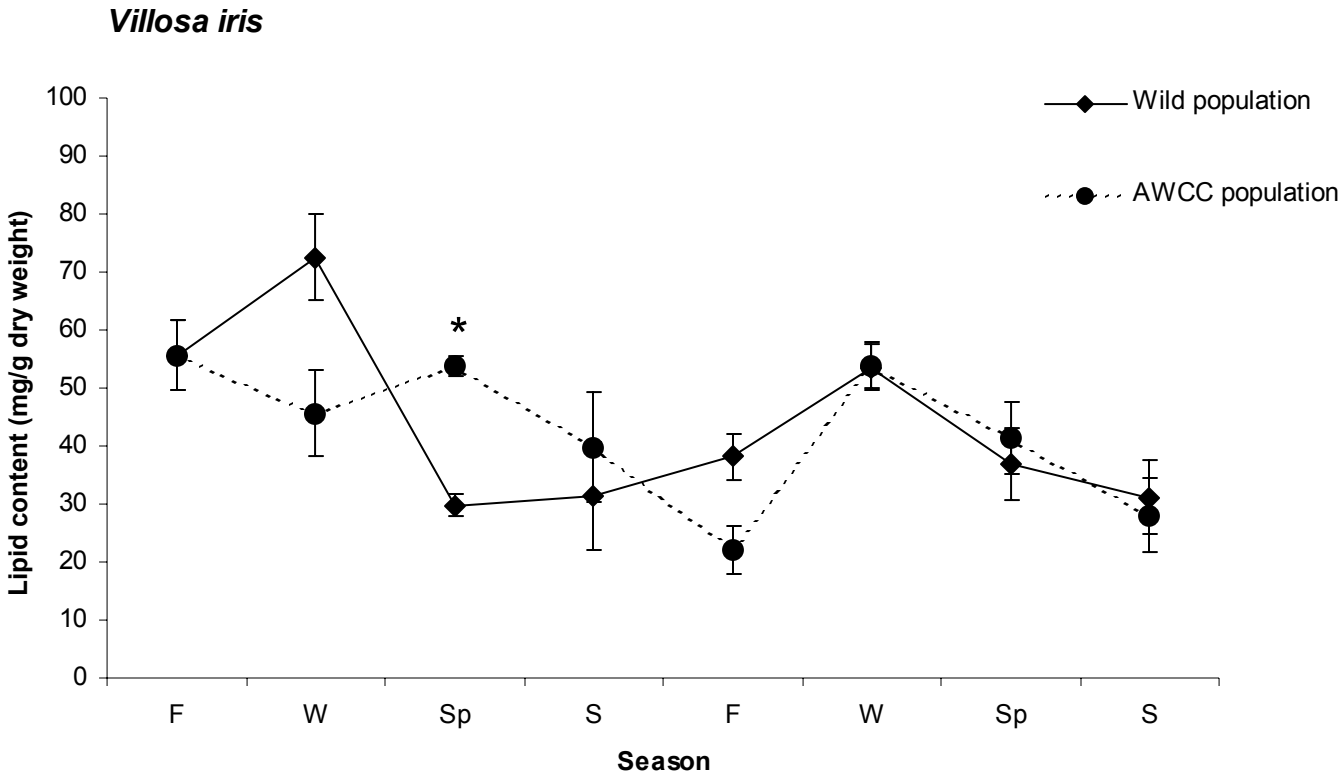

Figure 1.12. Comparison of mean lipid content (± 1 SE) of *V. iris* sampled from the North Fork Holston River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference.

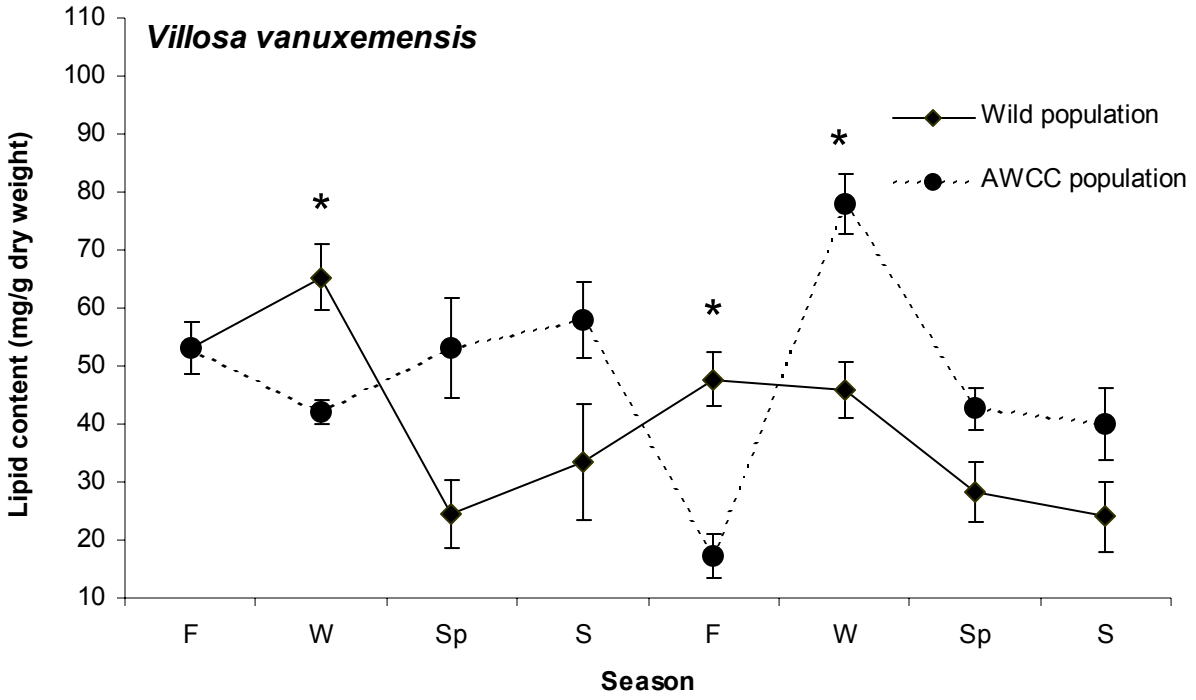

Figure 1.13. Comparison of mean lipid content (± 1 SE) of *V. vanuxemensis* sampled from the North Fork Holston River (n=6) and captive mussels (relocated August 2002) (n=6) at AWCC from fall 2002 to summer 2004 (F, fall; W, winter; Sp, spring; S, summer). * denotes significant difference.

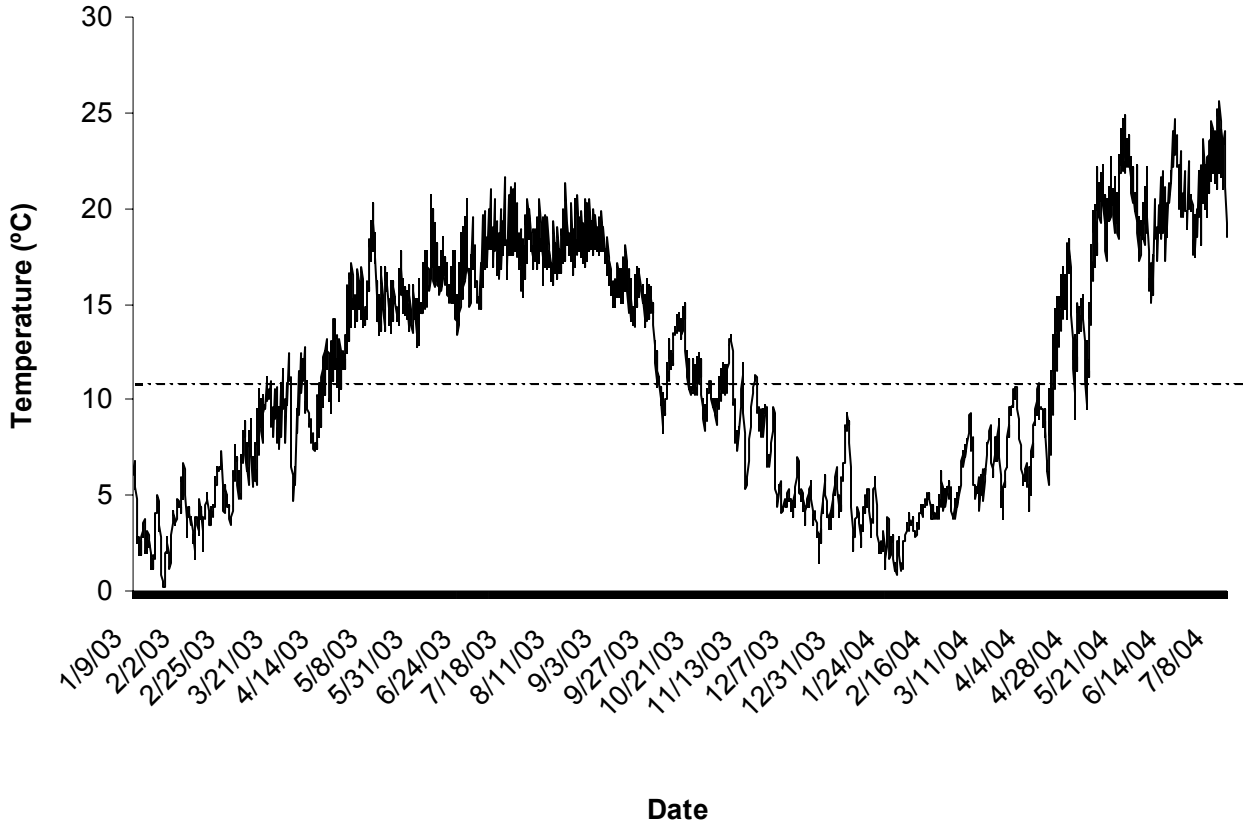

Figure 1.14. Daily mean temperature (ºC) of the raceway with captive mussels at AWCC during January 2003 to July 2004. Mean temperature (ºC) for duration of experiment indicated by dotted line.

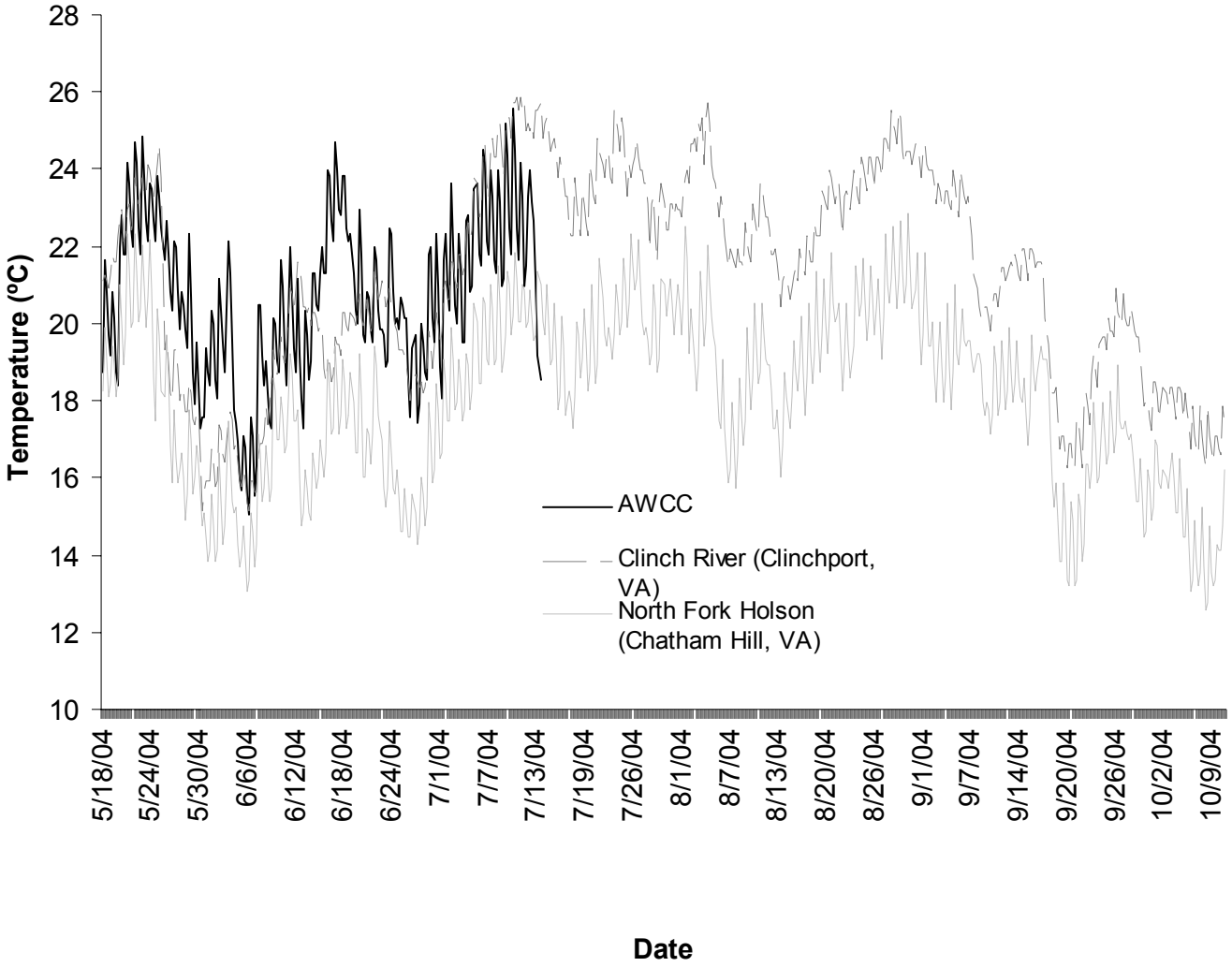

Figure 1.15. Daily mean temperature (ºC) of AWCC, Clinch River, and North Fork Holston during May to October 2004.

Chapter 2: Gametogenesis in Four Species of Freshwater Mussels in Virginia

INTRODUCTION

The reproduction biology of freshwater mussels has been well studied in a variety of species (Zale and Neves 1982, Holland-Bartels and Kammer 1989, Jirka and Neves 1992). However, detailed studies on the gametogenic cycle and reproductive traits of mussels have not yet been fully explored (Jones et al. 1986, Holland-Bartels and Kammer 1989, Haag and Staton 2003). Research has shown that there is a correlation between the gametogenic cycle and biochemical composition of bivalves (Bayne and Thompson 1970, Barber and Blake 1981, Okumus and Stirling 1998, Berthelin et al. 2000 b). Typically, the relationship between energy substrates and gametogenesis has been determined for marine bivalves (Barber and Blake 1981, Berthelin et al. 2000 a, b, Saucedo et al. 2002). In freshwater bivalves, this comparison has not been fully researched (Makela and Oikari 1995, Henley 2002). Since reproduction in freshwater mussels utilizes energy reserves, this likely accounts for certain fluctuations in the energy substrates of the rainbow mussel (*Villosa iris*), mountain creekshell (*Villosa vanuxemensis*), Tennessee clubshell (*Pleurobema oviforme*), and threeridge (*Amblema plicata*).

Before gametogenesis occurs, food reserves accumulate in the form of glycogen, protein, and lipids (Barber and Blake 1981). Research has shown that of the three energy stores, glycogen plays the greatest role in supplying energy for gametogenesis in bivalves (Okamus and Stirling 1998, Berthelin et al 2000 a, b). Gabbott and Bayne (1973) reported that oogenesis in female *Mytilus edulis* coincided with depletion in glycogen levels. The degree to which these energy substrates are used, and which are most important, will oftentimes vary among species and populations within the same species (Barber and Blake 1981, Saucedo et al. 2002). Nagabhushanam and Dhamne (1977) reported that glycogen level was highest prior to

gametogenesis and then declined during gametogenesis and spawning. It was also reported that protein and lipid levels were highest when gametes were in the mature stage and that levels declined upon spawning. Gabbott and Bayne (1973) also reported that glycogen and protein reserves were accumulated during summer and then subsequently utilized for gonad development.

The gametogenic stage of freshwater mussels has been assessed in the past by categorizing the stage observed during the time it was sacrificed (Holland-Bartels and Kammer 1989, Haggerty et al. 1995, Saucedo et al. 2002). Such quantitative analyses using stage evaluations are believed to give a more accurate assessment of the gametogenic stage than solely qualitative analyses (Haggerty et al. 1995). One method used to assess gametogenic stage is the Gamete Development Index (GDI) developed by Barber (1996), which has been used in subsequent studies (Henley 2002, Boyles 2004). The GDI method was deemed appropriate for my study and was used to make all gametogenic stage evaluations.

The Aquatic Wildlife Conservation Center (AWCC) is a state-owned facility operated by the Virginia Department of Game and Inland Fisheries (VDGIF) in Marion, Virginia. Currently this facility is for the conservation and propagation of freshwater mussels in southwest Virginia. Survival and reproduction of mussels on site is critical for the long-term goal of this facility. Determining the gametogenic stages of captive and wild mussels also will be useful in making comparisons with energy substrate levels and changes during the year. More importantly, documentation of mature gametes will determine whether this facility is capable of sustaining reproduction of captive mussels for multiple years. To address this question, the gametogenic stage of each species in captivity was compared with the stage found in the wild source populations on a seasonal basis for the duration of the 2 yr study.

METHODS AND MATERIALS

From September 2002 to July 2004, quarterly sample events were conducted in January, April, July, and October after the original sample date. All sample events occurred in the middle of each month so comparisons between years could be made. At each sample event a random selection of four mussels of each species from the source population and captive population had their gonads removed and preserved in Bouin's fixative (75 % picric acid, 20 % formalin, 5 % acetic acid) for histological analysis. This was accomplished by sectioning the visceral mass of the mussel to examine the gonads. These visceral masses were placed in embedding cassettes before being bathed in Bouin's fixative for 5 days. Then the cassettes were removed from the Bouin's fixative and placed in 70 % ethyl alcohol. Tissue was then cleared with xylene and embedded in paraffin blocks. Henley (2002) reported gametogenic stage did not vary within different areas of the gonad for *V. iris* or *Utterbackia imbecillis*. Therefore, each block was cut with a microtome, and a 5 μm section was taken at a random point within the gonad. Each section was affixed to a glass microscope slide and stained with Heidenhain's iron hematoxylin and eosin-orange G based on the methods of Humason (1979).

Four fields of view were randomly selected for each slide and examined under a compound microscope at 10 X. Within each field of view, the gametogenic stage was evaluated under four randomly placed points drawn on the ocular, following the methods of Chalkley (1943). This amounted to 16 points of observation for each slide. The gamete development index (GDI), developed by Barber (1996) and Henley (2002), was used to evaluate the gametogenic stage based on the following scale: 0, inactive; 1, early active; 2, late active; 3, mature; 4, spawned; and 5, resorbing (Table 2.1).

Gravidity was assessed for two long-term brooder species, *V. iris* and *V. vanuxemensis*, held at AWCC in July 2003 and 2004. Gravidity in short-term brooders, *A. plicata* and *P. oviforme,* was not assessed since both of these larger species were extremely difficult to open, and opening the shells could cause stress. It has also been reported that other species of short-term brooders, including *P. oviforme*, at AWCC experience less mortality when they are left untampered and undisturbed (Mike Pinder, VDGIF, pers. comm.).

Data Analysis

Results of the GDI values were compared between captive and source populations for each species at the seven sample events following the September 2002 collection of specimens. Sample size was small (n=4) for each species. To maximize replication, GDI values were not analyzed by gender since previous studies have reported that freshwater mussels are synchronous spawners (Zale and Neves 1982, Weaver et al. 1991, Haggerty et al. 1995). Comparisons between GDI values for each species and location at each sample event were made using an ANOVA in the SAS software package (SAS Institute Inc. 2002). Tukey's multiple comparison technique was then used to compare means for statistical differences at $P<0.05$.

RESULTS

In July 2003 and 2004, captive *V. iris* and *V. vanuxemensis* were examined for gravidity. Long-term brooders remaining in the raceways were separated into male and female mussels by shell characteristics. Of 14 female *V. vanuxemensis* checked in July of 2003, 79 % were gravid. In July of 2004, 50 % of 8 female *V. vanuxemensis* were gravid, with 3 gravid for during their second consecutive year at AWCC. The second long-term brooder, *V. iris,* had similar results; 82 % of 11 females in July of 2003 were gravid. In July of 2004, 60 % of 5 female *V. iris* were

gravid, with 2 gravid during their second consecutive year at AWCC. *Amblema plicata* and *P. oviforme* were not checked for gravidity, but during July 2003 and 2004, *P. oviforme* released conglutinates into the raceways. The number of gravid *P. oviforme* could not be obtained because determining which mussels had released conglutinates was not possible.

A total of 59 *V. vanuxemensis* were sacrificed and examined histologically for gametogenic stage, comprised of 86.4 % male, 10.2 % female, and 3.4 % undifferentiated. A total of 59 *V. iris* also were evaluated: 76.3 % male, 15.3 % female, 5.1 % undifferentiated, and 3.4 % hermaphroditic. During October 2003 and January 2004, samples of the few remaining *P. oviforme* from the original collection were combined with samples from the 2003 collection within each season for gametogenic analysis. Of these 70 *P. oviforme*, 42.9 % were male, 54.3 % were female, 1.4 % were undifferentiated, and 1.4 % were hermaphrodites. Lastly, 59 mussels of *A. plicata* were examined; 47.5 % male, 50.8 % female, and 1.7 % undifferentiated.

Trematodes were present in all four species of mussels, from the Clinch and North Fork Holston (NFHR) rivers. This trematode was identified as *Digenea* sp. and has been reported in mussels inhabiting the Clinch River, NFHR, and New River in Virginia (William Henley, Virginia Tech, pers. comm.). To account for trematodes as a possible confounding factor, comparisons were made between glycogen, protein, and lipid levels in infested and non-infested *V. iris* and *V. vanuxemensis*. Using a Wilcoxon two-sample test, there were no significant differences between infested and non-infested mussels of either species at any sample event (Tables 2.2, 2.3, 2.4, and 2.5). Trematodes were much more prevalent in long-term brooders from the NFHR. Trematode infestation rates for *V. vanuxemensis*, *V. iris*, *A. plicata*, and *P. oviforme* were: 34, 34, 5, and 3 %, respectively. Very few specimens infested with trematodes had mature gametes, and mussels were often so heavily infested that gametogenic stage, and

often gender, were indeterminable. Males also seemed more prone to trematode infestation. The following are the percentages of species by sex that were infested: *V. vanuxemensis* (85 % male, 10 % female, 5 % undifferentiated), *V. iris* (70 % male, 20 % female, 10 % undifferentiated), *A. plicata* (67 % male, 33 % undifferentiated, 0 % female), and *P. oviforme* (50 % male, 50 % female). No apparent discrepancies were observed between the number of trematode-infested mussels reported at AWCC and in the wild populations, since 52 % of those infested were sampled from the source populations, and 48 % were from the captive mussels at AWCC.

Results of the GDI values indicate that the four species of mussels held at AWCC and in the source populations were producing mature gametes. For *V. vanuxemensis* at AWCC, mature gamete production was evident from January to July during 2003 and 2004 (Figure 2.1). In the source population, this species did not produce any mature gametes prior to July of 2003 and 2004, except for a small percentage of mature gametes in January 2004 (Figure 2.1), indicating gametogenesis was occurring later in the wild. Small sample size (n=4) and one section per specimen may have also contributed to this obvious difference in gametogenesis between captive and wild *V. vanuxemensis*, especially in January 2003 and 2004. Mountain creekshells exhibited the greatest significant difference in GDI values between captive and source population mussels (Table 2.6). Significant differences were observed in April 2003 ($P<0.0001$), October 2003 ($P=0.0324$), and April 2004 ($P<0.0001$). *Villosa iris* was very similar in timing of reproduction to *V. vanuxemensis* at AWCC, since most mature gamete production occurred during July 2003 and 2004 (Figure 2.2). Mature gamete production of *V. iris* in the wild population during 2004 mimicked gametogenesis at AWCC closely, but the source population had more mature gametes in October 2003 than in July 2003 (Figure 2.2), again indicating that possibly gametogenesis was

occurring sooner in the captive populations. This resulted in the only significant difference in GDI values during October 2003 for *V. iris* (P=0.0036) (Table 2.6).

Results of the GDI values for *A. plicata* and *P. oviforme* in captivity and in the source populations were very similar. Mature gametes for both species were found during every sample event in captivity and in the wild, except for *A. plicata* during April 2004 at AWCC (Figures 2.3 and 2.4). No patterns of GDI values were observed in *A. plicata* (Figure 2.3), but the captive and source population of *P. oviforme* had the highest percentage of mature gametes in April 2003 and 2004 (Figure 2.4). The only significant differences between GDI values for these two species were for *A. plicata* in July 2003 (P=0.0314) and April 2004 (P=0.0002) (Table 2.6).

GDI values for each species at AWCC and in source populations did not appear to change between years. The only significant difference reported between GDI values in 2003 and 2004 was for *A. plicata* in April. GDI values also were found to be significantly different between individuals in the same treatment for all species at each sample event (P<0.001).

The wild population of *V. vanuxemensis* had high lipid levels in January 2003 and 2004 (Figure 1.13) that decreased with the production of mature gametes in April 2003 and July 2004 (Figure 2.1). Decreasing lipid levels with the production of mature gametes also observed with captive *V. vanuxemensis* in 2004 (Figures 1.13 and 2.1), and captive and wild *V. iris* in 2004 (Figures 1.12 and 2.2). High percentages of mature gametes in April 2003 and 2004 (Figure 2.4) for *P. oviforme* also coincided with low lipid reserves at this time of year (Figure 1.11). This indicates that perhaps lipids are fueling gametogenesis in these species. To exclude any differences in the utilization of lipids between sexes at each sample event, comparisons were made for each species. Although sample sizes were small (n=4), the only significant difference reported between lipid reserves in males and females at each sample event was for *A. plicata* in

July 2004 ($P<0.05$). No comparisons between glycogen and protein reserves in males and females were made since energy reserve trends did not coincide with trends in gametogenesis in this experiment.

DISCUSSION

Results of this study show that the conditions at AWCC did not compromise the ability of all four species of mussels to produce mature gametes in captivity. Mature gametes were present in all species during both years of captivity. The long-term brooders not only produced gametes, but also became gravid in 2003 and 2004, with some females becoming gravid for both years in captivity.

Definitive patterns in gametogenic activity of all four species was likely compromised by small sample size (n=4) and by sampling only once per season. In addition, the high costs and limited time available to complete this analysis required data to be collected from only one thin-section per specimen. At every sample event at each location, GDI values among mussels in the same treatment were significantly different from each other ($P<0.001$). This indicates that individual mussels within a species had gametogenic development occurring at different rates. This accounts for the presence of different gametogenic stages within species on individual sample dates. These findings are similar to those reported by Henley (2002), who found similar results in *Elliptio complanata*. In spite of these fluctuations, patterns were evident in each species. The species of long-term brooders had the largest percentage of mature gametes in July of 2003 and 2004, except for *V. iris* having a larger percentage of mature gametes in the wild in October 2003 (Figures 2.1 and 2.2). The specimens of *A. plicata* and *P. oviforme* stored mature

gametes year-round (Figures 2.3 and 2.4), with *P. oviforme* peeking in gamete production in April (Figure 2.4).

Previous studies suggest that development of mature gametes is temperature-dependent (Holland-Bartels and Kammer 1991). Makela and Oikari (1995) reported a delay in gonadal development in *Anodonta anatina* and *Pseudanodonta complanata* held in captivity at below normal temperatures. Water temperatures at AWCC from May to July were an average of 3.2°C warmer than in the North Fork Holston River during this time (Figure 1.15). It is likely that this warmer water temperature had an accelerated effect, with development of gonads occurring earlier in captive specimens compared to those in the wild. This is evidenced by no mature gametes in April 2002 and 2003 in *V. vanuxemensis* from the wild (Figure 2.1), while mature gametes were reported in AWCC specimens at this time of year (Figure 2.1). Significant differences in GDI values between locations for *V. vanuxemensis* in April 2003 ($P<0.0001$) and 2004 ($P<0.0001$) further support this hypothesis (Table 2.6). Also, it appeared that spawning was completed in *V. iris* at AWCC by October 2003 (Figure 2.2), whereas those in the wild continued to produce mature gametes into October 2003 (Figure 2.2). This difference in October 2003 was found to be significant ($P=0.0036$), and again may indicate a response to warmer water temperatures at AWCC (Table 2.6). These results concur with a study by Holland-Bartels and Kammer (1991), who reported that the long-term brooders *Lampsilis cardium* and *Potamilus alatus* reached full gonadal maturity from late May to mid-July.

Zale and Neves (1982) conducted a similar study with *V. iris* and *V. vanuxemensis*, sampling specimens weekly from April to October 1979, and semi-monthly from November 1979 to May 1980. Their study reported that both sexes of *V. vanuxemensis* peaked in percentage of mature gametes at the end of July, and spawned during the following 2 wk period.

This agrees with my study since the largest percentage of mature gametes was observed in mid-July, with spawning complete by mid-October. *Villosa iris* in their study were observed to be fully mature and spawn in mid-August. This also concurs with my study since the majority of mature gametes were observed in July in captive and wild specimens, except for the wild population having a greater percentage of mature gametes in October. However, since sampling for my study occurred every 3 mo, it is difficult to determine the exact dates when different stages of gametogenesis were occurring. Results from these two studies are similar and helps support the need for sampling on a more frequent basis.

Small sample size (n=4) could also be responsible for the significant differences in GDI values between captive and wild *V. vanuxemensis*. This small sample size could help explain why mature gametes were observed 7 months earlier in captive *V. vanuxemensis*. Additionally, one section per animal may not have been representative of the overall gametogenic stage in these species. Henley (2002) reported no difference in the gametogenic stage of *V. iris* or *U. imbecillis* when comparing different locations of their respective gonads. However, this is an area lacking in research and may not hold true for all species.

Interestingly, both species of short-term brooders seemingly stored mature gametes throughout the year, except for captive *A. plicata* in April 2004. When seasonal GDI values for each species were compared between 2003 and 2004, *A. plicata* in April exhibited the only significant difference reported ($P<0.0001$). Weaver et al. (1991) reported similar findings in *P. oviforme*, with mature sperm being held in the acini over the winter, and being subsequently released from late March through May. My study recorded mature gametes to be abundant in April for *P. oviforme*, with most mature gametes being released soon thereafter (Figure 2.4). There are several possible explanations for why these short-term brooders were storing mature

gametes year-round. Mature gametes could have been held in acini throughout the year for release in spring, as indicated by Weaver et al. (1991); oocytes were being held in the gonosinus for release in spring; or that trying to fit these two species into a standard reproductive mode may not be possible (William Henley, Virginia Tech, pers. comm.). Previous research suggests that breeding in short-term brooders may be more widespread throughout the year than previously believed (Matteson 1948, Garner et al. 1999). Matteson (1948) reported that males of *Elliptio complanata*, a short-term brooder, contained mature gametes throughout the year, and females of this species also stored mature gametes during fall and winter. Any of these possibilities may help explain the storage of mature gametes year-round in these two species.

Due to the storage of mature gametes year-round in both species of short-term brooders, it was difficult to overlay these data with seasonal energy reserves, especially for *A. plicata*. However, it seems that the peek in mature gamete production in April for *P. oviforme* (Figure 2.4) coincides with the drop in lipid levels reported in Chapter 1 (Figure 1.11). Again, patterns were obscure for long-term brooders, but the majority of data from Chapter 1 suggests that lipid levels peaked during January and decline through April and July (Figures 1.12 and 1.13). This pattern seems to fit the record of mature gamete production in April and July for both species (Figures 2.1 and 2.2). This indicates that lipids are fueling gametogenesis in these species, both in captivity and in the wild. Differences in lipid levels between sexes for each species did not appear to be a factor in utilizing this energy substrate for gametogenesis. The only significant difference in lipid levels between sexes was observed for *A. plicata* in July 2004 ($P<0.05$). However, since no trends in gametogenesis were seen with this species, this significant difference does not appear to have affected gametogenesis at this sample date.

Digenean trematodes are known to affect reproductive potential of mussels and possibly castrate freshwater bivalve hosts (Zale and Neves 1982, Heinonen et al. 1999). Trematode-infested bivalves can have an increased sensitivity to environmental stress, resulting in depleted energy stores (Holopainen et al. 1997, Heinonen et al. 1999). The results of my study indicated that *Villosa* species were more apt to contain these trematodes than either short-term brooder, with 40 % infestation for each *Villosa* species. Zale and Neves (1992) reported similar rates of infestation by an unidentified species of trematode in *V. vanuxemensis* (32 % infestation) and *V. iris* (23 % infestation). In addition, mature gametes were uncommon in mussels infested with trematodes in my study (Figure 2.5). Mature gametes comprised a total of 11 % for GDI values of all four species infested with trematodes. The majority of histological sections for mussels infested with trematodes was classified as inactive, and made up 47 % of total GDI values. Boyles (2004) had similar findings, with less than 10 % of trematode-infested gonads in *Actinonaias ligamentina* containing mature gametes. Therefore, infested mussels in the wild could be producing few mature gametes, and the effect of this trematode could significantly affect the reproductive success of these species in the source populations.

It is unlikely that this trematode has the ability to infest other mussels held in captivity at AWCC. Typically, digenean trematodes require a species-specific vertebrate host after an intermediate host (i.e., mussels) to complete its life cycle. Because vertebrate hosts are typically needed to ensure the completion of the trematode life cycle, it is doubtful this trematode could infest other captive mussels (William Henley, Virginia Tech, pers. comm.). In my study these trematodes also did not appear to be affecting the energy reserves of infested mussels. Boyles (2004) reported similar findings, with no significant difference in energy reserves in *A. ligamentina* infested by digenean trematodes.

Occasional functional hermaphroditism in freshwater mussels is fairly common and has been documented in 41 species (Grande et al. 2001, Henley 2002). The majority of mussel hermaphrodites are dominated typically by one sex, with limited tissue of the opposite sex (Haag and Staton 2003). This generality was evident in one of the *V. iris* hermaphrodites, where approximately 80 % of the reproductive tissue was male, and only 20 % was female. However, in the other *V. iris* hermaphrodite, gonadal tissue was approximately 50 % for each gender. In addition, sperm and eggs occurred within the same acini, which is considered to be a rare occurrence in freshwater mussels (William Henley, Virginia Tech, pers. comm.). The third hermaphrodite was a specimen of *P. oviforme*, with equal portions of male and female gonadal tissue. This species has not been recorded as hermaphroditic in previous studies.

All four species of freshwater mussels produced mature gametes at AWCC. Gametes were observed at or slightly before production of mature gametes in the wild source populations, likely because of warmer water temperatures at AWCC from May to July. In addition, *V. iris* and *V. vanuxemensis* became gravid at AWCC, suggesting that captive conditions there can fully support active gametogenesis in at least some mussels held in captivity.

CONCLUSIONS

V. vanuxemensis produced mature gametes in captivity at AWCC in April through July, and spawned by October.

V. iris produced mature gametes in captivity at AWCC in January through July, and spawned by October.

Pleurobema oviforme and *A. plicata* produced mature gametes at AWCC in April, with a large spawning event occurring between April and July.

Both species of short-term brooders, in the source populations and held in captivity, seemingly stored mature gametes throughout the year.

The following significant differences in seasonal GDI values were reported between captive and source populations: *A. plicata* in July 2003 ($P=0.0314$) and April 2004 ($P=0.0002$); *V. vanuxemensis* in April 2003 ($P<0.0001$), October 2003 ($P=0.0324$), and April 2004 ($P<0.0001$); and *V. iris* in October 2003 ($P<0.0036$).

The only significant difference in seasonal GDI values between years for the species was with *A. plicata* in April 2003 and 2004 ($P<0.0001$).

Warmer spring water temperatures at AWCC because of the lined pond may result in production of mature gametes and spawning slightly earlier than in source populations.

Digenean trematodes infested all species in this study, with infestation rates for *V. vanuxemensis*, *V. iris*, *A. plicata*, and *P. oviforme* of roughly 34, 34, 5 and 3 %, respectively.

Infestation rates in mussels were higher in males than females.

Mussels infested with digenean trematodes produced fewer mature gametes, with the inactive developmental stage dominating GDI values (47 %) for the four species combined.

Lipids appear to be the energy reserve supplying energy for mature gamete production in short-term and long-term brooders.

Hermaphroditism was rare in all species (<3.4 %), with the first report of a hermaphroditic specimen of *P. oviforme*.

Captive conditions at AWCC are seemingly conducive to the production of mature gametes, with some species (*V. iris* and *V. vanuxemensis*) becoming gravid in captivity in consecutive years.

RECOMMENDATIONS

The high costs of processing and preparing samples for histological analyses of gametogenesis made sampling more than four individuals per species very expensive. Ideally, this study should increase sample size to 10 individuals per sex per species and increase gonad sections per mussel for greater replication. Also, conducting such a study in the future may benefit from conducting sampling on a more frequent basis, such as monthly. As discussed in the recommendations for Chapter 1, experimentation with non-lethal methodologies for histological sampling would be of tremendous value in future studies, and more analysis of the relationship between energy reserves and gametogenesis should be conducted to determine whether lipids are in fact fueling gametogenesis.

Although this was not the first documentation of digenean trematodes in freshwater mussels, the effects of these parasites appear to be severe on reproductive ability. Since so few trematode-infested mussels were producing mature gametes, the negative effects of these trematodes on wild populations needs to be evaluated. Research on these trematodes should assess the full impacts and range of this trematode on all mussel species affected. Also, since the life cycle of digenean trematodes varies greatly among species, determining the life cycle and vertebrate hosts of this species could rule out the small possibility of this species infesting other captive species. This parasite has the potential to cause reproductive inhibition to already diminished populations of freshwater mussels.

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Table 2.1. Classification stages of the gamete development index (GDI) for evaluating gametogenic activity in male and female gonads of freshwater mussels. Adapted from Barber (1996) and Henley (2002).

Gametogenic Stage	Stage Classification	Description
0	Inactive	Acini are nonexistent or elongated, with walls consisting of undifferentiated germinal epithelium.
1	Early Active	Acini contain oogonia and primary oocytes, or spermatogonia, spermatocytes and spermatids, but no free oocytes or spermatozoa.
2	Late Active	Free oocytes or spermatocytes and spermatids predominate the acini; there are some spermatozoa.
3	Mature	Mature gametes (oocytes and spermatozoa) fill the acini.
4	Spawned	Acini contain spaces mostly devoid of gametes; acini walls may be broken. Hemocytes and phagocytes may be sparsely present.
5	Resorbing	Acini have a shrunken appearance and contain phagocytes and products of resorbtion. Gametes are refractory, and development is not evident.

Table 2.2. Mean (± 1 SE) glycogen, protein, and lipid content in mg/g dry weight of trematode-infested and uninfested *V. vanuxemensis* sampled from the wild source population (North Fork Holston River) from fall 2002 to summer 2004. P-values determined by Wilcoxon Two-Sample Test with *P<0.05 considered significant.

Glycogen					
Sample Date	**No Trematodes**	***n***	**Trematodes**	***n***	***p*-values**
Fall 2002	510.9 ± 60.1	4	385.9 ± 5.4	2	0.1134
Spring 2003	497.7 ± 48.0	4	481.2 ± 25.8	2	0.8261
Fall 2003	387.0 ± 36.6	4	259.5 ± 35.3	2	0.2994
Winter 2004	379.2 ± 71.3	5	411.2 ± 0	1	0.7697
Spring 2004	609.3 ± 0	5	538.3 ± 36.2	1	0.5836
Summer 2004	473.3 ± 146.1	5	385.0 ± 88.0	2	1.0000

Protein					
Sample Date	**No Trematodes**	***n***	**Trematodes**	***n***	***p*-values**
Fall 2002	429.0 ± 57.4	2	429.5 ± 25.6	4	0.8261
Spring 2003	412.4 ± 6.6	2	405.9 ± 16.7	4	1.0000
Fall 2003	369.5 ± 21.4	2	449.2 ± 17.9	4	0.1661
Winter 2004	424.0 ± 0	1	314.1 ± 33.3	5	0.5836
Spring 2004	376.8 ± 0	1	395.1 ± 17.4	5	1.0000
Summer 2004	340.3 ± 7.6	2	346.8 ± 25.1	4	1.0000

Lipid					
Sample Date	**No Trematodes**	***n***	**Trematodes**	***n***	***p*-values**
Fall 2002	48.1 ± 7.5	2	58.5 ± 3.2	4	0.7244
Spring 2003	40.2 ± 0	2	16.6 ± 5.4	4	0.1661
Fall 2003	44.4 ± 4.5	2	49.3 ± 6.6	4	0.8261
Winter 2004	66.3 ± 0	1	41.7 ± 2.0	5	0.2943
Spring 2004	16.6 ± 0	1	30.8 ± 5.0	5	0.2943
Summer 2004	16.3 ± 0	1	25.9 ± 7.6	4	1.0000

Table 2.3. Mean (± 1 SE) glycogen, protein, and lipid content in mg/g dry weight of trematode-infested and uninfested *V. vanuxemensis* sampled from the captive population (AWCC) from winter 2003 to summer 2004. P-values determined by Wilcoxon Two-Sample Test with *P<0.05 considered significant.

Glycogen					
Sample Date	**No Trematodes**	***n***	**Trematodes**	***n***	***p*-values**
Winter 2003	249.0 ± 0	1	393.8 ± 43.9	5	0.2943
Summer 2003	255.6 ± 0	1	481.0 ± 103.9	4	0.2943
Fall 2003	598.9 ± 178.5	2	396.3 ± 40.0	4	0.2994
Winter 2004	309.9 ± 78.9	2	307.5 ± 19.4	4	1.0000
Spring 2004	465.0 ± 0	1	462.5 ± 15.0	5	1.0000
Summer 2004	694.5 ± 107.4	2	605.9 ± 51.2	5	0.5184

Protein					
Sample Date	**No Trematodes**	***n***	**Trematodes**	***n***	***p*-values**
Winter 2003	371.4 ± 0	1	532.9 ± 57.8	4	0.3486
Summer 2003	425.2 ± 0	1	447.1 ± 11.1	5	1.0000
Fall 2003	357.9 ± 26.5	2	401.1 ± 23.3	4	0.5184
Winter 2004	406.3 ± 20.1	2	368.3 ± 36.0	4	0.8261
Spring 2004	402.8 ± 0	1	439.8 ± 19.7	5	0.5836
Summer 2004	323.4 ± 19.7	2	346.8 ± 25.1	4	1.0000

Lipid					
Sample Date	**No Trematodes**	***n***	**Trematodes**	***n***	***p*-values**
Fall 2002	37.2 ± 0	1	43.1 ± 2.3	4	0.3486
Summer 2003	57.9 ± 0	1	58.1 ± 7.4	5	0.5836
Fall 2003	15.7 ± 4.5	2	17.9 ± 5.7	4	1.0000
Winter 2004	85.7 ± 4.3	2	74.1 ± 13.8	4	0.7415
Spring 2004	45.6 ± 0	1	42.1 ± 4.8	5	1.0000
Summer 2004	41.7 ± 8.3	1	39.3 ± 8.1	4	0.8261

Table 2.4. Mean (± 1 SE) glycogen, protein, and lipid content in mg/g dry weight of trematode-infested and uninfested *V. iris* sampled from the wild source population (North Fork Holston River) from winter 2003 to spring 2004. P-values determined by Wilcoxon Two-Sample Test with *P<0.05 considered significant.

Glycogen					
Sample Date	**No Trematodes**	***n***	**Trematodes**	***n***	***p*-values**
Winter 2003	657.6 ± 412.2	2	392.7 ± 42.4	4	1.0000
Spring 2003	333.6 ± 123.6	3	362.3 ± 5.8	3	0.6806
Fall 2003	259.8 ± 5.5	2	576.7 ± 184.1	4	0.5184
Winter 2004	244.2 ± 36.8	2	384.2 ± 112.3	4	0.5184
Spring 2004	560.9 ± 0	1	608.3 ± 114.8	5	1.0000

Protein					
Sample Date	**No Trematodes**	***n***	**Trematodes**	***n***	***p*-values**
Winter 2003	507.6 ± 38.6	2	486.5 ± 46.5	4	1.0000
Spring 2003	396.7 ± 37.1	3	443.2 ± 13.1	3	0.4227
Fall 2003	446.7 ± 67.9	2	488.0 ± 57.4	4	0.8261
Winter 2004	461.5 ± 29.5	2	381.5 ± 40.0	4	0.2994
Spring 2004	396.4 ± 0	1	403.0 ± 18.6	5	1.0000

Lipid					
Sample Date	**No Trematodes**	***n***	**Trematodes**	***n***	***p*-values**
Winter 2003	45.8 ± 2.0	2	49.9 ± 2.6	3	0.7872
Spring 2003	45.1 ± 4.1	3	75.7 ± 2.6	2	0.2224
Fall 2003	16.1 ± 0.1	2	26.0 ± 1.3	3	0.2224
Winter 2004	41.4 ± 0	1	66.2 ± 0	1	x
Spring 2004	47.0 ± 0	1	46.2 ± 14.5	4	1.0000

x sample size too small to conduct statistics

Table 2.5. Mean (± 1 SE) glycogen, protein, and lipid content in mg/g dry weight of trematode-infested and uninfested *V. iris* sampled from the captive population (AWCC) from fall 2002 to winter 2004. P-values determined by Wilcoxon Two-Sample Test with *P<0.05 considered significant.

Glycogen					
Sample Date	**No Trematodes**	***n***	**Trematodes**	***n***	***p*-values**
Fall 2002	789.2 ± 46.1	2	383.5 ± 61.3	4	0.1661
Spring 2003	609.4	1	792.6 ± 51.9	5	0.2943
Summer 2003	393.6 ± 6.2	1	806.5 ± 209	4	0.5184
Fall 2003	263.1 ± 0	1	439.3 ± 95.9	5	0.5836
Winter 2004	465.6 ± 33.0	3	545.0 ± 106	3	0.6807

Protein					
Sample Date	**No Trematodes**	***n***	**Trematodes**	***n***	***p*-values**
Fall 2002	424.9 ± 90.0	2	512.9 ± 72.6	4	0.8261
Spring 2003	407.6 ± 0	1	455.0 ± 20.8	5	0.5836
Summer 2003	409.0 ± 11.0	2	504.2 ± 13.2	4	0.1661
Fall 2003	487.7 ± 0	1	447.7 ± 23.7	5	0.5835
Winter 2004	382.7 ± 3.8	3	335.6 ± 9.5	3	0.1413

Lipid					
Sample Date	**No Trematodes**	***n***	**Trematodes**	***n***	***p*-values**
Fall 2002	69.5 ± 0	1	51.0 ± 5.5	3	0.4370
Spring 2003	32.8 ± 0	1	29.2 ± 2.0	5	0.2943
Summer 2003	15.5 ± 0.7	2	39.4 ± 11.9	4	0.2994
Fall 2003	38.8 ± 0	1	38.0 ± 5.3	4	1.0000
Winter 2004	61.9 ± 2.1	3	45.1 ± 0.7	3	0.1413

Table 2.6. Results of ANOVA using Tukey's multiple comparison technique between GDI values in captive and source population mussels from September 2002 to July 2004. *P-values <0.05 represent significant differences in gametogenic stages.

Sample Date	***V. iris***	***V. vanuxemensis***	***P. oviforme***	***A. plicata***
September 2002	x	x	x	x
January 2003	1.0000	1.0000	1.0000	1.0000
April 2003	1.0000	<0.0001*	1.0000	0.3361
July 2003	0.9974	1.0000	0.9997	0.0314*
October 2003	0.0036*	0.0324*	1.0000	0.9315
January 2004	1.0000	1.0000	1.0000	1.0000
April 2004	1.0000	0.0001*	1.0000	0.0002*
July 2004	1.0000	1.0000	1.0000	1.0000

x no seasonal comparisons were made in September 2002

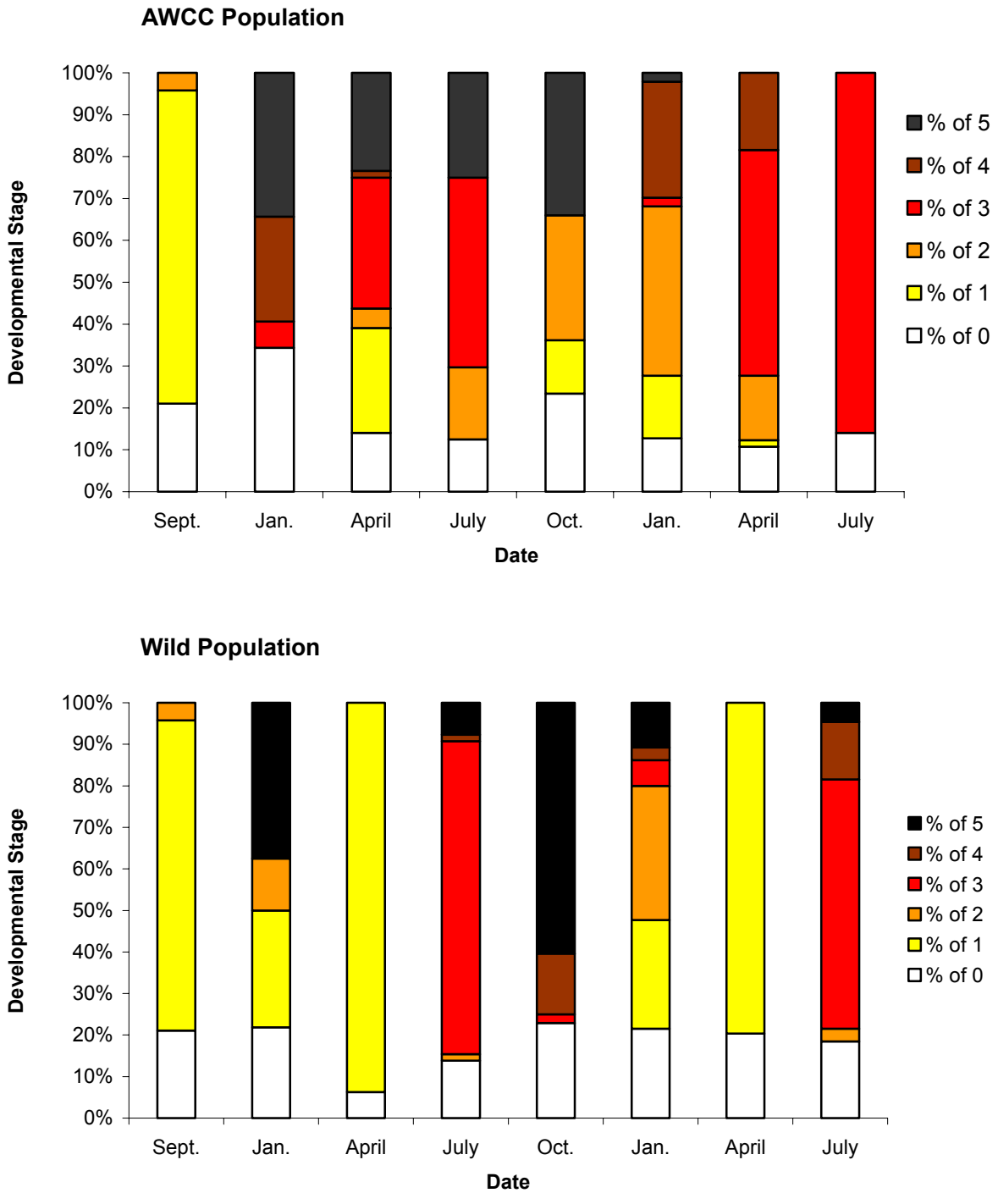

Figure 2.1. Gamete development index (GDI) of gonads of *Villosa vanuxemensis* (*n*=4/sample date) held in captivity at AWCC and from wild source population (North Fork Holston River) during September 2002 to July 2004, showing percent of observations identified in each developmental stage (0: inactive, 1: early active, 2: late active, 3: mature, 4: spawned, and 5: resorbing).

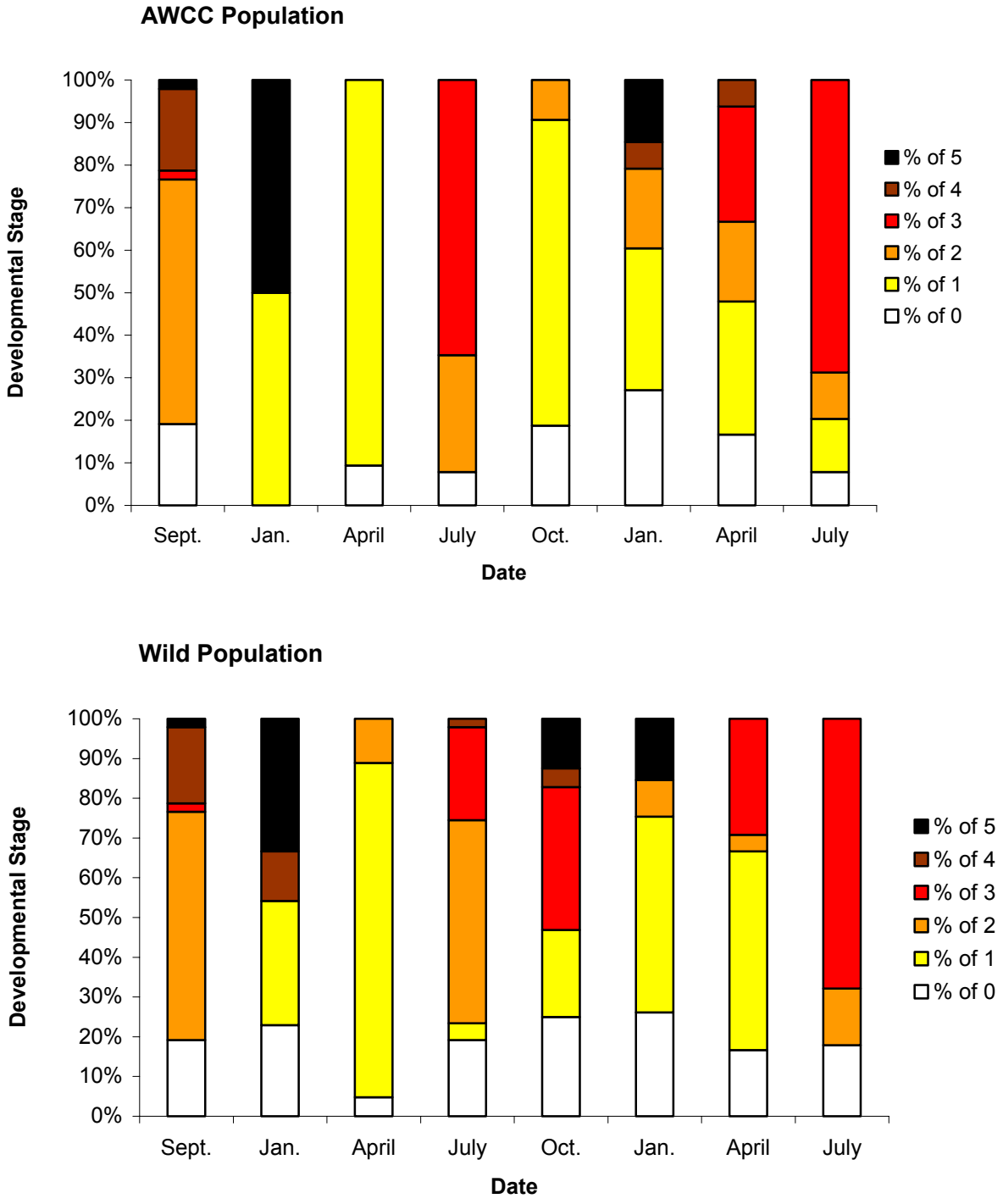

Figure 2.2. Gamete development index (GDI) of gonads of *Villosa iris* (*n*=4/sample date) held in captivity at AWCC and from wild source population (North Fork Holston River) during September 2002 to July 2004, showing percent of observations identified in each developmental stage (0: inactive, 1: early active, 2: late active, 3: mature, 4: spawned, and 5: resorbing).

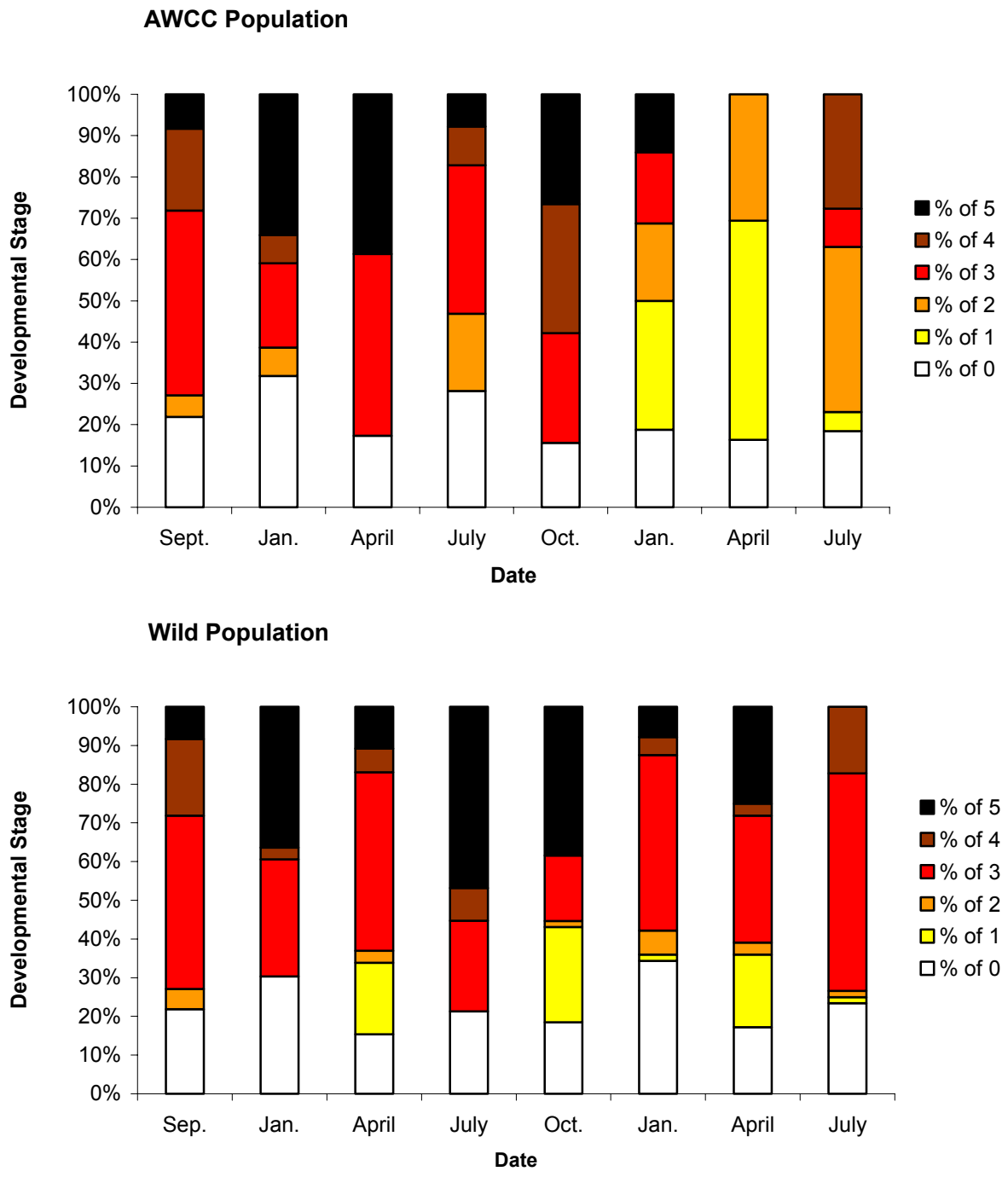

Figure 2.3. Gamete development index (GDI) of gonads of *Amblema plicata* (*n*=4/sample date) held in captivity at AWCC and from wild source population (Clinch River) during September 2002 to July 2004, showing percent of observations identified in each developmental stage (0: inactive, 1: early active, 2: late active, 3: mature, 4: spawned, and 5: resorbing).

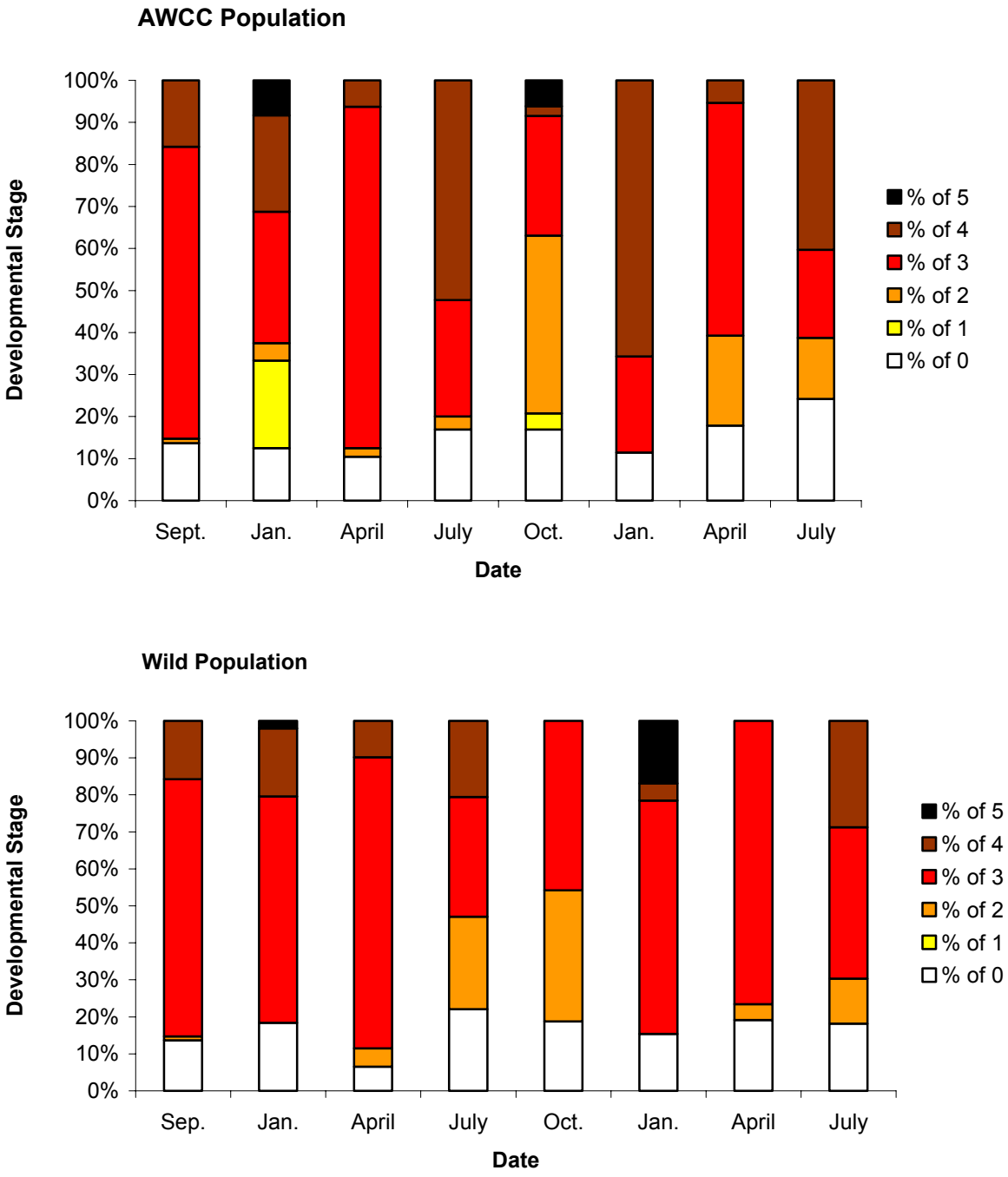

Figure 2.4. Gamete development index (GDI) of gonads of *Pleurobema oviforme* (*n*=4/sample date) held in captivity at AWCC and from wild source population (North Fork Holston River) during September 2002 to July 2004, showing percent of observations identified in each developmental stage (0: inactive, 1: early active, 2: late active, 3: mature, 4: spawned, and 5: resorbing).

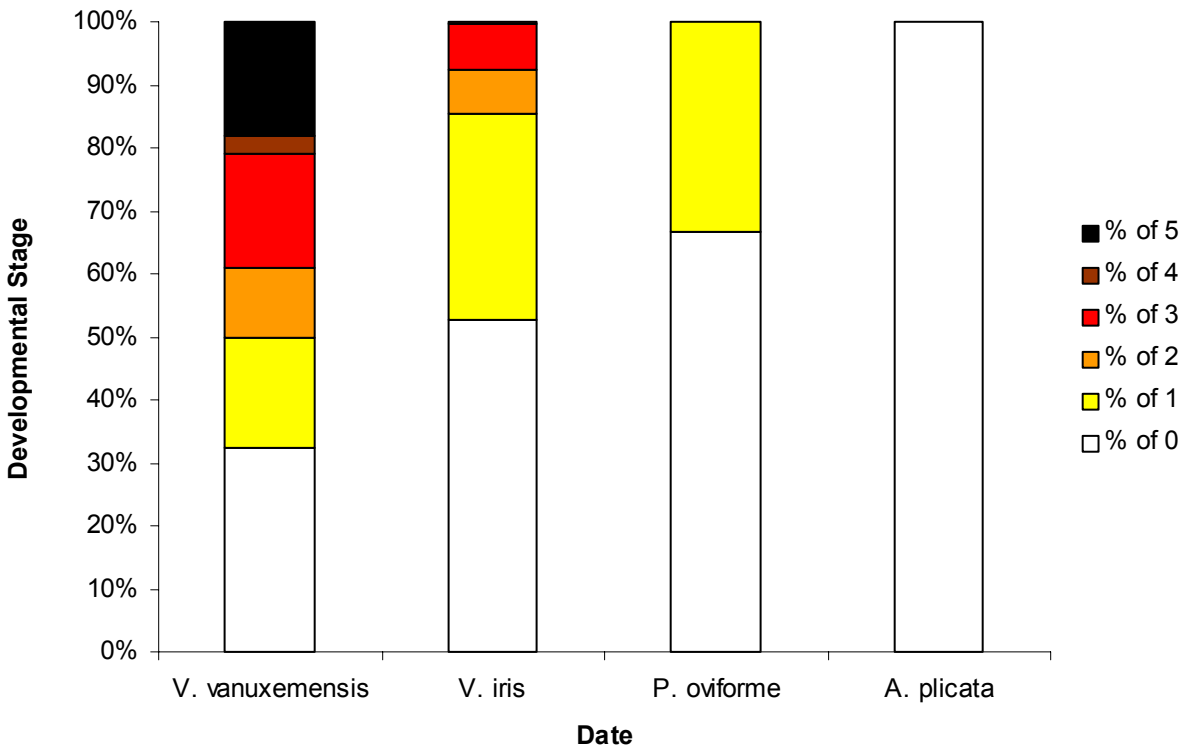

Figure 2.5. Gamete development index (GDI) of gonads infested with digenean trematodes for *V. vanuxemensis* (n=22), *V. iris* (n=17), *P. oviforme* (n=3), and *A. plicata* (n=2) from wild source populations and those held in captivity at AWCC from September 2002 to July 2004, with percent of observations identified in each developmental stage (0: inactive, 1: early active, 2: late active, 3: mature, 4: spawned, and 5: resorbing).

Chapter 3: Survival and Growth of Juvenile Freshwater Mussels Reared in Flow-Through Troughs and Round Tanks

INTRODUCTION

Passage of the Endangered Species Act in 1973 and subsequent listing of species brought public attention and interest to the decline of freshwater mussel populations. Since then, propagation efforts have been developed to restore populations of threatened and endangered mussels. Historically, no propagation success was documented with American species of freshwater mussels until Lefevre and Curtis (1912) found a young mussel in a tank following an infestation. Shortly thereafter, Dr. R.E. Coker and his successor, A. F. Shira, experimented with different techniques of artificial propagation such as floating crates, tanks, cement-lined ponds and earth ponds, only to find minimal survival (Howard 1922).

From the 1920's until the recognition of mussel species as endangered in 1976, very little attention was given to mussels and propagation efforts (Jenkinson and Todd 1997). Many recent studies have resulted in high survival rates compared to early studies of nearly total mortality. Hudson and Isom (1984) were the first to report success in raising juveniles in a laboratory setting. Since that time, many other experiments have had excellent success in propagating juveniles under controlled conditions (Beaty 1997, Hanlon 2000, Mummert 2001).

One such facility for propagating freshwater mussels is the Aquatic Wildlife Conservation Center (AWCC), established in 1998 by the Virginia Department of Game and Inland Fisheries (VDGIF) in Marion, Virginia. Raceways renovated with flow-through culture systems have allowed this facility to culture juveniles and release them to natal rivers. Juvenile production and rearing at the AWCC have had promising results. Hanlon (2000) had excellent success, with one experiment yielding 50 % survival of wavy-rayed lampmussels (*Lampsilis fasciola*) after 72 days, and another

experiment yielding 82.2 % survival after 90 days. Zimmerman (2003) had only mediocre results using *L. fasciola* and oystermussels (*Epioblasma capsaeformis*) due to escapement, fathead minnow (*Pimephales promelas*) predation, and mortality from turbellarian worms (*Macrostomum* sp*)*. Because of variability in culture results and problems encountered in grow-out systems, there is need for further investigation of AWCC's ability to raise juvenile mussels.

As sedentary organisms, freshwater mussels depend on water flow to transport oxygen and food particles. Very few studies have been conducted on growth and survival of juvenile mussels using flow as a variable (Kirby-Smith 1972). Water velocity has been shown to have huge influences on the growth rates of marine bivalves, but flow rate, the volume of water flowing through a given area, has yet to be tested. Cahalan et al. (1989) reported that the feeding processes of some bivalves may in fact be facilitated by local flow regimes. Water flowing too slowly inhibits growth for lack of water exchange, and water flowing too fast prevents mussels from being able to remove particles from the water column (Kirby-Smith 1972, Cahalan et al. 1989). It was also reported that growth rates of bay scallops (*Argopecten irradians*) were influenced by the combination of food concentration and flow effects, and were not attributed to one factor alone (Cahalan et al. 1989). Cahalan et al. (1989) noted that most studies dealing with food concentrations and bivalves have been done in static water systems where flow regimes have not been considered.

When designing a grow-out system for juvenile, substrate size also plays an important role in survival and growth. Previous research has typically focused on the effects of fine sediment on juvenile mussels, and relatively little research has been conducted on various substrate sizes (Rogers 1999, Hanlon 2000, Zimmerman 2003). Substrate size in a culture system can affect juvenile mussels in a variety of ways, including ability to feed and escape predation and high flow. Research results conflict on which substrate size is ideal for juvenile culture systems. Rogers (1999) reported that

juvenile rainbow mussels (*Villosa iris*) reared in fine substrate (<120 µm) did the poorest, with 7 % survival and only 0.86 mm in length after 16 wk, while juveniles in a mixed sediment (>1400 µm) had 26 % survival and grew 1.06 mm in length. Juveniles raised in fine sand (500-800 µm) also had significantly higher survival than those in fine sediment (<120 µm). However, Beaty and Neves (2004) reported that neither growth nor survival was affected by rearing juveniles of *V. iris* in two sizes of fine substrate (<120 µm and 120-600 µm). With these seemingly conflicting results, there is need for more research on the effects of substrate sizes on juvenile mussel survival and growth.

The goal of freshwater mussel propagation is to achieve maximum growth and survival over an extended period of time. In order to achieve high survival and growth rates, captive rearing conditions like those at AWCC need to be well suited for juveniles and easy to maintain. To obtain these suitable conditions, aspects of a culture system need to be altered and tested repeatedly until an optimal combination of substrate size, flow, and type of grow-out container is determined. Other potentially important aspects of culturing juveniles are sampling frequency and diet requirements. The effects of sampling frequency at AWCC was studied by Hanlon (2000), but due to extensive mussel predation, results were inconclusive. O'Beirn et al. (1998) reported that growth decreased as sampling frequency increased with *L. fasciola*, whereas Zimmerman (2003) reported that unsampled *E. capsaeformis* and *L. fasciola* had better survival than those that were sampled every 2 wk. Beaty (1997) observed lower survival in juveniles of *V. iris* that were sampled, compared to juveniles left unsampled.

Few diet studies with juvenile mussels have been published, and diet requirements for juveniles are only partially known (Yeager et al. 1994, Gatenby and Neves 2002, Beck and Neves 2003). It has been shown that juveniles can feed on bacteria and detritus (Yeager et al. 1994, Gatenby et al. 1996), but that algae is their primary food source (Gatenby et al. 1996, et al. 1997, Beck and Neves 2003). Some studies suggest that juvenile mussels selectively feed and do so primarily on the

basis of particle size (Beck and Neves 2003). Parker et al. (1998) reported that adult freshwater mussels may not selectively feed, but rather consume what is in greatest abundance. At AWCC, the diets of juveniles need to be determined since the water pumped into grow-out containers is passed through a series of mesh screens (50 and 100 μm) before entering the grow-out systems. The finer the mesh used, the less incoming sediment accumulates in the grow-out containers, and the easier to sample juveniles. The size of algae that juveniles consume at AWCC is currently unknown. Therefore, very small mesh sizes have not been installed for fear that they will screen out certain nutritionally important algal species.

Two separate experiments were undertaken during summers 2003 and 2004 at AWCC. The first experiment was completed in summer 2003 and compared the effects of three flow rates on survival and growth of *V. iris* and *E. capsaeformis* in flow-through troughs. The second experiment was completed in summer 2004 and compared the effects of three substrate sizes on survival and growth of *V. iris* in round flow-through tanks. To assess the effects of sampling frequency on survival and growth, and to potentially find a way to minimize sampling time, another variable was incorporated in Experiment 2. Survival and growth of sampled to unsampled juveniles of *V. iris* were compared after a 10 wk period.

Since the growth and survival of freshwater bivalves has been determined to be directly related to amount of food available (Foe and Knight 1986), the amount and genera of algae present in the water at AWCC was analyzed seasonally, with focus on summer levels in particular. The gut contents of juveniles were examined at the end of each experiment to determine which algal genera were being consumed. Also, since detritus is important to the growth and health of juveniles (Coker et al. 1921, Yeager et al. 1994), incoming sediment samples were taken from the grow-out systems in Experiment

1 and analyzed for percent organic and inorganic matter to determine the composition of incoming sediment at AWCC.

A group of juveniles of *V. iris* from the same infestation used for Experiment 2 also was reared at the Freshwater Mollusk Conservation Center (FMCC) located at Virginia Tech in Blacksburg, Virginia. This center also serves as a mussel propagation facility for southwest Virginia. Space was not available to complete replicates for the experiment at the FMCC, but the information gained from this was helpful in comparing the survival and growth rates at two different propagation facilities.

Species Selection

The rainbow mussel, *Villosa iris*, and the endangered oystermussel, *Epioblasma capsaeformis*, were chosen for this experiment for several reasons. First, *E. capsaeformis* have habitat requirements similar to other endangered species, making them good substitutes for habitat evaluations (Gatenby et al. 1997, Hanlon 2000). Second, both species are known to exist either historically or currently in the Holston River watershed. Third, gravid females of both species can be found throughout most of the year. Finally, both species have been successfully cultured at the FMCC and AWCC.

METHODS AND MATERIALS

Experiment 1: Growth and Survival of Juveniles Reared at Three Flow Rates

This experiment was carried out using PVC flow-through troughs at AWCC. This type of grow-out system had been tested with varying degrees of success (Hanlon and Levine 2001, Zimmerman 2003). However, modifications to the general design of these troughs were made to improve upon previous results. Compared to previous designs these troughs were larger, contained more substrate, and utilized a catchment container to monitor escapement.

Water for this experiment was pumped into an indoor, covered raceway at AWCC from the polyethylene-lined holding pond. All water for this experiment was screened through 125 µm mesh before being held in a 378 L Rubbermaid reservoir. The 125 µm mesh screen was exchanged for a 200 µm mesh screen at 4 wk, after heavy rains continually clogged the 125 µm screen with incoming sediment. Water exited the reservoir through a 3.2 cm bulkhead that was approximately 10 cm from the bottom of the reservoir. The water was fed through a 7.6 cm PVC pipe with five T joints every 2 m (Figure 1). Approximately 2 m of 7.6 cm PVC pipe was then extended from each T joint at a 90 º angle. Three 5.0 x 5.0 x 1.9 cm T joints were used to carry the water to a 1.9 cm ball valve that regulated water flow into each trough.

Each trough was considered an individual replicate, with five replicates for the low (1 L/min), medium (3 L/min) and high (7 L/min) rates of flow (Figure 3.1). The troughs were constructed of a 133 cm piece of PVC pipe, 20.3 cm in diameter, cut in half longitudinally with caps on both ends (Figure 3.2). Water exited the system via a standpipe located 8 cm from the end of the trough. Standpipes were constructed by drilling a hole through the bottom of the trough to accommodate either a 1.3 cm male adapter for the low and medium rates of flow, or a 1.9 cm male adapter for the high rate of flow. The male adapters were then fitted with a sufficient length of either 1.3 or 1.9 cm pipe to bring the height of the standpipe to 6.4 cm. The male adapters were fastened with a female adapter on the underside of the trough and was made water tight by applying silicone caulk to exposed seams.

In previous studies, these flow-through troughs had no means to record escapement out the standpipe. Juvenile mussels have been shown to be very mobile, with the potential to move great distances when pedal-feeding (Reid et al. 1992). With no means to account for escapement, this makes it difficult to attribute mortality to escapement or to other factors related to the design of the grow-out system. To account for any emigration, each trough was fitted with a separate catchment container

placed directly below the outflow pipe. Each container was made by suspending a 15.2 cm diameter, 8.9 cm high piece of PVC pipe inside a 9 L plastic bucket (Figure 3.3). The bottom of the PVC pipe was fitted with a 125 µm mesh screen to catch escaping juveniles. After the juveniles had grown to a sufficient size, all mesh screens were replaced with 300 µm mesh to allow unwanted debris to pass through.

Given the limited amount of water at AWCC for mussel culture, this experiment based its three rates of flow on the amount of water available. These rates of flow were considered appropriate since most juvenile systems at AWCC utilize 1 L/min of water, and anything greater than 7 L/min was impractical in a water-limited facility. Water exiting the standpipe was collected for 1 min in a graduated plastic container and measured for volume to determine the rate of flow. The desired rate of flow was maintained by simply adjusting the ball valve at the head of each trough. Ball valves were cleaned of incoming sediment, and rates of flow were checked every three to four days to ensure flow accuracy.

Course limestone sand, 1000-2500 µm in size, was used since it was the substrate of choice in similar experiments (Hanlon 2000, Zimmerman 2003). Color and size differences made this substrate suitable for locating and extracting juveniles in the troughs. About 1.5 L of course limestone sand was placed in each trough, after it had been sieved twice to remove all particles <1000 µm. Once evenly distributed in the trough, the depth of the substrate was approximately 2 cm.

Production of Juveniles

Juveniles of *V. iris* and *E. capsaeformis* were produced at the FMCC. Six gravid females of *V. iris* were collected from the Little River at the Route 610 bridge in Tazewell County, Virginia. Approximately 16 rock bass (*Ambloplites rupestris)*, ranging in size from 7.6 to 20.3 cm, were collected by boat electrofishing from Claytor Lake, Virginia, and used as host fish. Three gravid *E.*

capsaeformis were collected from the Clinch River in Scott, County, Sneedville, TN. Approximately 60 to 70 banded sculpin (*Cottus carolinae*) were used as host fish for this species and were collected by backpack electroshocker from the South Fork Holston River near AWCC.

Glochidia for each mussel species were removed from the gravid females by using a 3.8 cm, 18-gauge sterile hypodermic needle. Water was then injected from a 10-cc syringe into the marsupium, flushing out the glochidia into a petri dish. A few glochidia of each species were exposed to a couple of grains of salt (NaCl), to ensure a snapping response, indicating that they were mature and usable. The host fish and glochidia then were placed in a cooler with a sufficient amount of water to cover the fish. The water in the cooler was then agitated with air stones to keep the glochidia suspended. The gills of the fish were checked for glochidial attachment every few minutes until the fish had become sufficiently infested, after 15 min. Infested fish were separated by species and placed in 38 L aquarium tanks. Once the juvenile mussels were shed from the host fish, the bottoms of the tanks were siphoned every two days into a 120 μm mesh sieve. These juveniles were then placed in 500 mL Tupperware containers where they were fed five drops of a fine sediment (< 50 μm) slurry and 100 mL of *Neocloris oleoabundans* at approximately 60,000 cells per mL once every 2 days. The juveniles were held in these containers until the desired length of approximately 300 μm was achieved.

Previously at AWCC, flatworms of the genus *Macrostomum* have been observed feeding on juvenile mussels (Hanlon 2000, Zimmerman 2003). *Macrostomum* sp. are known to be extremely voracious predators, and the presence of these organisms in a juvenile mussel experiment could result in depredation of many individuals (Sickel 1998, Zimmerman and Neves 2003). Keeping these microscopic organisms out of a juvenile culture experiment, where unfiltered river water is used, is difficult. Therefore, to minimize predation by flatworms, *V. iris* and *E. capsaeformis* were grown out

at the FMCC for 15 days to reach a minimum mean length of 350 µm. Juvenile mussels at least 300 µm in length are seemingly too large for *Macrostomum* sp. to ingest (Jess Jones, FMCC, pers. comm.).

Release of Juveniles

Juveniles were transported to AWCC on June 26, 2003, in 2 L Tupperware containers with dechlorinated well water. Hanlon (2000) reported that June was the best month for release of juvenile *L. fasciola* and *E. capsaeformis* at AWCC. The end of June was selected for this experiment since water temperatures and food availability would be suitable in the South Fork Holston River. Each container of juveniles was acclimated to the water temperature and chemistry of AWCC water by slowly adding small amounts of hatchery water to the containers over the course of 1 hr. A total of 3000 *E. capsaeformis* (15 days old) and 1500 *V. iris* (15 days old) were used for this experiment. Each trough received 200 randomly selected *E. capsaeformis* and either 100 or 200 randomly selected *V. iris*. Troughs 1, 9, and 14 received 200 juveniles of *V. iris*, while the remainder received 100 juveniles of *V. iris* (Figure 3.1). Juveniles for this experiment were spread evenly throughout the length of the trough to avoid density dependent growth (Steg 1997, Hanlon 2000). Because juveniles of these two species are morphologically distinguishable, they were mixed together in each trough.

Trough Sampling

Sampling for this experiment was conducted every 2 wk, following the initial release of the juveniles into the troughs on June 26, 2003. On each sampling event, all 15 troughs were sampled. Juveniles were separated from the substrate by emptying the contents of the trough into a series of 1000, 800, and 200 µm vertically-stacked sieves. The 1000 µm sieve retained the majority of limestone sand, while the 200 µm sieve retained the juveniles. The contents of all three sieves were thoroughly rinsed with hatchery water to flush the juveniles into the 200 µm sieve.

The limestone sand used for this experiment began to break up into fine particles after only 2 wk in the troughs. Contents of the 200 µm sieve therefore yielded not only the juveniles, but also a large amount of fine limestone sand. To separate the juveniles, a technique developed at AWCC was used. This separation technique relies on the same principle as an elutriator, but is far less labor intensive and cuts down on the time juveniles are tumbled, possibly minimizing stress and loss of juveniles. This technique involved placing the contents of the 200 µm sieve into a 4 L, 10.2 cm high circular Rubbermaid container. Approximately 1 L of water was placed in the container and swirled in a counter-clockwise motion. The agitated water in the container caused the less dense contents, including the juveniles, to become suspended. The water was then quickly poured into a series of 400, 300, and 200 µm sieves to further separate the juveniles from any unwanted floating debris. Testing of this method consistently revealed that repeating this method six times separated all juveniles from the limestone sand. This process was repeated eight times for each trough to ensure that all juveniles were retrieved. The juveniles were then placed in a petri dish and observed with a stereo-zoom microscope to quantify survival and growth. Shell length and width were recorded using an ocular micrometer on a subsample of ten individuals per species from each trough.

All substrate, including that caught in the 800 and 200 µm mesh sieves, was returned to respective troughs to ensure that any missed juveniles were not discarded. Once counted and measured, the juveniles were allowed to settle back into the substrate for approximately 30 min before water flow was resumed.

Escapement Sampling

Catchment containers were sampled twice weekly to prevent clogging and subsequent overflow. Each suspended PVC container was removed from the larger containers, rinsed thoroughly, and its contents placed in a petri dish. A stereo-zoom microscope was used to count and measure

juveniles. Again, all contents of the petri dishes were returned to their respective troughs to ensure that any missed juveniles would not be discarded. All contents of the petri dishes were allowed to settle back into the trough without water flow for 30 min.

Experiment 2: To Determine a Suitable Substrate Size and Sample Frequency for Juvenile Mussels in 4 L Round Flow-Through Tanks

The flow-through troughs used in summer 2003 were extremely difficult to sample because of their large size and the large amounts of substrate contained in each trough. Additionally, each trough required a great deal of maintenance to ensure water flows were adequate and nothing was overflowing. Because of this, Experiment 2 was conducted in 4 L round Rubbermaid flow-through tanks. This grow-out system was much smaller, easier to sample, and needed minimal maintenance. Also, this system has been used with excellent success at AWCC (Mike Pinder, VDGIF, pers. comm.), and will likely be used in the future at AWCC for propagation purposes.

The three sizes of substrate used for this experiment were fine sediment (which was allowed to naturally accumulate in the dishes), and 200 mL of either 500-850 µm limestone sand or 1000-2500 µm limestone sand. Water for this experiment was screened through a 50 and 100 µm mesh filter before entering a 946 L reservoir, which gravity-fed the water into the grow-out units. Water entered these tanks via a horizontal piece of 5.1 cm diameter PVC pipe. Six 3.8 cm T-joints spaced approximately every 75 cm along the horizontal PVC main water line were used to distribute the water. Water was then fed downward in a piece of 1.3 cm PVC pipe to supply three rows of tanks set on wooden stairs (Figure 3.4). Water entering the tanks was regulated through a 1.3 cm PVC ball valve that could be adjusted to maintain flow. Finally, a 1.3 cm female adapter fed into a 4 mm reducer, with a rubber tube transporting the water from the reducer into a small hole cut into the side of

the tank to produce a circular flow. A 2.5 cm high standpipe was placed into a male adapter and served as exit for the water (Figure 3.1). This resulted in each 4 L tank holding 2.7 L of water. A 150 µm mesh basket was placed beneath the standpipe and filtered all out-going water to ensure that escaped juveniles were caught and returned to the tank.

Juveniles at the FMCC were placed into one 38 L tank supplied with a mixture of recirculating well and dechlorinated city water This tank received 266 mL of fine sediment (<150 µm) every 2 wk, and 300 mL of cultured *Neocloris oleoabundans* at approximately 60,000 cells per mL were provided every day. Water temperature in this system was maintained at 21°C for the duration of the experiment.

Production of Juveniles

Six gravid *V. iris* were collected from the North Fork Holston River in Chatham Hill, Smyth County, Virginia in early June of 2004. Approximately 16 *A. rupestris* were collected by backpack electroshocker from Tom's Creek in Montgomery County, VA, and used as host fish. Glochidia from the six *V. iris* collected from the North Fork Holston and glochidia from two *V. iris* collected from Indian Creek, also collected in early June, were mixed together to ensure an adequate infestation. Mixing of glochidia from two different drainages was decided since these juveniles were going to be used only for research purposes and not released back into the wild. This infestation occurred at the FMCC and followed the same methods described for Experiment 1.

Release of Juveniles

A total of six *A. rupestris* survived infestation and produced 24,043 juveniles of *V. iris*. Newly transformed juveniles were then transported to AWCC on June 29 in Rubbermaid containers. Juveniles were acclimated to water conditions at AWCC for an hour by slowly adding hatchery water to the containers before being released. A total of 10,500 juveniles was divided into 700 juveniles per

round tank, with five tanks per treatment to be sampled every 2 wk (Figure 3.4). A total of 4,800 juveniles also were divided into 400 juveniles per tank, for a total of four tanks per treatment that would remain unsampled until the end of the experiment (Figure 3.4). A total of 8,743 juveniles remained and were placed into the grow-out system at the FMCC.

Tank Sampling

Sampling of the tanks at AWCC occurred every 2 wk after the initial release date of June 29. All fifteen tanks were sampled on each sample date. Before any juveniles from the tanks were removed, the contents of the 150 µm mesh basket were examined, and juveniles were enumerated and measured. Each tank was sampled using the same method as Experiment 1, with water being agitated in a counter-clockwise motion and emptied into a series of 1000, 400, and 200 µm sieves for a total of eight times. Total survival in each tank was recorded, and a subsample of ten individuals per tank had their shell lengths measured. The 12 unsampled tanks were sampled for the first and only time on September 8. During each sample event, the mesh bags of the unsampled dishes were emptied back into their respective tanks without being enumerated or measured (except at week 10). This was done to minimize handling and potential stress in this experiment.

During each sample event, the time needed to extract, count, and measure the juveniles from each of the three treatments also was recorded. This was to determine whether certain substrate sizes were easier to process when sampling juveniles. This step was taken to ensure that if survival and growth was not significantly different in the three treatments, than processing time could be used as a factor in deciding which substrate to use.

Villosa iris kept at the FMCC also were sampled every 2 wk, on the day immediately following sampling at AWCC. All juveniles were siphoned from the tank into a 200 µm sieve and examined for total survival. A subsample of 20 juveniles also had shell lengths measured to record growth. After

sampling, all juveniles were returned to the tank with new sediment and allowed to settle for approximately 6 hr before having the water flow resumed.

Water Chemistry

During scheduled sampling dates, a water sample was collected from the ponds that fed both experiments at AWCC and the single experiment at the FWCC. Approximately 2 L of this water was collected in Nalgene bottles and transported on ice to the FMCC for analysis. A Hach™ DR/2000 spectrophotometer was used to measure alkalinity, hardness, nitrate, nitrite, and ammonia in all water samples. Temperature, pH, and dissolved oxygen were measured throughout both experiments at AWCC with a Hydrolab unit. Experiment 1 also had dissolved oxygen monitored continuously in one randomly selected trough from each rate of flow with a YSI Model 50B DO meter. Chlorophyll A levels were not measured in this experiment since they were barely detectable in previous analyses of hatchery water (Zimmerman 2003).

Incoming Sediment Determination

During each sampling event at AWCC, incoming sediment (< 200 μm in Experiment 1 and < 50 μm in Experiment 2) that had accumulated in the grow-out systems was washed out of the substrate and collected in a 19 L bucket. This sediment-laden water was then diluted to the 18 L mark with sediment-free water. The contents of the bucket were then stirred, and a 1 L sample of the slurry was collected to estimate levels of incoming sediment accumulation. Total organic and inorganic matter calculations were taken from this incoming sediment during each sample event for Experiment 1 using dry weight and muffle-furnace ashing. Dry weight was calculated by first preheating five Whatman GF/C fiberglass filters (47 mm) in a muffle furnace at 480°C to remove any organic matter. A suction pump was used to filter 200 mL of the slurry through the pre-weighed filters. This volume was used because it was approximately the largest amount of slurry that could pass through an individual filter

without becoming clogged. Filters and samples were dried in an oven at 60°C for 24 hr or until a stable weight was reached. A muffle-furnace set at 480°C burned the samples for 24 hr, to measure ash content (inorganic residue). The samples were placed in a desiccator until they had cooled to 20°C and were weighed. The weight of ash was subtracted from the dry weight of the slurry to give weight of organic matter. Once the organic and inorganic matter in the 200 mL subsample was determined, this value was used to calculate the total organic and inorganic matter in each trough. All incoming sediment loads were expressed in mg/100 cm^2 so comparisons between experiments could be made. Since the ratio of organic to inorganic matter remained relatively unchanged throughout Experiment 1, only total incoming sediment accumulation was determined in Experiment 2.

Gut Content Analysis

The gut contents of juveniles at the end of Experiment 1 and 2 were analyzed to determine which algal genera were being consumed by juveniles. On the last sampling date of Experiment 1, two *E. capsaeformis* juveniles were selected from each trough, for a total of 30 juveniles. At the end of Experiment 2, 12 *V. iris* were randomly selected from each treatment, for a total of 36 juveniles. All juveniles were rinsed with distilled water before being placed in appropriately labeled 100 mL glass bottles. Each bottle had 50 mL of distilled water and 2 mL of acid Lugol's solution to preserve algae in the guts.

Gut analysis was carried out by pouring each treatment into a Petri dish and locating the juveniles with a dissecting microscope at 20 X magnification. A Pasteur pipette was then used to select approximately half the juveniles and to place them on a microscope slide. The juveniles were then crushed with the rear end of a pair of tweezers to expose the guts and ingested algae. Each slide then underwent seven different linear transects to standardize analyses. Algal genera and size ranges of

algae were noted for each of the three treatments using an Olympus light microscope at 100 X magnification. An algal key was used to aid in the identification of genera (Prescott 1978).

Algae Density and Composition

No previous analysis has been completed to determine the genera and concentrations of algae in the water at AWCC, and the amount of organic/inorganic matter in the incoming sediment. To better understand what types of food are available for captive mussels, seasonal 1 L water samples were taken in January, April, July, and August of 2003 and 2004. These water samples were stored in Nalgene bottles and preserved with 10 mL of acid Lugol's solution (Vollenweider 1969). These samples were then stored in a cool, dark file cabinet until analyses were completed (Vollenweider 1969). During analysis, a 100 mL Utermohl's settling chamber was used for 48 hr to concentrate the algae. An inverted Olympus light microscope at 300 X magnification was then used along with a dichotomous key (Prescott 1978) to identify algal genera in each sample. A square grid in the eyepiece was placed on a random point, and the genera within the grid were identified and enumerated. This process was continued until a total of approximately 300 individual algal cells were counted. The conversion formula was calibrated to the microscope magnification by having 130 correct for the number of algal cells in one transect of the counting chamber grid system, and having 98.174 correct for the number of algal cells in 100 ml. The following formula was then applied to determine the number of algae cells in one mL of the sample:

[(130/number of grids) x number of algal cells x 98.174] /100

Juvenile mussels may do poorly in culture systems because of inadequacies in food type and abundance (i.e., algae types and concentrations). To assess such a possibility, seasonal water collection and algae identification/enumeration also occurred at the Clinch River (Clinchport, VA) and North Fork Holston River (Chatham Hill, VA). Water samples were taken in October of 2003 and

January, April, and July of 2004. The same methods of analysis were used as on AWCC water samples. This allowed for seasonal comparisons of algae between AWCC and these rivers with resident mussel populations.

Data Analysis

Data analysis for both experiments consisted of comparing rates of survival of juveniles among each of the three flow rates or substrate types at a selected sampling event for all species. Likewise, length comparisons were made among mussels in each of the three flow rates or substrate types at a selected sampling event for all species. Two-week comparisons were of interest, to determine whether any trends were evident throughout the course of this experiment. However, the last sample event for each experiment held the greatest implication since overall survival and growth was the main objective.

All survival comparisons were tested using a Poisson distribution test, and all length comparisons were made using a Tukey's student range test. The Poisson distribution test was used since it compares independent occurrences of an event that happen randomly over time and assumes that the occurrence of one event does not change the probability of a future event. Tukey's student range test was used because it compares more than two sample means, testing the largest and smallest sample means. P-values less than 0.05 were considered to be significant for both tests, and all statistical analyses were conducted using SAS software (SAS Institute 2002).

RESULTS

Experiment 1: Growth and Survival of Juvenile Mussels Reared at Three Flow Rates

Survival

Some obvious trends in survival of *E. capsaeformis* were evident at the end of the experiment (Figure 3.5). The 1 L/min flow yielded better survival than the other two rates of flow at every sampling event. Similarly, the 3 L/min flow had better survival than the 7 L/min flow during every sample event, except at 4 wk. At the end of the experiment, the 1 L/min flow resulted in 18 % survival; the 3 L/min flow, 16 % survival; and the 7 L/min flow, 11 % survival. Statistical comparisons were made only at 29 days and at the end of the experiment (56 days). Using the Poisson distribution test, the only significant differences in survival rate at 29 days were between the 1 L/min flow and the 7 L/min flow ($P=0.0008$), and the 1 L/min flow versus 3 L/min flow ($P<0.0001$). Similar results were observed at 59 days, with significant differences between the 1 L/min flow and the 7 L/min flow ($P<0.0001$), and between the 1 L/min flow and the 3 L/min flow ($P<0.0001$). No significant differences occurred between the 3 L/min flow and the 7 L/min flow.

The same overall survival trends were observed with *V. iris* during the first 2 wk of the experiment. However, between 2 and 4 wk, *V. iris* suffered high mortality, and after 6 wk, no live *V. iris* remained (Figure 3.6). The last sampling event that yielded measurable data was at 2 wk, with survival rates of 47, 28, and 8 % for the 1 L/min, 3 L/min, and 7 L/min rates, respectively. With such poor results, only one statistical comparison was made at the 2 wk sample event. The limited results for this species were similar to those of *E. capsaeformis*; significant differences were observed between the 1 L/min flow and the 7 L/min flow ($P<0.0001$), and between the 1 L/min flow and the 3 L/min flow ($P=0.0003$).

Growth

Differences in lengths of *E. capsaeformis* in these three treatments were less apparent (Figure 3.7) than those reported for survival. After 2 wk, juveniles raised in the 7 L/min flow achieved the greatest length throughout the remainder of the experiment. At the end of the experiment, mean lengths of *E. capsaeformis* in the 1 L/min, 3 L/min, and 7 L/min rates were 594, 609, and 656 µm, respectively. Again, statistical analyses were only conducted at 28 and 56 days. Using Tukey's student range test to compare lengths, the only significant difference at 28 days was between the 3 L/min flow and 7 L/min flow ($P<0.05$). At the end of the experiment, there were significant differences between mean lengths in the 1 L/min flow and the 7 L/min flow ($P<0.05$), and the 1 L/min flow and 3 L/min flow ($P<0.05$).

Evidence of unhealthy *V. iris* juveniles was observed after the first three sampling events (Figure 3.8). During the first month, *E. capsaeformis* grew from a mean length of 376 µm to 428 µm at 4 wk. During this same time period, *V. iris* grew from a mean length of 352 µm to only 383 µm at 4 wk. No statistical analysis was conducted on *V. iris* growth due to poor survival.

Water Quality

Water chemistry values were within the range of those typically considered suitable for juvenile mussel culture; total ammonia, 0.0-0.08 mg/L; hardness, 76-100 mg/L; alkalinity, 62-88 mg/L; nitrate, 1.5 to 2.9 mg/L; and nitrite, 0-0.008 (Table 3.1). Mean temperature during this experiment was 17.6ºC, and ranged between 15.2 and 19.0ºC (Figure 3.9).

Mean dissolved oxygen values for each treatment were as follows: 1 L/min, 5.85 mg/L; 3 L/min, 7.16 mg/L; and 7 L/min, 7.79 mg/L (Table 3.2). There were significant differences among all three flows ($P<0.05$).

Incoming Sediment

Due to the above-average rainfall in summer 2003, incoming sediment accumulated quickly in the reservoir and troughs during this experiment. Incoming sediment accumulated so rapidly during summer 2003 that the 125 and 200 µm meshes used to filter water entering the reservoir had to be cleaned every two to four days. Any incoming sediment that passed through the mesh screen and accumulated in the bottom of the reservoir was removed weekly.

Over the course of this 8 wk experiment, incoming sediment (organic + inorganic matter) accumulations in the 1 L/min, 3 L/min, and 7 L/min flow rates averaged 0.73 ± 0.01, 1.21 ± 0.02, and 1.67 ± 0.03 mg/100 cm^2, respectively. T tests showed that there were significant differences in incoming sediment accumulation among all three flows ($P<0.05$). Even though the amount of incoming sediment differed significantly among all three troughs, percent organic and inorganic components remained relatively consistent throughout the summer (Figure 3.10). The majority of incoming sediment was inorganic matter, with the 2, 4, 6, and 8 wk sample events containing 76.5, 72.5, 73.4, and 74.2 % inorganic matter, respectively. The 3 L/min and 7 L/min flow rates had an increase in inorganic matter after July 10, which is likely due to many intense rain storms after this date (Figure 3.10).

Escapement

During Experiment 1, a total of 59 juveniles of *E. capsaeformis* and 2 of *V. iris* were captured in the catchment containers. These data reinforce the presumption that the *V. iris* juveniles used in this experiment were not healthy since they were not moving in the troughs as extensively as those of *E. capsaeformis*. These containers not only showed that juveniles were very mobile and could escape these flow-through systems, but also showed that juveniles remained mobile for at least 74 days. Only the number of *E. capsaeformis* escapees was analyzed, since only 2 *V. iris* were observed leaving these

troughs. The number of escaping juveniles increased as rate of flow increased. The 1 L/min, 3 L/min, and 7 L/min flow rates yielded a total of 9, 22, and 28 juveniles of *E. capsaeformis,* respectively, after 8 wk. Juveniles of *E. capsaeformis* were most mobile between 15 and 29 days of age, with a total of 40 individuals escaping during this time frame. Escapement was minimal throughout the rest of the experiment, with only 8 individuals escaping between 30 and 59 days. However, the last 2 wk of this experiment yielded a slight increase in escapement, with 11 individuals escaping between 60 and 74 days of age. Those juveniles in the containers after 44 days of age were measurably larger than those in the troughs, but not significantly larger (Table 3.3).

Gut Content Analysis

Several problems arose in the gut content analysis. First, the juveniles used for this experiment had extremely strong shells that were possibly calcified prior to preservation. Calcification made the preferred method of using a cover slip to crush the juveniles inadequate. Second, previous juvenile gut content analyses had been done using an epiflourescent microscope, which illuminates chlorophyll in algae, making identification of easier. Since an epiflourescent microscope was not available, a binocular light microscope was used. Much of the algae observed had its chlorophyll already digested, or was digested seemingly after it had died. As a result, complete quantification of algal genera was not possible, and as much as two to three times the amount of algae observed was not identifiable. However, some useful quantifiable and qualitative data were obtained.

The mean lengths of juveniles used for this analysis from the l L/min, 3 L/min, and 7 L/min flow rates were 566, 565, and 554 µm, respectively, with no significant differences (ANOVA; P=0.5858). Only algal genera were identified in this analysis, but guts showed that juveniles from this experiment also were consuming various types of bacteria and considerable detritus. Overall, three taxonomic groups of algae (green algae, diatoms, and euglenoids) were present in the gut contents of

juveniles in each of the three treatments. A comparison of results between the three flow treatments yielded an obvious trend (Table 3.4). Juveniles of *E. capsaeformis* reared in the 1 L/min flow had the fewest number of algal cells (33) in their gut contents and the least algal diversity of gut contents examined from all three treatments, with only nine genera identified. Juveniles reared in the 3 L/min and 7 L/min rates of flow yielded approximately the same number of algae cells in their gut contents, 90 and 89 cells, respectively. However, juvenile gut contents in the 7 L/min flow had 12 genera of algae compared to 10 genera for juveniles reared in the 3 L/min flow.

The diatom *Navicula* dominated all three treatments, making it the smallest (5 μm) and most abundant size range of algae consumed. The largest algae ingested were *Pinnularia* and other pennate diatoms, which ranged in size from 30 to 40 μm. However, it is likely that these algae were ingested lengthwise, in which case they were only approximately 5 μm in diameter. All other genera in the guts ranged in size from 10 to 20 μm.

Experiment 2: To Determine a Suitable Substrate Size and Sample Frequency for Juvenile Mussels in 4 L Round Flow-Through Tanks

Survival

Throughout this experiment, juveniles in the fine sediment treatment had the lowest survival, and coarse sand consistently had the highest survival. After 10 wk, the fine sediment, fine sand, and coarse sand had survival rates of 9.4, 36.3, and 36.4 %, respectively, with a 27.4 % overall survival rate (Figure 3.11). Statistical comparisons were made using a Poisson distribution test at every sample event. Survival of juveniles among all three treatments was significantly different ($P<0.05$) from each other at all sample events, except for the fine and coarse sand treatments, which yielded no significant difference at 8 ($P=0.4530$) and 10 wk ($P=0.9368$) .

The dishes that were left unsampled for 10 wk had a significantly higher overall survival of 40.2 %; 12.8 % better survival than those dishes that were sampled every 2 wk (ANOVA; $P<0.05$). The fine sediment, fine sand, and coarse sand treatments in the unsampled dishes had survival rates of 27.5, 51.9, and 41.1 %, respectively (Figure 3.11). A Poisson distribution test at 10 wk revealed that the three treatments were significantly different from each other ($P<0.001$).

Villosa iris juveniles that were reared at the FMCC had better survival than the *V. iris* reared at AWCC for the first 6 wk (Figure 3.12). Between 8 and 10 wk, survival decreased dramatically with < 1 % survival at 8 wk (Figure 3.12). After this sample event, the experiment at the FMCC was discontinued due to poor survival. However at 6 wk, juveniles at the FMCC had significantly greater survival (55.8 %) ($P<0.05$), than the juveniles (46.2 %) sampled at AWCC.

Growth

The fine sediment treatment, although consistently showing the lowest survival in the sampled dishes, had the best growth at each sample event (Figure 3.13). The fine sediment, fine sand, and coarse sand had mean lengths of 887, 834, and 755 µm, respectively, at the end of the experiment. Using Tukey's student range test to compare lengths at each sample event, the only significant differences were between the fine sediment and coarse sand ($P<0.05$) at 4 wk, and between the fine sediment and coarse sand ($P<0.05$) at 6 and 8 wk, and fine sand and coarse sand ($P<0.05$) at 6 and 8 wk. There were no significant differences in lengths among substrate treatments at the end of the experiment.

Juveniles in the unsampled dishes had comparable growth among the three treatments at the end of the experiment, with the fine sediment, fine sand, and coarse sand having mean lengths of 856, 852, and 857 µm, respectively. The total mean growth at 10 wk was 855 µm in the unsampled dishes, slightly greater than the total mean growth of 825 µm in the sampled dishes (Figure 3.14). Using a

Tukey's student range test at 10 wk, no significant differences in growth occurred among the three treatments.

The *V. iris* held at the FMCC exhibited consistently lower growth than those cultured at AWCC (Figure 3.14). At 6 wk, juveniles at the FMCC had a mean length of 492 µm, compared to 675 µm for those at AWCC. Poor condition and subsequent mortality was anticipated since mussels between 6 and 8 wk at the FMCC did not grow over this 2 wk period. Empty shells had an mean length of 486 µm, indicating these mussels likely died shortly after 6 wk.

Water Quality

Water chemistry values at AWCC were similar to those in summer 2003, with all values from AWCC and the FMCC within the range typically considered suitable for juvenile mussels. At AWCC, total ammonia ranged from 0.0-0.09 mg/L, hardness from 80-144 mg/L, alkalinity from 22-87 mg/L, nitrate from 0.0 to 2.6 mg/L, and nitrite from 0-0.008 (Table 3.5). Water temperatures during summer 2004 were significantly higher (ANOVA; $P<0.0001$) than those in summer 2003, averaging 21.1ºC with a range of 18.1 to 23.8ºC (Figure 3.9).

At the FMCC, total ammonia ranged from 0.0-0.18 mg/L, hardness from 190-232 mg/L, alkalinity from 131-183 mg/L, nitrate from 0.4 to 1.3 mg/L, and nitrite from 0-0.005 (Table 3.6). No trends were observed for values at AWCC or FMCC, with no obvious effects of water chemistry at either facility.

Incoming Sediment

Incoming sediment accumulated more in the tanks that held substrate than those without (Figure 3.15). The average accumulation of incoming sediment in the fine sediment, fine sand, and coarse sand treatments was 0.32 ± 0.09, 0.42 ± 0.04, and 0.47 ± 0.04 mg/100 cm^2, respectively. No

significant differences in the amount of incoming sediment among the three treatments were observed (ANOVA; P=0.2884).

As expected, the unsampled dishes accumulated significantly more incoming sediment (ANOVA; P<0.0001) than those that were sampled every 2 wk (Figure 3.15). The fine sediment, fine sand, and coarse sand treatments contained mean incoming sediment loads of 1.50 ± 0.13, 2.01 ± 0.29, and 1.77 ± 0.17 mg/100 cm^2, respectively. However, there were no significant differences in the amount of incoming sediment among the three treatments (ANOVA; P=0.2884).

Escapement

The round flow-through tanks experienced far more escapement than did the flow-through troughs in Experiment 1. A total of 1566 juveniles escaped the grow-out units that were sampled every 2 wk (Table 3.7). For the fine sediment, fine sand, and coarse sand treatments, the total escapement was 640, 531, and 395 juveniles, respectively. Escapement varied between sample events, with the highest escapement of over 23 % at 6 wk. During every sample event, the mussels in the catchment container had a smaller mean shell length than those in the grow-out units. Juveniles in the catchment containers also were significantly smaller (P<0.05) than those in the grow-out units on every occasion except at 6 wk. At 10 wk, escapement enumerated in the unsampled dishes was far less than that in the sampled dishes; only 13 juveniles were collected, 5 in both the fine sediment and fine sand treatments, and 3 in the coarse sand treatment.

Sampling Effort

Mean time needed to sample the tanks containing fine sand and coarse sand substrate was nearly 30 min. Mean sample time for the fine sediment treatment was 24 min/tank. Even though the fine sediment treatments could be sampled faster, there were no significant differences among sample times (ANOVA; P=0.7334).

Gut Content Analysis

The mean lengths of the juveniles used for this analysis from the fine sediment, fine sand, and coarse sand treatments were 926, 798, and 867 µm, respectively, and were not significantly different (ANOVA; P=0.1792). Similar to Experiment 1, considerable detritus was found in the gut contents of juveniles raised in the fine sediment and fine sand treatments. However, less detritus was observed in the juveniles from the coarse sand treatment. Four taxonomic groups of algae (blue-green algae, cryptophytes, diatoms, and green algae) were present in the gut contents of juveniles. Fine sediment and fine sand had very similar results in terms of number of genera present, number of cells present, and relative abundance (Table 3.8). Juvenile gut contents examined from the fine sediment and fine sand treatments yielded 83 and 78 cells, respectively, and contained 11 and 10 genera, respectively. However, juvenile gut contents from the coarse sand treatment yielded only 11 algal cells and six genera (Table 3.8). In the fine sand treatment, the three most abundant genera in decreasing order were: *Coelastrum*, *Chlorella*, and *Navicul*a. The fine sediment treatment had the same results except that *Navicula* was the second most abundant genus, and *Chlorella* was the third. Lastly, in the coarse sand treatment, the most abundant genera in decreasing order were: *Coelastrum*, *Navicula*, and *Chlorella*. The majority of algal cells consumed in Experiment 2 were approximately 5 to 10 µm in size.

Algae Density and Composition

For the duration of this experiment, 10 samples were taken from water supplying grow-out systems at AWCC. In total, five phyla and 26 genera were present in the water at AWCC (Appendix 1). Five additional genera (*Coelastrum*, *Gyrosigma*, *Nitzchia*, *Surirella*, and *Trachelamonas*) were found in gut contents of juveniles that were not found during analysis of water samples (Tables 3.4 and 3.8).

Mean algal densities over a two-year period at AWCC were 1120, 1617, 2971, and 1272 cells/mL, for winter, spring, summer and fall, respectively. The total mean density of algae in the water supplying AWCC was 2119 cells/mL. Algal density peaked in late August of 2004 at 3660 cells/mL and experienced its lowest level of 576 cells/mL in January of 2003 (Appendix 1). The number of algal genera in the water remained relatively constant throughout the study, and averaged about 12 genera per sample event. The four most dominant algal genera in decreasing order were *Chlorella*, *Chlamydomonas*, *Navicula*, and *Scenesdesmu*s (Appendix 1). Green algae were the most abundant, making up 65 % of the total algae reported, and diatoms next in abundance, with 33 % of all algae.

Due to time restraints, seasonal algal analysis of water taken from the Clinch River (Clinchport, VA) and North Fork Holston River (Chatham Hill, VA) was only done for 1 yr. Algal analysis for both rivers had mean densities less than that reported at AWCC and experienced far less seasonal variability. Five phyla and 25 genera were present in water samples from the North Fork, with a mean algal density of 1602 cells/mL (Appendix 2). The Clinch River water samples contained four phyla and 23 genera, with a mean algal density of 1209 cells/mL (Appendix 3).

DISCUSSION

Experiment 1: Growth and Survival of Juvenile Mussels Reared at Three Flow Rates

For unknown reasons, *V. iris* survived poorly in this experiment, and propagation attempts at the FMCC were largely unsuccessful. In addition to this poor survival and inability to raise healthy *V. iris* at the FMCC during summer 2003, personnel at AWCC also reported poor results in culturing this species. These results indicate that this species may have had a poor year-class during summer 2003, as results at the culture facilities do not reflect typical rearing success. Potential reasons for this poor

year-class may be related to the harsh winter in 2002-2003, the above-normal rains and flooding in 2003, or the below-normal temperatures experienced throughout spring and summer 2003. Although a colder than average summer, temperatures stayed above 15ºC (Figure 3.9), which Beaty and Neves (2004) reported was the point at which juveniles exhibit growth. These cool temperatures and high flows, which could have introduced high sediment loads, may have stressed gravid females or affected the maturation of glochidia. Therefore, mortality of this species at AWCC is likely related to the health of these individuals prior to culture at AWCC, and not attributed to conditions at the facility.

Overall survival, and growth to some extent, was not particularly good in comparison to previous experiments at AWCC. Interestingly, the highest survival occurred in the lowest flow, but greatest growth was observed in the highest flow. Beaty (1997) reported that survival rates of *V. iris* were higher in lower flow velocities of an artificial stream grow-out system and was likely due to less physical disturbance. My results agree with that study and indicate that juvenile mussels benefit from flowing water; however, water flowing at too high a rate (3 L/min or 7 L/min) may disturb the juveniles and reduce overall survival.

Results of this experiment could also be attributable to several other variables. Incoming sediment loads accumulating in the troughs during the experiment may have negatively affected the juveniles. The lowest flow treatment with the highest overall survival of 18 % also contained the least incoming sediment (0.73 mg/100 cm^2). These findings are contrary to those of Zimmerman (2003), who reported that the greatest survival was in high incoming sediment treatments at AWCC. High incoming sediment levels defined by Zimmerman (2003) ranged between 5.5 and 7.0 mg throughout the course of that experiment, and were expressed in weight, not weight per unit volume. Given the bottom area of her troughs, this would equal incoming sediment levels of 1.83 to 2.33 mg/100 cm^2. High values reported for her experiment were above even the highest incoming sediment accumulation

in my 7 L/min troughs (1.67 mg/100 cm^2). Hanlon (2000) also reported that survival at AWCC was negatively affected by excessive incoming sediment which possibly affected pedal-feeding of the juveniles.

Other studies have debated the importance of fine sediment in the growth and development of juvenile freshwater mussels. Adult giant floaters (*Pyganodon grandis*), when covered with 45 cm of fine sediment, experienced between 85 and 100 % mortality (Imlay 1972). Most researchers have reported that small amounts of fine sediment are beneficial to young juvenile mussels and improve growth and survival rates by providing nutritional supplements and possibly serve as a grinding substrate (Hudson and Isom 1984, Gatenby et al. 1996). Although it is generally accepted that excessive incoming sediment accumulation negatively affect juveniles, the threshold of how much sediment can be tolerated has not been determined (Brim-Box and Mossa 1999, Henley et al. 2000). Zimmerman (2003) concluded that incoming sediment loads up to 3.33 mg/100 cm^2 were not lethal to juveniles. It is likely that the accumulations of 1.21 to 1.67 mg/100 cm^2 of incoming sediment in my 3 L/min and 7 L/min flow treatments negatively affected juvenile survival in this experiment. This much incoming sediment likely filled the interstitial spaces in the substrate used by the juveniles and reduced interstitial flow, possibly interfering with feeding and respiration. Those juveniles able to survive the high incoming sediment loads may have received additional nutrition from these high deposited sediments, resulting in greater growth in the 3 L/min and 7 L/min treatments.

It is difficult to determine the significance, if any, of dissolved oxygen levels in the growth and survival of these juveniles, since the effects of incoming sediment accumulation were probably of greater influence. As expected, dissolved oxygen values increased with the higher flows and differed significantly among the three treatments ($P<0.05$), since the water entered these troughs with greater velocity and agitation. Although a minimum dissolved oxygen value for juvenile mussels has not been

defined, Havlik and Marking (1987) recommended 5 mg/L for adult mussels. My mean values, ranging from 5.85 to 7.79 mg/L, were comparable to past measurements at AWCC. It is possible that those juveniles that survived in the 3 L/min and 7 L/min treatments were benefited by the higher oxygen levels, thus resulting in the significantly higher growth rates between these two treatments and the 1 L/min treatment.

Inclusion of a catchment container on a flow-through system to capture emigrating juveniles is essential. This research concurs with previous work, showing that juveniles are extremely mobile and are capable of moving up and out of a 6.4 cm standpipe upwards of 74 days of age (Reid et al. 1992). Hanlon (2000) also reported that not only were juveniles very mobile during their first 2 mo of age, but those that escaped were significantly larger. In my experiment, those juveniles that escaped after 45 days had a greater mean length than those in the troughs, but at no time were significant differences observed. The higher rates of flow had more escapement (22 juveniles in the 3 L/min and 28 juveniles in the 7 L/min), when compared to only 2 juveniles in the 1 L/min. High incoming sediment loads could have caused the juveniles to exit these troughs. However, it may be that these emigration results simply reflect juveniles being flushed from the troughs by the higher flow rates.

Typically, small amounts of substrate have been used in grow-out systems to facilitate sampling. Zimmerman (2003) used only 2 mm of coarse limestone sand in flow-through troughs, and Hanlon (2000) used 5-10 mm of coarse limestone sand in several grow-out experiments. Yeager et al. (1994) reported finding all juveniles of *V. iris* in a study within the top 1 cm of sediment. However, Neves and Widlak (1987) reported juveniles 0-3 yr of age up to 8 cm in the sediment. I used 2 cm of substrate in the troughs to allow juveniles to bury deeper in order to escape flow and possible sediment accumulation, and to give juveniles more space to move. However, this volume of substrate also made sampling difficult and time consuming.

Gut Content Analysis

Clearly the juvenile mussels at AWCC were consuming primarily algae, and lesser amounts of bacteria and detritus. The majority of the algae consumed was approximately 5 µm in size. This size range agrees with results of Yeager et al. (1994), who reported that *V. iris*, 5 to 8 d old, primarily consuming algae 2 to 5 µm in size. Beck and Neves (2003) also reported that *V. iris* of all age groups favored algal cells from 2.8-8.5 µm in size, as opposed to larger cells (22.3-44.5 µm).

Juvenile mussels in Experiment 1 ingested algal taxa in the following decreasing order of abundance: *Navicula*, *Cymbella*, *Synedra*, and *Chlorella* (Table 3.4). Mean length of *Navicula* was 5 µm in the gut contents, possibly consumed in greater quantities not just due to size, but also because of habitat occurrence. *Navicula* species are primarily benthic, move on the substrate, and thrive in muddy environments, where juvenile mussels are pedal-feeding. Given the high rainfall during summer 2003, and the subsequent high incoming sediment load in the troughs, these occurrences may explain why *Navicula* cells were abundant in the guts. A variety of other algae also were present in lower abundance in the gut contents such as *Diatoma*, *Gomphonema*, *Gyrosigma*, and *Surirella*, indicating that pedal-feeding was occurring at 74 days of age (Table 3.4).

Experiment 2: To Determine a Suitable Substrate Size and Sample Frequency for Juvenile Mussels in 4 L Round Flow-Through Tanks

The overall survival of juveniles in this experiment was greater than that obtained in Experiment 1. Mean survival in sampled and non-sampled tanks after 10 wk was 33.8 %. If the fine sediment treatment is excluded, overall survival was 41.8 % (Figure 3.12), which is comparable or better than many previous experiments with *V. iris* juveniles (Figure 3.12) (Beaty 1997, Mummert 2001, Zimmerman 2002). Mean length of juveniles after 10 wk (840 µm) was considered average

growth, since other studies at AWCC have had similar or greater lengths at 90 days (Hanlon 2000, Zimmerman 2003).

Results of this study show that juveniles reared in fine or coarse sand have better overall survival than those reared solely in fine sediment. In the case of unsampled dishes, juveniles in fine sand had significantly higher survival than those in coarse sand at 10 wk. These results agree with those of Rogers (1999), who reported that juveniles in fine sand (500-800 μm) had significantly higher survival (23.1 %) than those reared in fine sediment (<120 μm) after 16 wk. It has been suggested that the presence of substrate allows juveniles to feed interstitially and to attach to the substrate with byssal threads (Yeager et al. 1994). In my experiment, juveniles without sufficient substrate were possibly unable to burrow or attach to anything. Poorer survival and greater juvenile escapement in the fine sediment treatment could be the result of juveniles being unable to attach to substrate. O'Beirn et al. (1998) also reported that juveniles held without sediment would not feed and closed up in flowing water. Poor survival in the fine sediment treatments and the depressed growth in the juveniles in the catchment containers could have resulted due to juveniles remaining closed and not feeding.

Interstitial feeding could also explain why juveniles in the unsampled dishes had significantly higher survival in the fine sand than those in the coarse sand. The much larger grain size in the coarse sand treatments may have allowed more of the incoming sediment to settle through the sand, packing the interstitial spaces with incoming sediment to a greater extent and inhibiting feeding. With most of the incoming sediment accumulating on the surface of the tighter packed, fine sand, the juveniles may have been able to escape the incoming sediment by moving deeper in the fine sand to feed.

Juveniles reared in the fine sediment treatment in the sampled dishes exhibited the poorest survival. Additionally, this treatment also contained the least amount of incoming sediment and juveniles in these tanks exhibited highest growth. Even juveniles in the unsampled dishes containing

only fine sediment did not differ significantly in growth from the fine and coarse sand treatments. Expectation was that treatments with the poorest survival would also exhibit the poorest growth, but this was not the case. Beaty and Neves (2004) had similar results, in which juveniles cultured in fine sediment (<120 μm) grew slightly larger than those in coarse sediment (120-600 μm). One possible reason for my results is that transported incoming sediment in the substrate-free tanks was constantly agitated with circular flow, much more so than the incoming sediment contained in either the fine sand or coarse sand treatment. Rogers (1999) hypothesized that fine sediment, which is more loosely packed, such as in the fine sediment treatments, could facilitate feeding of juvenile mussels. However, the greater growth of juveniles in the unsampled dishes and comparable growth in the unsampled dishes is more likely related to survival of the most robust juveniles. Under stressful conditions, with no way to secure themselves, the least fit mussels probably died, leaving only the largest and healthiest mussels to colonize the sediments.

At AWCC, Zimmerman (2003) concluded that incoming sediment levels of up to 3.33 mg/100 cm^2 were not lethal to mussels but did reduce growth. In Experiment 2, the best survival (51.9 %) occurred in the fine sand, unsampled dishes, which also had the highest mean incoming sediment load (2.01 mg/100 cm^2). These results are contrary to Experiment 1, where the best survival was in troughs with the least incoming sediment accumulation. High accumulations of incoming sediment of up to 2.01 mg/100 cm^2 did not appear to affect growth rates in this experiment. Mean length of mussels reared in the unsampled dishes was significantly greater (855 μm) in 1.76 mg/100 cm^2 of incoming sediment, than those in the sampled dishes (825 μm) with 0.40 mg/100 cm^2 mg of incoming sediment (ANOVA; $P<0.05$) (Figure 3.14).

Historically, research at AWCC has tried to strike a balance between juvenile sampling, which may cause stress and potential loss of juveniles, and no sampling, which causes excessive incoming

sediment deposition that can negatively affect juveniles. Sampling the mussels in this experiment every 2 wk appeared to negatively affect survival and growth over the course of 10 wk, because overall survival was 12.8 % greater and mean lengths were 30 µm larger in the unsampled dishes. These findings are contrary to those reported by Hanlon (2000). In his experiment, *V. iris* left unsampled had less growth than those that were sampled. Although not measured in his experiment, it is likely that incoming sediment loads were high since the water was not pre-filtered. O'Beirn et al. (1998), on the other hand, reported results similar to my experiment; sampled juveniles of *L. fasciola* had growth rates lower than those that were sampled.

Since juveniles in the treatment without substrate exhibited the poorest survival, sampling time has little significance. As expected, mean sampling time required for the fine sediment treatments was 4 min faster than both the fine and coarse sand treatments, because extracting the juveniles only required thoroughly rinsing the dishes. In this experiment, the time saved was not worth compromising the potential of significantly higher survival with substrate in the tanks.

The open, round tanks and troughs contained some turbellarian worms (*Macrostomum* sp.), but no predation was observed in either experiment. Culturing juveniles to 300 µm in length for Experiment 1 likely avoided the effects of this predator. In Experiment 2, screening out debris <50 µm and the fast growth of juveniles in these round tanks likely contributed to minimal mortality from turbellarian worms.

The reason for the mass mortality event of *V. iris* juveniles at the FMCC between 6 and 8 wk is unknown. All water chemistry values were within accepted ranges during the experiment, and the quality of juveniles was not an issue since the same batch did well at AWCC. Chlorine in the grow-out system, which was not measured in this experiment, was monitored by other researchers and reported to be absent when measured with a Hach™ DR/2000 spectrophotometer. However, after this

experiment concluded, a residual chlorine level of 0.0003 mg/L was detected using a chlorine titrator. The immediate effects of such an insignificant amount of chlorine is not likely to have an impact on juvenile survival. However, evidence of chronic exposure may have been observed between 6 and 8 wk, when juveniles experienced increased mortality (Rachel Mair, FMCC, pers. comm.).

The round flow-through tank system used in this experiment was a much better grow-out unit than the flow-through troughs used in Experiment 1. Overall survival at 8 wk for the sampled dishes was 22 % greater than the survival results at the end of Experiment 2 at 8 wk. Additionally, these round tanks were smaller, contained less substrate, and required far less maintenance, making them a better system than used in Experiment 1. Also, water moving in a circular motion in these troughs likely allows juveniles more time to consume suspended food particles since the water is retained longer in this system than in the linear flow-through troughs.

Gut Content Analysis

Similar to Experiment 1, juvenile mussels consumed considerable detritus and algae approximately 5 µm in size. Juvenile mussels in the fine sand and fine sediment treatments ingested algal taxa in similar amounts, with *Coelastrum* cells most abundant, and *Chlorella* and *Navicula* either second or third in abundance (Table 3.8). However, the coarse sand treatment was quite different; *Chlorella* was most abundant, *Chloroccocum* was second, and three other genera *(Navicula*, *Pinnularia*, and *Synedra*) tied for third (Table 3.8).

Gut content analysis on the juveniles from the coarse sand treatment indicated that these juveniles were feeding less than juveniles in the other treatments, since few cells of various genera were consumed. In addition, it was apparent that detritus amounts were far less in the juveniles from the coarse sand treatment, suggesting these juveniles were not feeding at the same rates in the other

treatments. These results may partially explain why juveniles in this treatment were smaller (755 μm) than juveniles in either the fine sediment (834 μm) or fine sand (887 μm) treatments.

Algal Density and Composition

Mean algal cell densities (2119 cells/mL) over this 2-year period at AWCC were greater than those reported in either the North Fork Holston River (1602 cells/mL) or Clinch River (1209 cells/mL). Even though the Clinch River and North Fork Holston River were not sampled in late summer, all rivers were sampled in mid-July of 2004, and AWCC water contained approximately 2000 cells/mL more than either river. In a similar study, Boyles (2004) estimated that the yearly mean algal cell densities in raceways used to hold freshwater mussels at WSSNFH were 1903 cells/mL.

Little is known of the dietary requirements of freshwater mussels (Parker et al. 1998). However, it is believed that diets consisting of greater algal variety are better than diets of low variety in terms of juvenile survival and growth (Gatenby et al. 2003). If this is the case, then the five phyla and 31 genera present in the AWCC water samples are likely helping to support the high juvenile growth and survival documented in previous studies. The two dominate phyla, green algae and diatoms, also support the hypothesis that high quality food is available for mussels at AWCC. Diatoms are a very important food source since they are composed of a large percentage of polyunsaturated fatty acids and have been shown to store oils, two components crucial to juvenile development (Gatenby et al. 1997). The two taxa in greatest abundance at AWCC, *Chlorella* and *Chlamydomonas*, are nutritionally less important than diatoms, but have good nutritional value with total lipid content of approximately 13 to 20 % (Gatenby et al. 1997). Overall, algal densities and species composition appear to be excellent at AWCC, with suitable algal densities, and nutritionally important species.

CONCLUSIONS

After 8 wk, *E. capsaeformis* exhibited the best survival (18 %) in 1 L/min flow, with significant differences in survival between 1 L/min flow and 7 L/min flow ($P<0.0001$), and 1 L/min flow and 3 L/min flow ($P<0.0001$).

After 8 wk, *E. capsaeformis* exhibited the greatest mean length (656 μm) in 7 L/min flow, with significant differences in growth between 1 L/min flow and 7 L/min flow ($P<0.05$), and between 1 L/min flow and 3 L/min flow ($P<0.05$).

Incoming sediment accumulation in grow-out systems at AWCC was comprised of approximately 74 % inorganic and 26 % organic matter.

Incoming sediment loads of up to 2.01 mg/100 cm^2 seemingly did not compromise the survival and growth of juveniles of *V. iris*.

Juveniles of *E. capsaeformis* and *V. iris* consumed principally algae, with lesser amounts of bacteria and detritus.

Juveniles of *E. capsaeformis* in flow-through troughs, consumed the diatom *Navicula* in greatest abundance, probably due to its small size (5 μm) and benthic habitat.

Juveniles of *V. iris* reared in fine sand and fine sediment consumed principally *Coelastrum* in flow-through round tanks.

Mean algal cell densities at AWCC were 1120, 1617, 3582, and 1272 cells/mL, respectively, for winter, spring, summer and fall.

Green algae constituted 65 % of the algal diversity reported; diatoms were 33 % of remaining algae at AWCC.

After being sampled every 2 wk for 10 consecutive weeks, juveniles of *V. iris* reared in coarse sand had the best survival (36.4 %), with juveniles reared in fine sediment and fine sand having survival rates of 9.4 and 36.3 %, respectively.

After 10 wk of remaining unsampled, juveniles of *V. iris* reared in fine sand had the best survival (51.9 %), with juveniles reared in fine sediment and coarse sand having survival rates of 27.5 and 41.1 %, respectively.

Sampling of juveniles every 2 wk seemingly decreased their growth and survival when compared to those left unsampled for 10 wk.

Round flow-through tanks had more escapement of juveniles than did flow-through troughs, with juveniles in the outflow basket being smaller than those in the grow-out unit.

RECOMMENDATIONS

The round, flow-through tanks used in Experiment 2 were a more effective grow-out system, since survival of juveniles exceeded that in flow-through troughs used in Experiment 1. Circular water flow in these round flow-through tanks also increased the residence time of algae, allowing juveniles more opportunity to feed. Additionally, the round tanks facilitated sampling since they were more time efficient and much easier to clean than flow-through troughs. The only disadvantage to a round tank system is high juvenile escapement. Densities of up to 1750 juveniles per 1000 cm^2 in such a system seem viable since survival and growth seemingly were not affected. After using both grow-out systems, flow-through troughs are not recommended for future use at AWCC.

When using round flow-through tanks, fine limestone sand (500-800 μm) is recommended as substrate, with approximately 1 L/min of flow. Future propagation efforts should not use only fine sediment in grow-out units because survival was much less in this treatment. Sampling grow-out systems infrequently, so long as incoming sediment levels do not exceed approximately 2 mg/100 cm^2, is recommended. Since sedimentation levels are difficult to assess unless measured, leaving round tanks unsampled for up to 10 wk in an average rainfall summer may be an easier way to determine sampling frequency. However, summers with above-average rainfall may necessitate sampling prior to 10 wk, since incoming sediment would accumulate more quickly.

The results of these experiments also support the need for a catchment container since significant numbers of juveniles tend to escape from both types of grow-out units. Since growth was less in the juveniles that had escaped, removing juveniles from catchment containers at least weekly would minimize the reduced growth of escapees.

Filtering the water of particles < 50 μm in juvenile culture systems could potentially remove nutritionally important algal species. Even though the majority of algal genera consumed were 5 μm

sizes of up to 50 µm were found in gut contents. The size range of algal genera consumed by juveniles > 3 mo old at AWCC could be larger in size than 5 µm and may be useful information to document. Additionally, taking water samples and analyzing the algal contents more frequently than seasonally would clarify yearly patterns in cell densities and composition available to all mussels at the facility.

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Table 3.1. Summary of water chemistry values (n=5) taken from the pond during Experiment 1 in 2003 .

Date	pH	DO (mg/L)	Ammonia (mg/L)	Hardness (mg/L)	Alkalinity (mg/L)	Nitrate (mg/L)	Nitrite (mg/L)
6/26	8.99	7.64	0	86	64	2.8	0.005
7/10	8.84	7.2	0.08	84	88	2	0.008
7/24	9.15	7.7	0.07	76	70	2.9	0.005
8/7	8.34	7.25	0	90	62	1.5	0.001
8/21	8.67	6.87	0	110	63	2.2	0
MEAN	**8.8**	**7.33**	**0.03**	**89.2**	**69.4**	**2.3**	**0.004**

Table 3.2. Dissolved oxygen values (n=11) in mg/L measured with a YSI Model 50B DO meter from one replicate in each flow treatment of Experiment 1 in 2003.

	Flow Rate		
Date	1 L/min	3 L/min	7 L/min
6/26	6.06	8.22	8.50
6/30	6.45	7.50	8.46
7/4	6.10	7.17	7.50
7/7	5.90	7.30	7.60
7/14	6.06	6.36	7.16
7/17	5.70	6.60	7.78
7/21	6.45	7.10	8.20
7/28	5.75	6.50	7.76
7/31	5.70	7.80	8.20
8/4	5.34	7.60	7.70
8/12	4.80	6.66	6.80
MEAN	**5.85**	**7.16**	**7.79**

Table 3.3. Total number and mean lengths of juveniles of *E. capsaeformis* in the catchment containers for each flow treatment during Experiment 1.

Age (Days)	1 L/min	3 L/min	7 L/min	Percent Escapement	$\bar{X}$ of Escapees (µm) (± SE)	$\bar{X}$ of Juveniles (µm) (±SE)	Difference (µm)	Sig. Diff. (P<0.05)
15-29	7	18	15	1.30	382.6 ± 4.49	388.45 ± 5.94	5.85	-
30-44	1	2	0	0.16	381.0 ± 18.48	427.74 ± 8.60	46.74	-
45-59	0	2	3	0.72	549.2 ± 7.19	518.27 ± 12.19	30.93	-
60-74	1	0	10	1.58	627.6 ± 20.28	619.55 ± 11.32	8.05	-
Totals	9	22	28					

Table 3.4. Relative abundance (%) of algae in the gut contents of *E. capsaeformis* juveniles in each flow treatment after 56 days, from June 26 to August 21, 2003.

	Relative Abundance (%)		
Algae (Order/Genus)	**1 L/min**	**3 L/min**	**7 L/min**
Green Algae (Chlorophyta)			
Chlamydomonas	12.1	2.2	2.2
Chlorella	21.2	1.1	7.9
Oocystis		1.1	10.1
Euglenoids (Euglenophyta)			
Trachelomonas	3.0		
Diatoms (Bacillariophyceae)			
Cyclotella		1.1	
Cymbella		15.6	13.5
Diatoma	6.1	4.4	4.5
Gomphonema	3.0	3.3	2.2
Gyrosigma			3.4
Melosira			2.2
Navicula	39.4	53.3	40.4
Nitzschia			1.1
Pinnularia	6.1	3.3	
Surirella			2.2
Synedra		14.4	10.1
Unidentified pennate diatom	9.1		
Total Cell Density	33	90	89
No. of Genera	8	10	12

Table 3.5. Summary of water chemistry readings (n=4) taken from the reservoir of the recirculating system at FMCC during Experiment 2.

Date	Ammonia (mg/L)	Hardness (mg/L)	Alkalinity (mg/L)	Nitrate (mg/L)	Nitrite (mg/L)	Temp. (ºC)
7/15/04	0	190	157	0.4	0	21
7/28/04	0	205	159	0.5	0.005	21
8/11/04	0.18	232	131	0.7	0.001	21
8/25/04	0	210	183	1.3	0.005	21
MEAN	**0.045**	**209.25**	**157.5**	**0.725**	**0.00275**	21

Table 3.6. Summary of water chemistry readings (n=6) taken from water entering the grow-out system at AWCC for Experiment 2.

Date	Ammonia (mg/L)	Hardness (mg/L)	Alkalinity (mg/L)	Nitrate (mg/L)	Nitrite (mg/L)
6/15/04	0.09	80	64	2.1	0.003
7/13/04	0	77	42	2.6	0.002
7/27/04	0	93	78	0.5	0.01
8/10/04	0	144	67	0	0
8/24/04	0	87	87	0.4	0.008
9/7/04	0	85	76	1.3	0.004
MEAN	**0.015**	**94.33**	**69**	**1.15**	**0.0045**

Table 3.7. Total number and mean lengths of juveniles of *V. iris* in the catchment containers for each substrate treatment in Experiment 2 for sampled tanks and after 10 wk for unsampled tanks.

Age (Days)	Fine Sediment	Fine Sand	Coarse Sand	Percent Escapement	$\bar{X}$ of Escapees (µm) (± SE)	$\bar{X}$ of Juveniles (µm) (±SE)	Difference (µm)	Sig. Diff. (P<0.05)
14	220	93	78	6.93	406.7	464	57.3	*
28	15	1	12	0.54	462.1	538	75.9	*
42	57	60	68	4.22	661.8	675	13.2	–
56	287	335	194	23.09	763.7	804	40.3	*
70	61	42	43	5.78	774.1	825	50.9	*
70[+]	5	5	3	0.67	783.0	855	72.0	*
Totals	645	536	398					

[+] unsampled dishes

Table 3.8. Relative abundance (%) of algae in the gut contents of *E. capsaeformis* juveniles in each flow treatment after 70 days, from June 29 to September 7, 2004.

	Relative Abundance (%)		
Algae (Order/Genus)	**Fine Sand**	**Coarse Sand**	**Fine Sediment**
Blue-Green Algae (Cyanoprokaryota)			
Oscillatoria	1.2		3.8
Cryptophytes (Cryptophyta)			
Chroomonas	1.2		
Diatoms (Bacillariophyceae)			
Cocconeis			1.3
Cyclotella			1.3
Cymbella	4.8		
Gomphonema	3.6		2.6
Navicula	9.6	9.1	30.8
Pinnularia	1.2	9.1	1.3
Synedra		9.1	1.3
Green Algae (Chlorophyta)			
Chlorella	30.1	54.5	15.4
Chlorococcum	2.4	18.2	
Coelastrum	38.6		41.0
Oocystis	2.4		
Scenesdesmus	4.8		
Tetraedron			1.3
Total Cell Density	83.0	11.0	78.0
No. of Genera	11	5	10

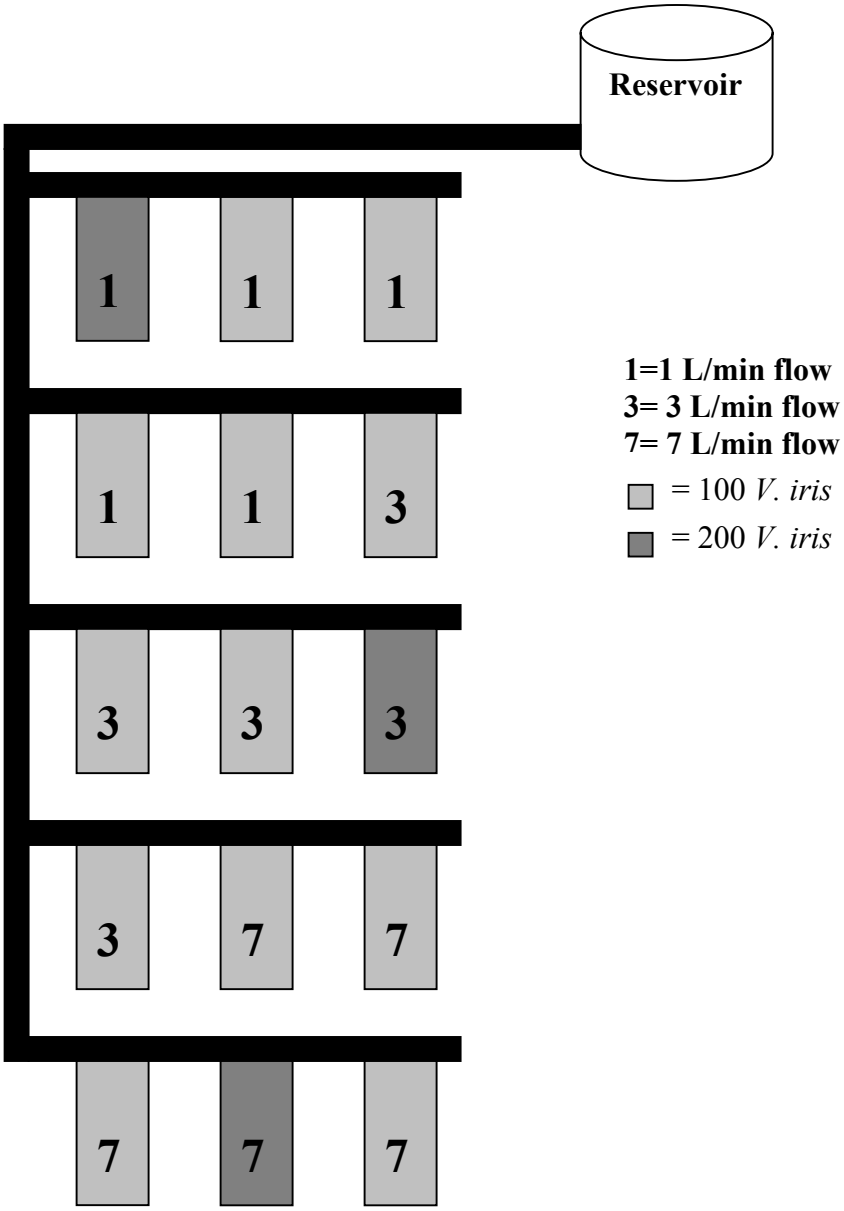

Figure 3.1. Schematic design of 15 flow-through troughs used in Experiment 1. All species were randomly assigned to troughs. Each trough contained 200 *E. capsaeformis* and either 200 or 100 *V. iris* as indicated by color scheme.

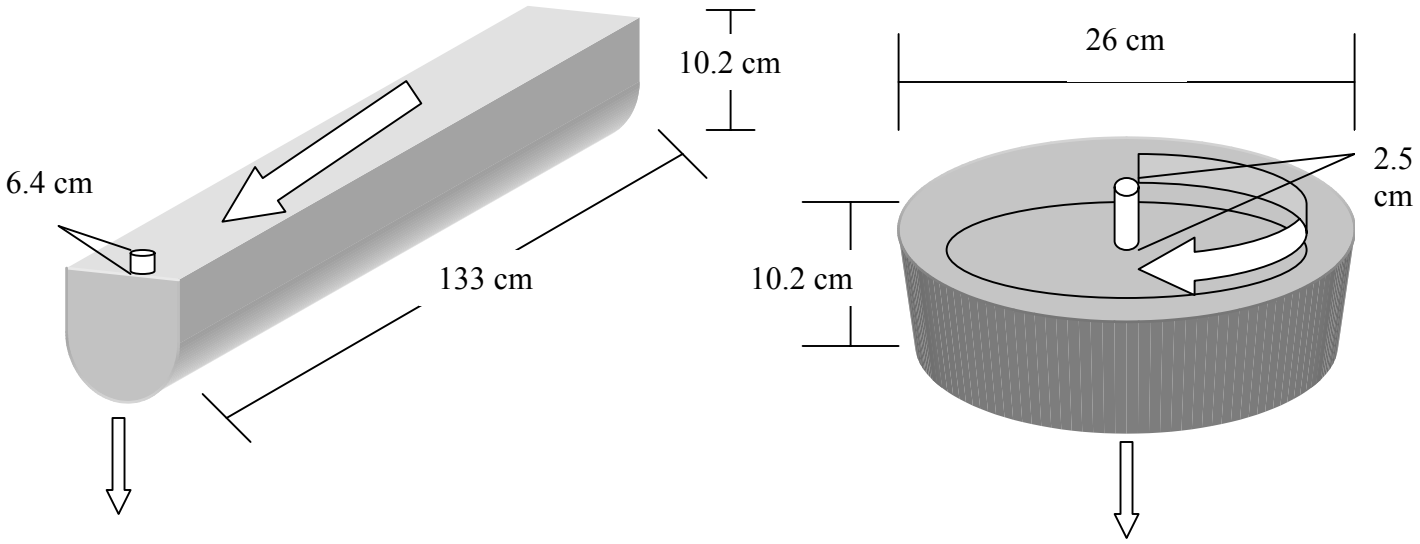

Figure 3.2. Trough used in Experiment 1 and round tank used in Experiment 2. Arrows indicate direction of water flow in each unit.

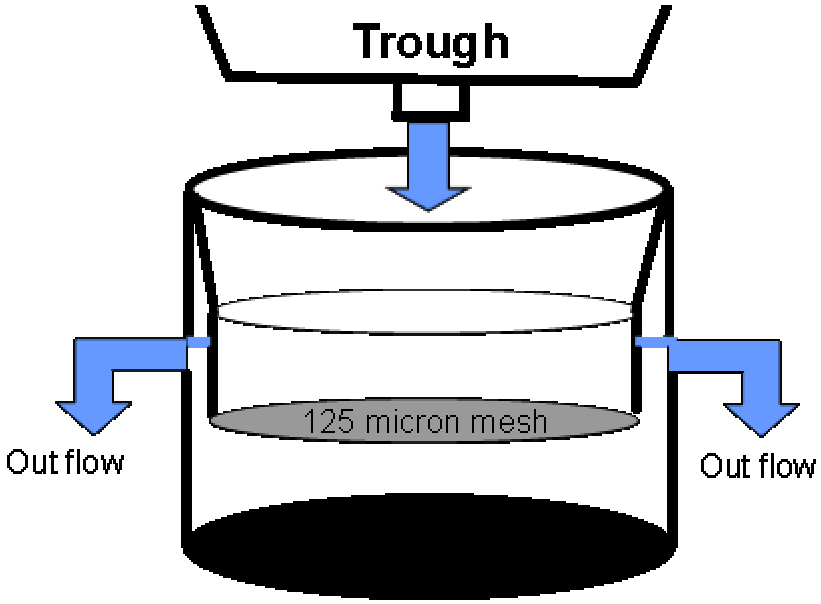

Figure 3.3. Catchment container used to capture escaping juvenile mussels from flow-through troughs in Experiment 1. Arrows indicate direction of water flow out of trough and out of holes cut into catchment container.

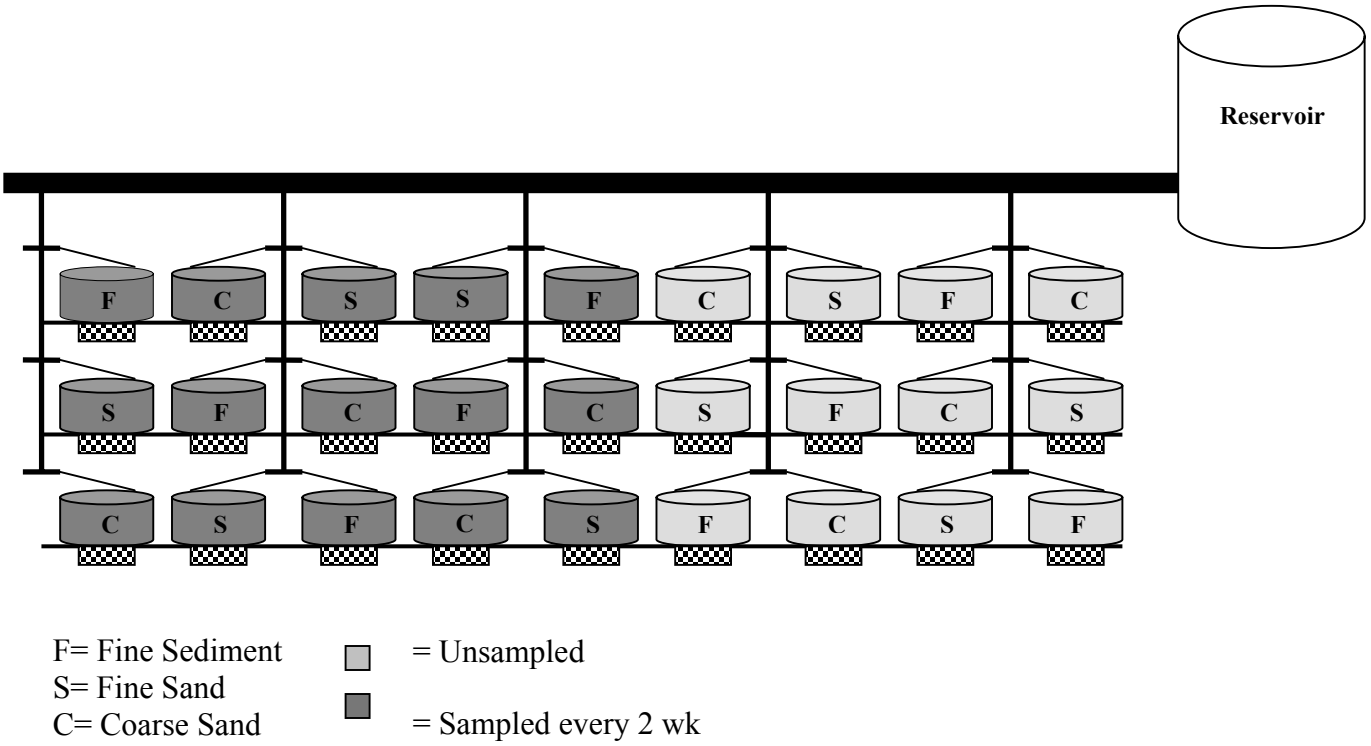

Figure 3.4. Schematic design of 27 flow-through round tanks used in Experiment 2. All treatments (indicated by letters in illustration) were randomly assigned to tanks. Each unsampled tank had 400 *V. iris*, and each sampled tank had 700 *V. iris*, as indicated by color scheme.

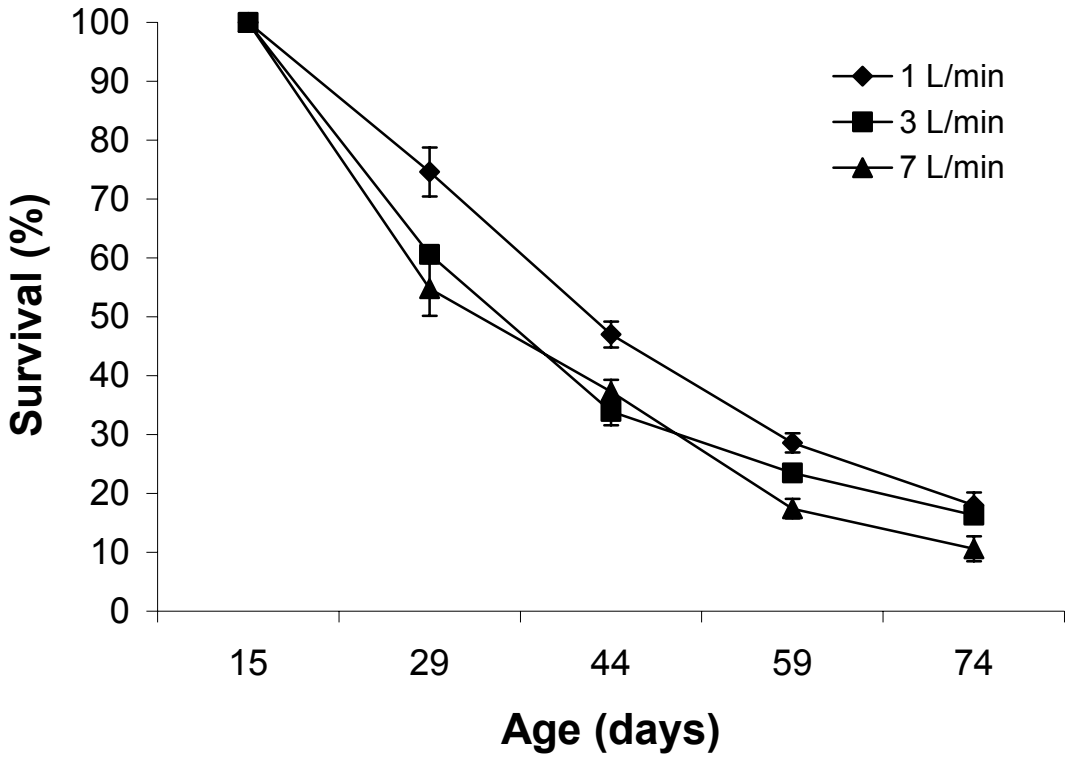

Figure 3.5. Mean survival (%) of *E. capsaeformis* reared in flow-through troughs at three rates of flow (1 L/min, 3 L/min, and 7 L/min) in Experiment 1. A Poisson distribution test showed significant differences between 1 L/min and 3 L/min treatments and between 1 L/min and 7 L/min treatments, at 29 and 59 days old.

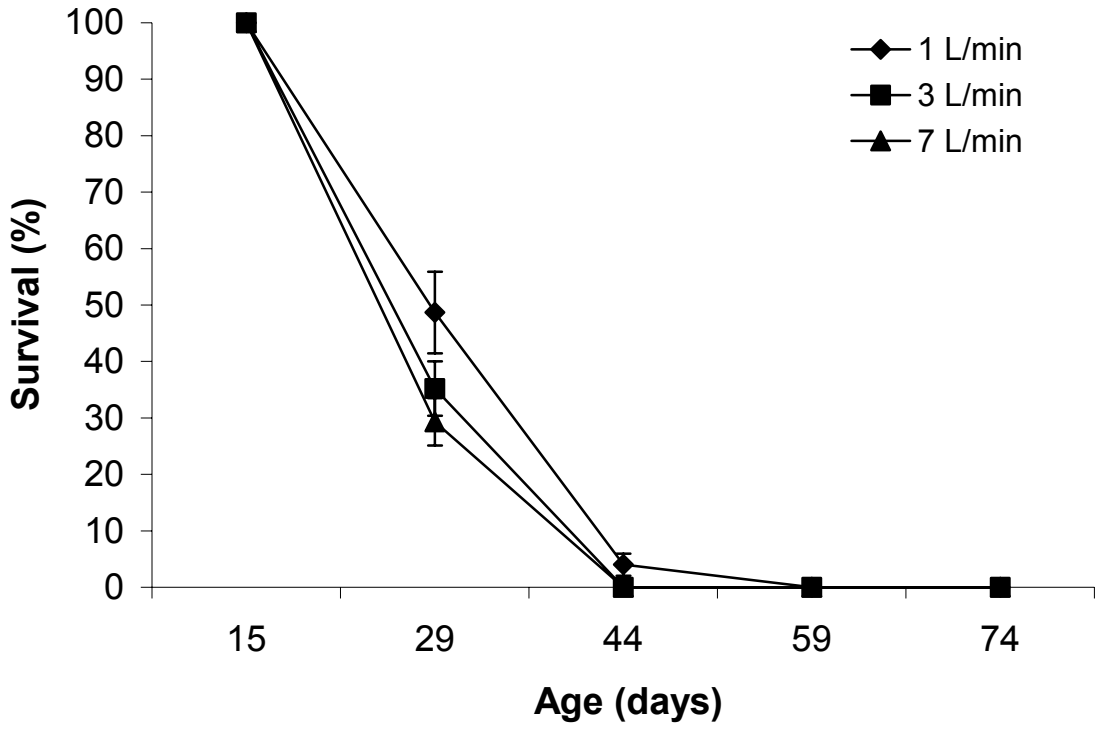

Figure 3.6. Mean survival (%) of *V .iris* reared in flow-through troughs at three rates of flow (1 L/min, 3 L/min, and 7 L/min) in Experiment 1. A Poisson distribution test showed significant differences between 1 L/min and 3 L/min treatments and between 1 L/min and 7 L/min treatments at 29 days old.

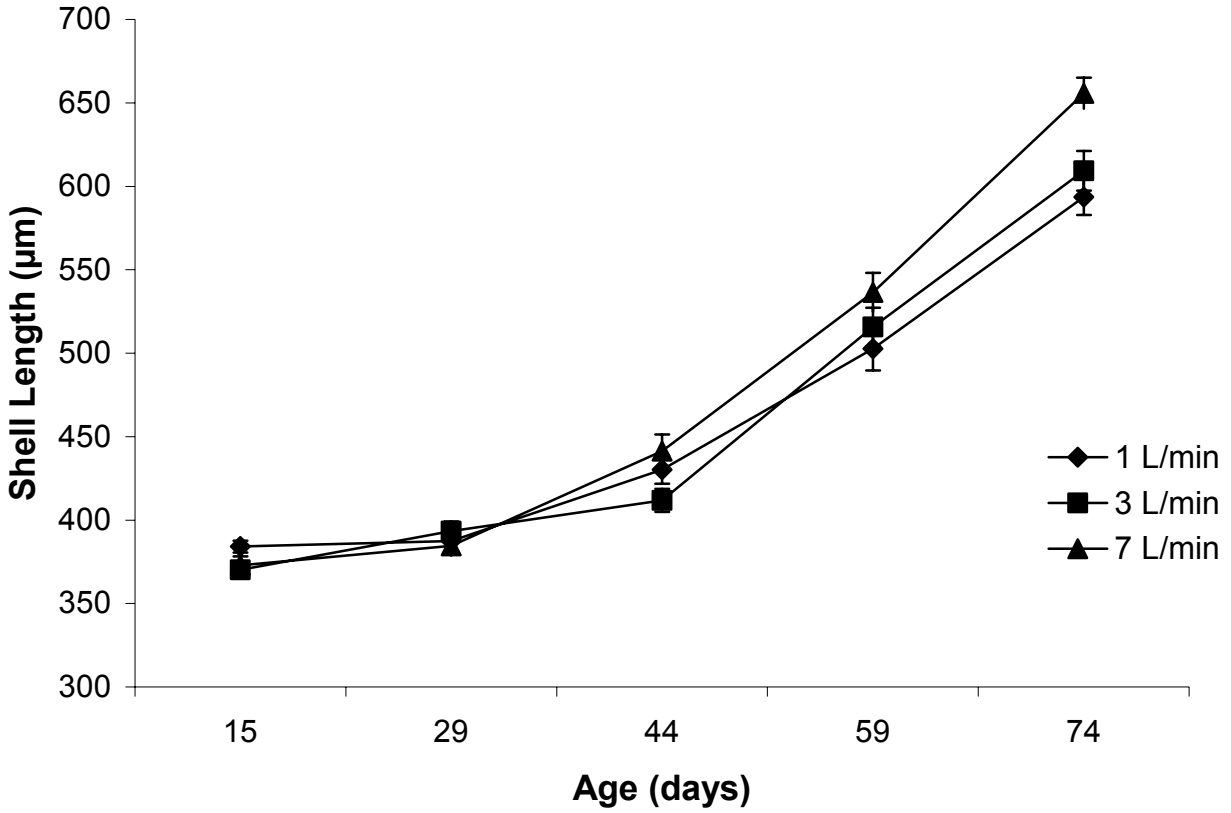

Figure 3.7. Mean shell lengths of *E. capsaeformis* reared in flow-through troughs at three rates of flow (1 L/min, 3 L/min, and 7 L/min). Tukey's student range test showed significant differences between the 3 L/min and 7 L/min treatments at 29 days old, and between 1 L/min and 3 L/min and 1 L/min and 7 L/min treatments at 59 days old.

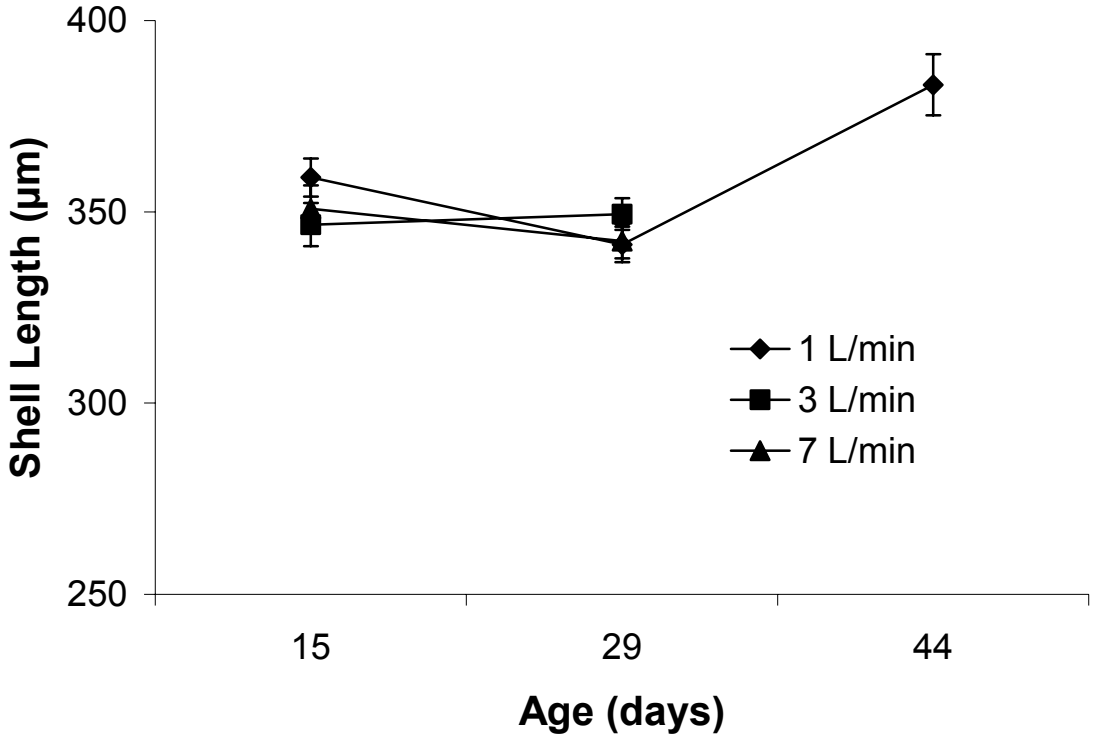

Figure 3.8. Mean shell lengths of *V. iris* reared in flow-through troughs at three rates of flow (1 L/min, 3 L/min, and 7 L/min). No statistical analysis was done because of excessive mortality.

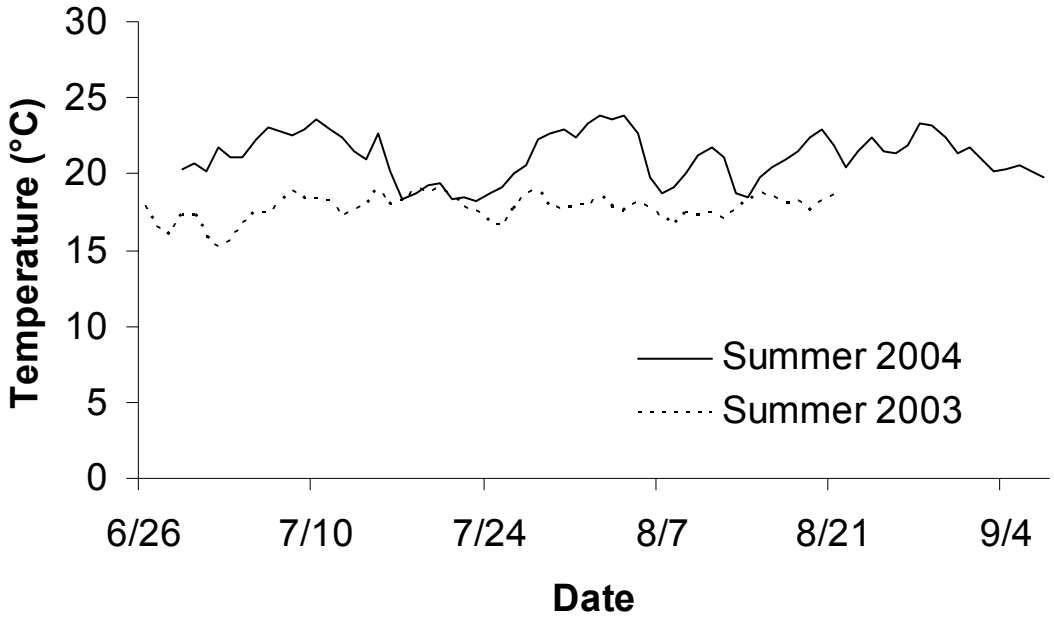

Figure 3.9. Daily mean water temperature in flow-through troughs over the 56 day study period (Experiment 1) from June 26 to August 21, 2003 and in round flow-through tanks over the 70 day study period (Experiment 2) from June 29 to September 7, 2004 for Experiment 2. Temperatures were significantly different between summer 2003 and 2004 (ANOVA; $P<0.0001$).

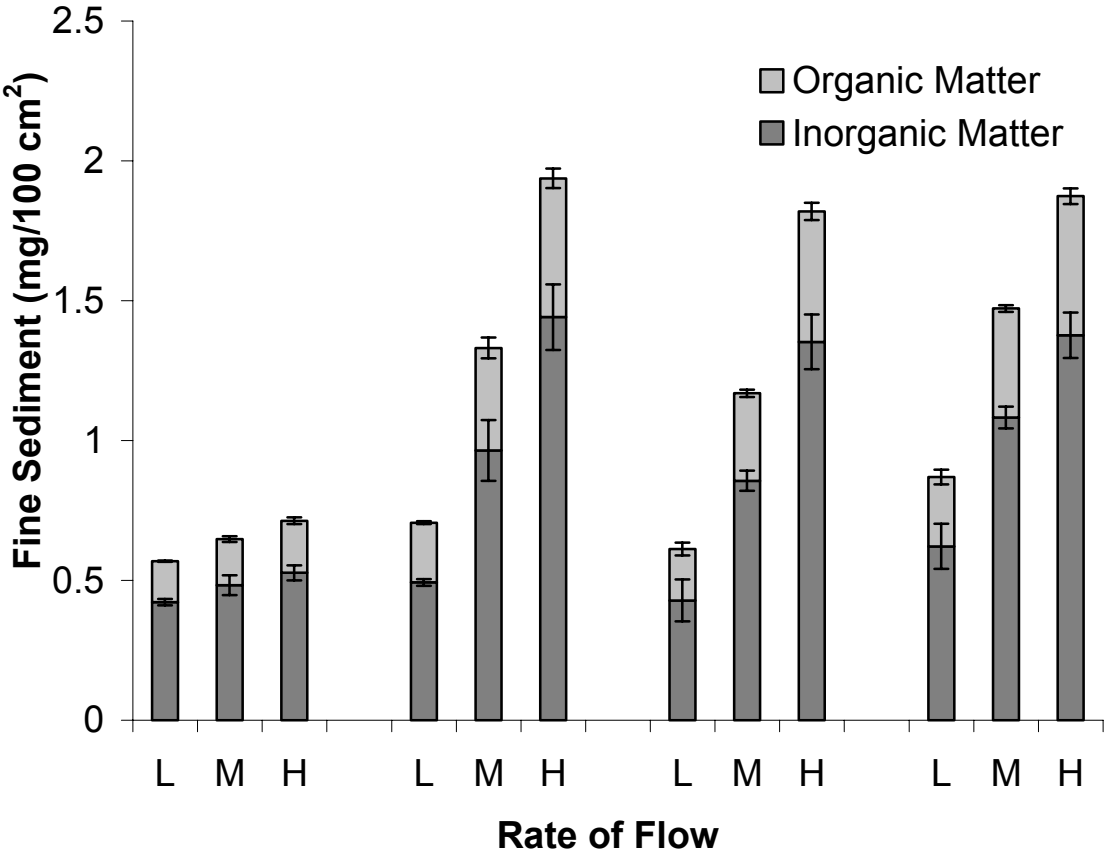

Figure 3.10. Mean organic and inorganic matter in mg/100 cm^2 of incoming sediment in low (L), medium (m), and high (H) rates of flow (5 replicates per treatment) for 4 sample dates during Experiment 1.

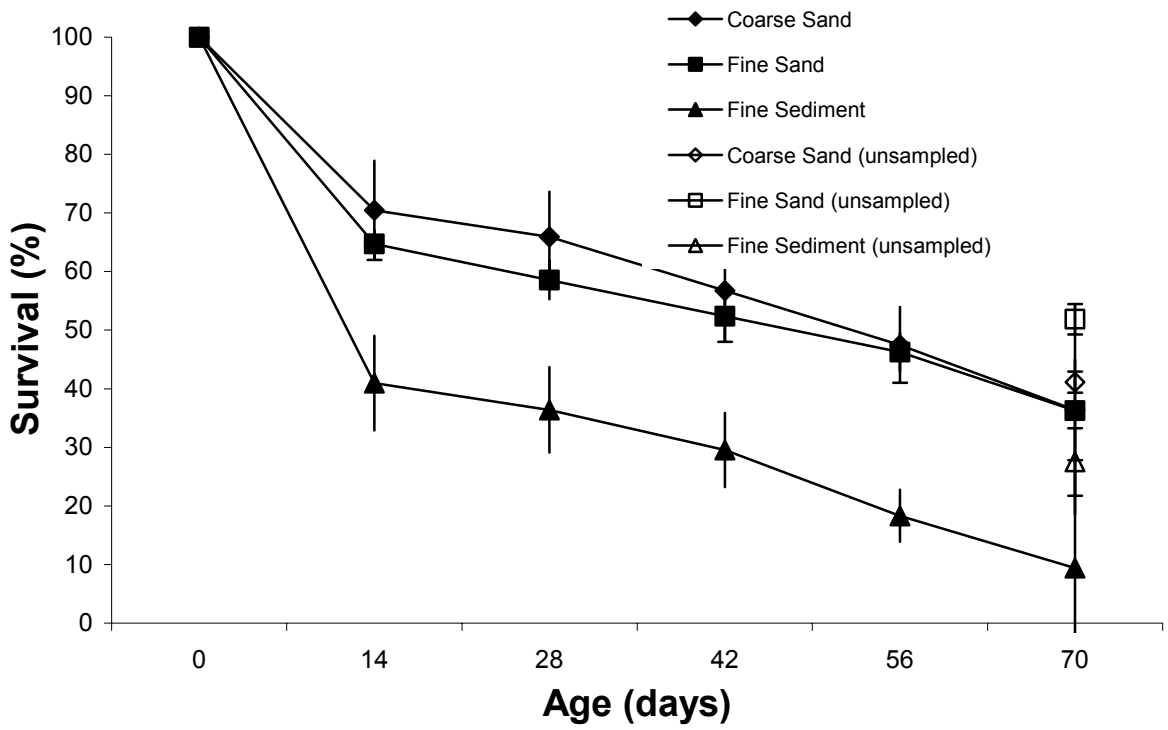

Figure 3.11. Mean survival (%) of sampled and unsampled juveniles of *V. iris* reared in flow-through tanks and with three different substrate sizes (fine sediment, fine sand, and coarse sand) in Experiment 2. A Poisson distribution test showed significant differences among all three treatments in the tanks that were sampled at every sample event, except between fine and coarse sand at 8 ($P=0.4530$) and 10 wk ($P=0.4368$). All three treatments in the unsampled dishes were significantly different from each other at 10 wk ($P<0.0001$).

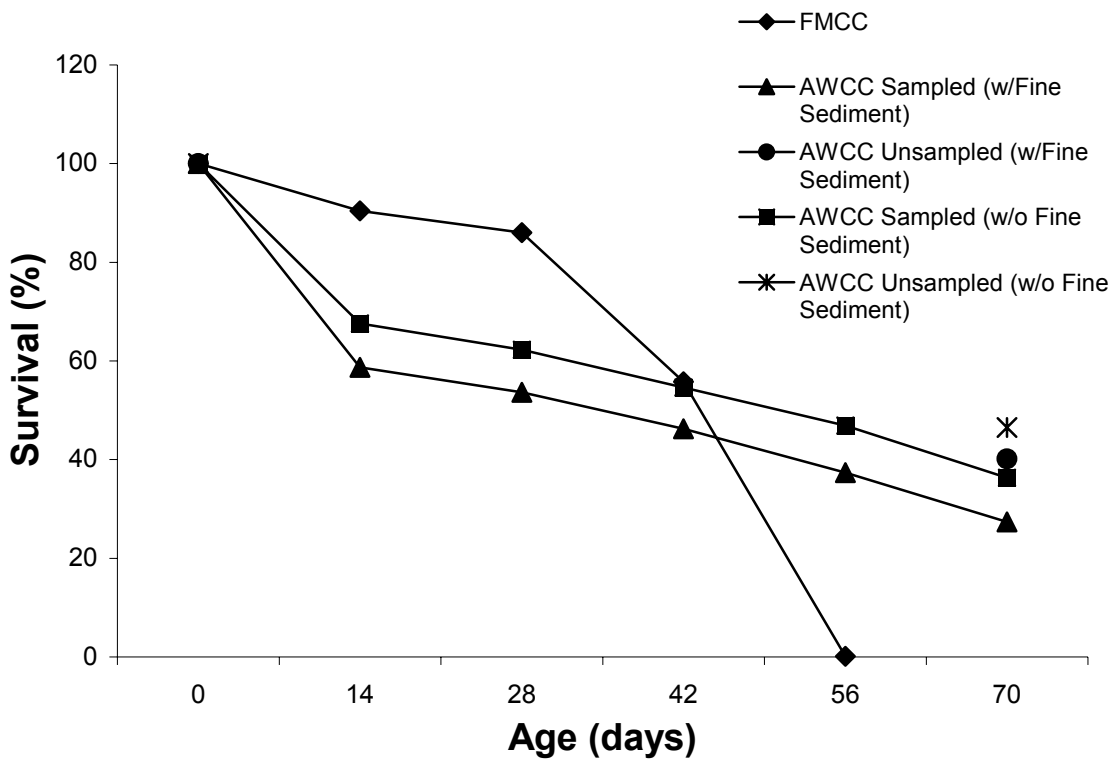

Figure 3.12. Mean survival (%) of juvenile mussels held at the FMCC, and sampled and unsampled juveniles of *V. iris* including and excluding fine sediment treatment reared in flow-through tanks in three different substrate sizes (fine sediment, fine sand, and coarse sand) in Experiment 2.

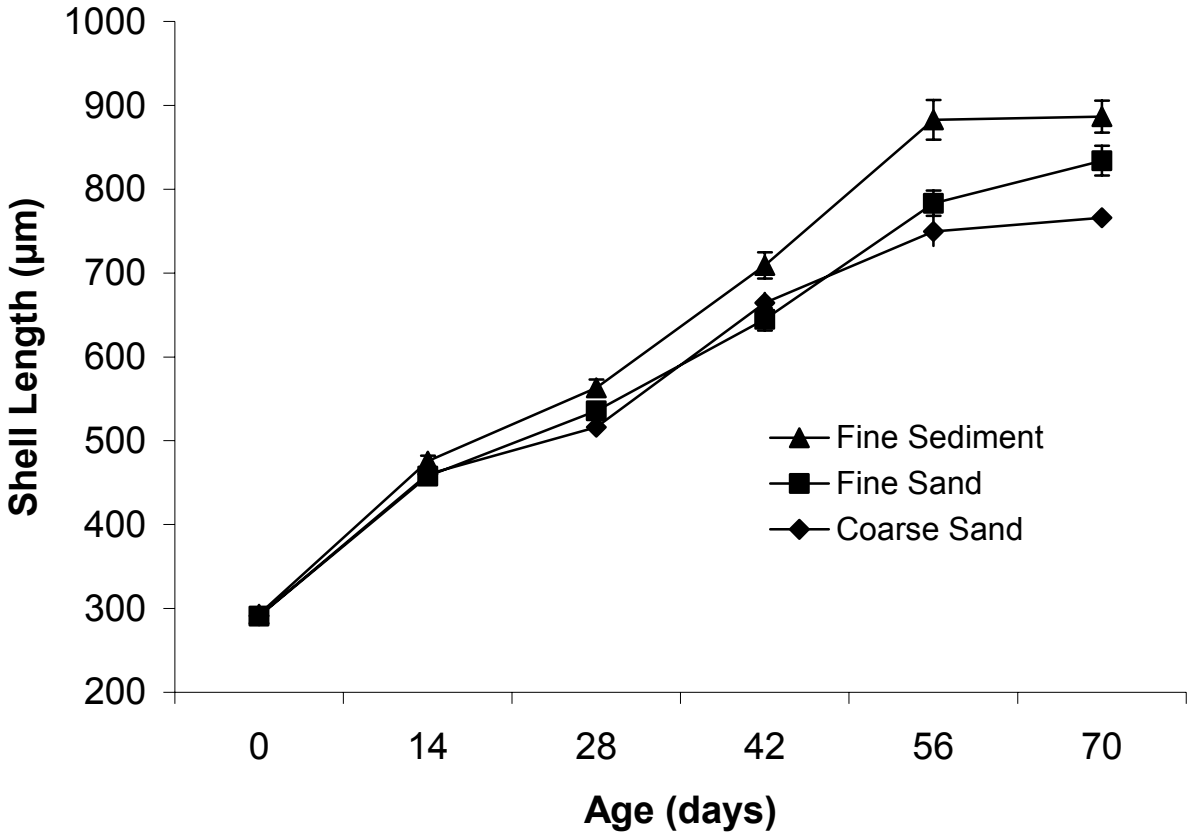

Figure 3.13. Mean shell lengths of sampled juveniles of *V. iris* reared in flow-through tanks in three different substrate sizes (fine sediment, fine sand, and coarse sand) in Experiment 2. Tukey's student range test showed significant differences between the fine sediment and coarse sand at 4 wk, and between the fine sediment and coarse sand, and fine sand and coarse sand at 6 and 8 wk, respectively ($P<0.05$).

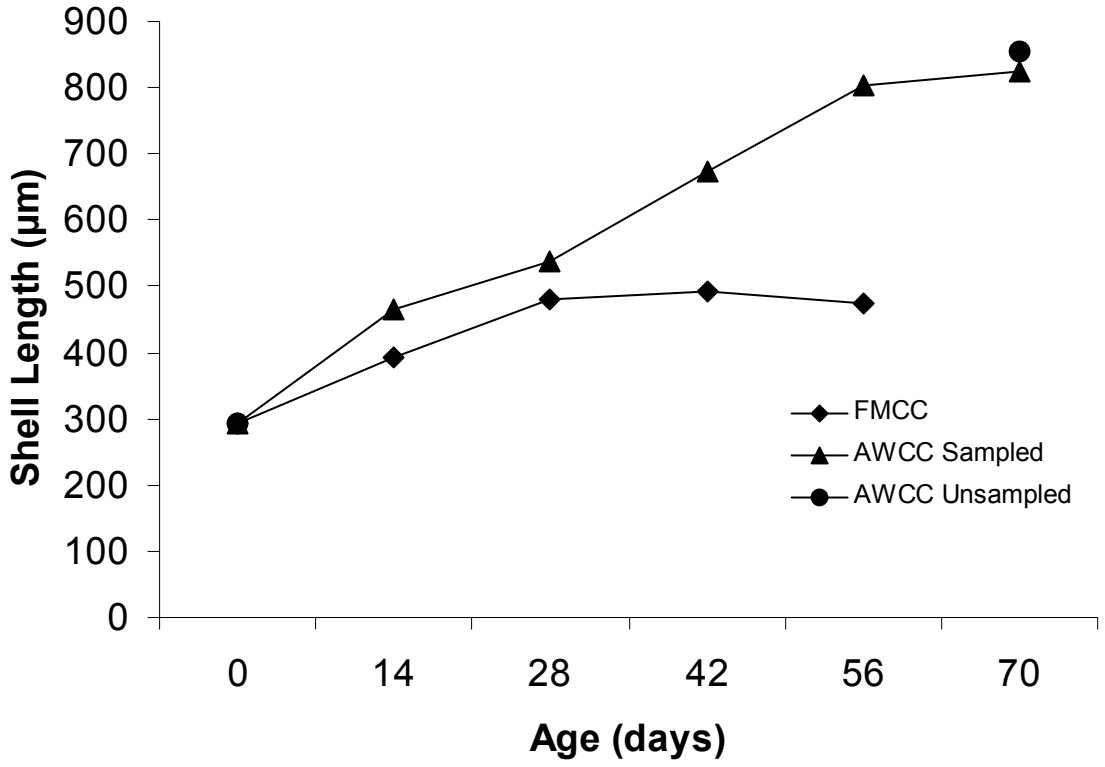

Figure 3.14. Mean shell lengths of *V. iris* juveniles reared at the FMCC, and in sampled and unsampled flow-through tanks in three different substrate sizes (fine sediment, fine sand, and coarse sand) in Experiment 2 at AWCC.

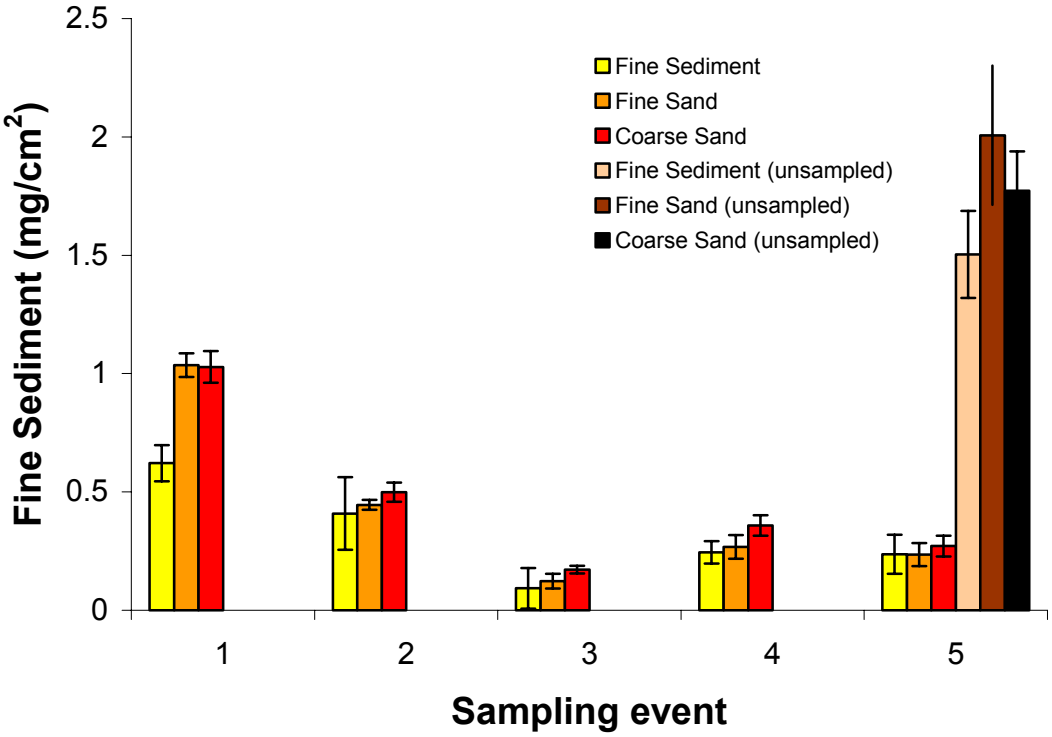

Figure 3.15. Mean of incoming sediment in mg/100 cm^2 in each of the three substrate treatments for sampled and unsampled round tanks at 4 sample events in Experiment 2.

Appendix 1. Algae cell counts from water entering grow-out systems at AWCC from January 2003 to September 2004.

	Algae Characteristics			Sample Date			
Algae (Order/Genus)	**1**	**2**	**3**	**1/9/03**	**4/18/03**	**7/13/03**	**8/21/03**
Blue-Green Algae (Cyanoprokaryota)							
Chroococcus	C	B	I	1			
Oscillatoria	F	B,P	X	5			
Cryptophytes (Cryptophyta)							
Chroomonas	U	P	I			3	
Diatoms (Bacillariophyceae)							
Cocconeis	U	B,P	I			5	3
Cyclotella	U	P	I	1			
Cymbella	U	B,P	I	6	16	17	
Diatoma	U	B,P	I	10	1	9	3
Fragilaria	C	P	X			10	3
Gonphonema	U	B,P	I	3		2	
Melosira	U	P	I		7	10	1
Navicula	U	B	I	65	34	53	50
Pinnularia	U	B	I	3	2	2	
Synedra	U	P	I	7	3		
Unidentified pennate diatom						2	
Euglenoids (Euglenophyta)							
Phacus	U	P	I				
Green Algae (Chlorophyta)							
Ankistrodesmus	U	P	I				
Chlamydomonas	U	P	I	17	96	297	322
Chlorella	U	P	I	60	65	66	170
Chlorococcum	U	B,P	I				
Gloeocystis	C	P	I				
Oocystis	U	P	I				2
Pandorina	C	P	I				
Scenedesmus	C	P	I		51	56	50
Selenastrum	U	P	I	1			
Tetraedron	U	B,P	I	2			
Trochiscia	U	P,G	I				2
Ulothrix	F	B	X		6	9	
Total				181	281	541	606
No. of Genera				13	10	14	10
# Grids Counted				40	30	20	20
Cell/mL				577.5	1195.4	3452.3	3867.1

1. Form is the classification of unicellular (U), colonial (C), or filamentous (F) algae

2. Habitat is the classification of mostly planktonic (P) or mostly benthic (B) algae

3. Ingestibility is the classification of ingestible (I) algae and algae not likely to be ingested (X) by adult mussels

Table format designed by Boyles (2003)

Appendix 1 (continued). Algae cell counts from water entering grow-out systems at AWCC from January 2003 to September 2004.

	Sample Date					
Algae (Order/Genus)	**10/13/03**	**1/16/04**	**4/23/04**	**7/13/04**	**8/10/04**	**9/7/04**
Blue-Green Algae (Cyanoprokaryota)						
Chroococcus						
Oscillatoria	12					
Cryptophytes (Cryptophyta)						
Chroomonas		31		39	1	
Diatoms (Bacillariophyceae)						
Cocconeis	7	5	17	7	9	8
Cyclotella		4	2	5	4	3
Cymbella	14	2	18	5	2	
Diatoma	1	3	2	3	1	
Fragilaria	3		27			18
Gonphonema		7	33	4	15	45
Melosira	2		24			
Navicula	36	60	96	27	64	65
Pinnularia		17	11		3	2
Synedra	5			433	4	
Tetraedron						
Unidentified pennate diatom						
Euglenoids (Euglenophyta)						2
Phacus						
Green Algae (Chlorophyta)			8			
Ankistrodesmus	45	47			5	3
Chlamydomonas	140	169	205	187	353	182
Chlorella				10		
Chlorochoccum						60
Gloeocystis	2					
Oocystis					8	
Pandorina	32	111	36	6	10	135
Scenesdesmus						
Selenastrum				1		
Trochiscia						
Ulothrix						
Total	299	456	479	727	479	523
No. of Genera	12	11	12	12	13	11
# Grids Counted	30	35	30	25	35	40
Cell/ml	1272.0	1662.8	2037.8	3711.4	1746.7	1668.7

Appendix 2. Algae cell counts from the North Fork Holston River (Chatham Hill, VA) from November 2003 to July 2004.

	Algae Characteristics			Sample Date			
Algae (Order/Genus)	**1**	**2**	**3**	**10/10/03**	**1/15/04**	**4/22/204**	**7/8/04**
Blue-Green Algae (Cyanoprokaryota)							
Oscillatoria	F	B,P	X	16			
Diatoms (Bacillariophyceae)							
Anomoeoneis	U	B,P	I				1
Cocconeis	U	B,P	I		3		4
Cyclotella	U	P	I	1	1	3	4
Cymbella	U	B,P	I	8	9	24	3
Diatoma	U	B,P	I	2	7	5	2
Fragilaria	C	P	X				7
Gonphonema	U	B,P	I	9	43	6	2
Melosira	U	P	I	3		15	
Navicula	U	B	I	67	95	104	39
Nitzschia	U	B,P	I			2	
Pinnularia	U	B	I	3	9	5	8
Synedra	U	P	I	11	3	3	3
Dinoglagellate (Dinophyta)							
Peridinium	U	P	I	1			
Euglenoids (Euglenophyta)							
Peridinium	U	P	I	1			
Green Algae (Chlorophyta)							
Ankistrodesmus	U	P	I	3			
Chlamydomonas	U	P	I	34	10	41	3
Chlorella	U	P	I	93	100	81	339
Cosmarium	U	B,P	I	1			
Desmococcus	C	P	I				18
Monocilia	F	B,P	X	4			
Oocystis	U	P	I	11			
Pandorina	C	P	I		16		
Scenedesmus	C	P	I		12		4
Ulothrix	F	B	X	8			
Total				276	308	289	437
No. of Genera				18	12	11	14
# Grids Counted				20	30	25	30
Cell/mL				1761.2	1310.3	1475.4	1859.1

1. Form is the classification of unicellular (U), colonial (C), or filamentous (F) algae
2. Habitat is the classification of mostly planktonic (P) or mostly benthic (B) algae
3. Ingestibility is the classification of ingestible (I) algae and algae not likely to be ingested (X) by adult mussels

Table format designed by Boyles (2003)

Appendix 3. Algae cell counts from the Clinch River (Clinchport, VA) from November 2003 to July 2004.

	Algae Characteristics			Sample Date			
Algae (Order/Genus)	**1**	**2**	**3**	**10/10/03**	**1/15/04**	**4/22/04**	**7/15/04**
Blue-Green Algae (Cyanoprokaryota)							
Chroococcus	C	B	I				2
Cryptophytes (Cryptophyta)							
Chroomonas	U	P	I		1		2
Diatoms (Bacillariophyceae)							
Cocconeis	U	B,P	I	2	7	2	10
Cyclotella	U	P	I	1	1	3	6
Cymbella	U	B,P	I	4	5	12	4
Diatoma	U	B,P	I	3	4	4	1
Fragilaria	C	P	X			2	4
Gonphonema	U	B,P	I	9	12	13	3
Melosira	U	P	I			5	9
Meridion	U	B,P	I			6	
Navicula	U	B	I	55	63	77	45
Pinnularia	U	B	I	3	5	3	4
Rhizosolenia	U	P	I	1			
Synedra	U	P	I		6	1	5
Euglenoids (Euglenophyta)							
Euglena	U	P	I	1			
Green Algae (Chlorophyta)							
Ankistrodesmus	U	P	I	1			
Chlamydomonas	U	P	I	52	18	21	18
Chlorella	U	P	I	97	169	287	161
Desmococcus	C	P	I		6		8
Oedogonium	F	B,P	X		1		
Oocystis	U	P	I	3			
Pediastrum	C	P	I				16
Scenedesmus	C	P	I				12
Total				232	298	436	310
No. of Genera				13	13	13	15
# Grids Counted				35	30	35	35
Cell/mL				846.0	1267.8	1589.9	1130.4

1. Form is the classification of unicellular (U), colonial (C), or filamentous (F) algae
2. Habitat is the classification of mostly planktonic (P) or mostly benthic (B) algae
3. Ingestibility is the classification of ingestible (I) algae and algae not likely to be ingested (X) by adult mussels

Table format designed by Boyles (2003)

VITA

Aaron J. Liberty was born on August 13, 1979 in Laconia, New Hampshire. He graduated from Spaulding High School in Rochester, NH in June of 1997 and attended Unity College in Maine from 1997 to 2001. While at Unity College, he earned a Bachelor of Science in Conservation Law and was introduced to fisheries science. After an internship working with the Anadromous Fish Restoration Program at the N.H. Fish and Game Department, and working for the Department of Ecology at Montana State, he decided to enroll in the fisheries graduate program at Virginia Tech. He earned his M.S. in Fisheries and Wildlife Science in December of 2004.